STUDIES ON THE MECHANISM OF INDUCTION OF HUMAN SULFOTRANSFERASE BY METHOTREXATE

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LIST OF ABBREVIATIONS

SULT	Sulfotransferase
hSULT	Human Sulfotransferase
rSULT	Rat Sulfotransferase
SULT1A1/P-PST	Simple Phenol-sulfating Sulfotransferase
SULT1A3 /M-PST	Monoamine-sulfating Sulfotransferase
SULT2A1/DHEA-ST	Dehydroepiandrosterone Sulfotransferase
SULT1E1/EST	Estrogen Sulfotransferase
CYP	Cytochrome P450
DHFR	Dihydrofolate Reductase
GST	Glutathione S-transferase
UGT	UDP-glucuronyltransferases
MTX	Methotrexate
FR	Folic Acid
RA	Retinoic Acid
CDCA	Chenodeoxycholic Acid
DEX	Dexamethasone

CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
PB	Phenobarbital
DMSO	Dimethyl Sulfoxide
PVDF	Polyvinylidene Difluoride
DIG	Digoxigenin
NR	Nuclear Receptor
DBD	DNA Binding Domain
RXRα	Retinoid X receptor alpha
AhR	Aryl Hydrocarbon Receptor
CAR	Constitutive Androstane Receptor
PXR	Pregnane X receptor
FXR	Farnesoid X receptor
LXRα	Liver X Receptor alpha
VDR	Vitamin D Receptor
PPARα	Peroxisome Proliferators Activated Receptor alpha
ERRα	Estrogen Related Receptor alpha
TR	Thyroid Receptor
HRE	Hormone Response Element
PBRE	Phenobarbital Response Element
DR	Direct Repeat
IR	Inverted Repeat

ER	Everted Repeat
PAPS	3'-Phosphoadenosine 5'-Phosphosulfate
DHEA	Dehydroepiandrosterone
PNPS	*p*-nitro-phenyl Sulfate
MTT	3-(4 to 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
FBS	Fetal Bovine Serum
RIP	Receptor Interacting Protein
ERAPs	Estrogen Receptor Associated Proteins
TRAP	Thyroid Receptor Associated Protein
SF1	Steridogenic Factor 1
C/EBPα	CAAT/Enhancer Binding Protein alpha
CARLA	Coactivator-depenent Receptor Ligand Assay
EMSA	Electrophoretic Mobility Shift Assay
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction

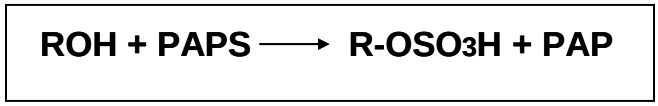
Chapter I

Introduction

1.1 Sulfotransferase

1.1.1 General

Sulfotransferases (SULTs) belong to one of the major families of phase II drug metabolism enzymes (Coughtrie 2002). They catalyze the transfer of a sulfuyl group to hydroxyl-containing molecules.



The co-substrate for all SULTs is 3′-phosphoadenosine 5′- phosphosulfate (PAPS). Sulfation (sulfuryl transfer) is widely observed in various biological processes. Various biological signaling molecules, including hormones, neurotransmitters, peptides and proteins, can be sulfated. Depending whether they are soluble or not, SULTs can be classified into membrane-associated or cytosolic SULT (Strott 2002). The membrane associated SULTs sulfate large molecules including carbohydrate, peptide, and protein. They are mainly related with posttranslational modification of carbohydrates, peptides, and proteins. The addition of a sulfonate group to carbohydrates can transform a common structural motif of a carbohydrate into a unique recognition site for a specific receptor (Hooper, Manzella et al. 1996). For many proteins, tyrosine sulfonation is important for

biological activity and correct cellular processing (Vishnuvardhan and Beinfeld 2000). The cytosolic SULTs sulfate small molecules such as hydroxyl-containing xenobiotics, steroids, bioamines and therapeutic drugs. The substrate specificities of cytosolic SULTs are very broad. Most hydroxyl-containing compounds (phenols and alcohols) are substrates for one of the SULT isoforms. Sulfation can change biological functions of endogenous signaling molecules because the sulfated molecules are usually unable to bind to their receptors and become inactive (Roy, Lavrovsky et al. 1999). For instance, the genomic action of steroid hormones is inhibited by sulfoconjugation because the sulfates of steroid hormones are unable to bind to their cognate nuclear receptors (Hahnel, Twaddle et al. 1973).

SULTs also catalyze the sulfation of a broad range of xenobiotics. Sulfation of xenobiotics is mainly associated with detoxification: biotransformation of a relatively hydrophobic xenobiotic into a more water-soluble sulfuric ester that is readily excreted. Nevertheless, it is noteworthy that a number of compounds can be converted into highly reactive intermediates by sulfation and can then act as chemical carcinogens and mutagens by covalently binding to DNA (Miller 1994; Glatt 1997). Detoxification or bioactivation depend on the electrophilic reactivity of the individual sulfuric ester products formed. Most sulfation products are stable enough for excretion, while other sulfuric ester products can be reactive toward nucleophilic sites on DNA, RNA, and protein, and so become involved in the initiation of carcinogenesis and other toxic responses.

1.1.2 Induciton of Cytochrome P450s

Phase I and phase II drug metabolizing enzymes are usually inducible by endogenous and xenobiotic compounds. Cytochrome P450s are the most well studied drug metabolizing enzymes. The induction of cytochrome P450 has long been a major driving force to attract many scientists into P450 research. Nuclear receptor mediated induction mechanisms of drug metabolizing enzymes are mostly revealed from the study of P450. Mammals contain at least 17 distinct P450 gene families. P450 gene families 1-4 (CYP1 – CYP4) code P450s that metabolize xenobiotics and endogenous lipophilic substrates. Other mammalian P450 gene families typically do not metabolize xenobiotics. Families 1 – 4 can be regulated by endogenous hormones, cytokines, and structurally diverse xenobiotics whereas other P450 gene families typically are not induced by xenobiotics (Waxman 1999). Induction of P450 gene expression is primarily through receptor-dependent mechanisms.

CYP1 genes are stimulated through the aryl hydrocarbon receptor (AhR) (Hankinson, Brooks et al. 1991; Gonzalez, Liu et al. 1993). The AhR becomes activated by binding an aromatic hydrocarbon ligand in the cytosol. The activated receptor then translocates to the nucleus, heterodimerizes with the nuclear factor Arnt, binds to DNA enhancer sequences upstream of CYP1, and stimulates target gene transcription. This mechanism explains the induction of CYP1 genes by a large number of polycyclic aromatic hydrocarbons.

CYP2B genes are regulated through the orphan nuclear receptor, constitutive active receptor (CAR) (Honkakoski, Zelko et al. 1998). This discovery advanced our understanding of the mechanism of phenobarbital (PB) induction. PB was the first cytochrome P450 inducer discovered (Sueyoshi and Negishi 2001). CAR binds to the PB

responsive element (PBRE) in CYP2B genes as a heterodimer with the RXR. The natural ligand for CAR is androstanes (Forman, Tzameli et al. 1998). Endogenous inhibitory steroids related to androstanol and androstenol bind to CAR and maintain it in an inactive state. In the presence of PB or PB-like inducers, the binding of inhibitory androstanes to CAR is abolished and receptor activity is thereby derepressed. CAR can directly transactivate a PBRE-linked reporter gene in transfected cells in the absence of PB inducers and inhibitory androstanes (Honkakoski, Zelko et al. 1998).

CYP3 gene families can be induced by a broad range of steroids and antibiotics, including both glucocorticoids and anti-glucocorticoids. The orphan nuclear receptor, pregnane X receptor (PXR), was found to be responsible for most of these inductions (Cui, Thomas et al. 2005). PXR belongs to the nuclear hormone receptor family. Like non-steroid hormone receptors, PXR binds as a heterodimer with RXR to a hormone response element (HRE). PXR is activated by a large number of endogenous and exogenous compounds including steroids, antibiotics, antimycotics, bile acids, and components of the herbal antidepressant, St. John's wort (Kliewer and Willson 2002). PXR serves as a generalized sensor of hydrophobic toxins. PXR is highly expressed in the liver and intestine (Kliewer, Moore et al. 1998; Kliewer and Willson 2002). This seems to be an indication that intestine, like liver, is an important organ for detoxification. PXR can also be activated by Phenobarbital (PB), suggesting that the effects of PB on CYP genes may be mediated by multiple receptors (Lehmann, McKee et al. 1998; Sueyoshi and Negishi 2001).

CYP4A enzymes catalyze the oxidation of fatty acids. CYP4A genes can be transcriptionally activated by different acidic drugs and xenobiotics including

hypolipidemic fibrate drugs, phthalate ester plasticizers and other environmental pollutants (Rao and Reddy 2001). These inducers were first found to induce peroxisomal enzymes and are classified as peroxisome proliferator chemicals (PPCs) (Yeldandi, Rao et al. 2000). The receptor protein responsible for these inductions was named peroxisome proliferator-activated receptorα (PPAR)(Savas, Hsu et al. 2003; Nishimura, Yamauchi et al. 2005). PPARα mediated CYP4A6 induction is dependent on upstream enhancer elements in the CYP4A6 gene (Palmer, Hsu et al. 1994). Like CAR and PXR, PPARα binds as a heterodimer with RXR to the peroxisome proliferator response elements (PPREs).

CYP7A enzymes catalyze hydroxylation of cholesterol. This reaction is the first and rate-limiting step for the catabolism of cholesterol to bile acids (Jelinek, Andersson et al. 1990). CYP7A gene is stimulated by cholesterol and suppressed by bile acids (Russell 1992). Bile acids suppress CYP7A promoter activity through the farnesol X receptor (FXR). Bile acids, such as chenodeoxycholic acid (CDCA), lithocholic acid, and deoxycholic acid, are the natural ligands for FXR (Goodwin, Jones et al. 2000; Song, Echchgadda et al. 2001). FXR is also designated as BAR (bile acid receptor). The induction of CYP7A gene expression is mediated through liver X receptor-α (LXRα). LXRα is activated through the binding of oxysterol (Lehmann, Kliewer et al. 1997).

1.1.3 Induction of Sulfotransferases and Mechanism

Induction of SULTs, though, hve not been well studied compared to other drug metabolizing enzymes, such as P450s, UDP-glucuronyltransferases (UGTs), and glutathione S-transferases (GSTs). SULT regulation and induction were not regarded

until the 1980s (Clarke, Adams et al. 1982). Hormonal regulation and mRNA expression have been systematically studied by Dr.Curtis Klaassens' research team using male and female rat livers (Liu and Klaassen 1996; Liu and Klaassen 1996; Liu and Klaassen 1996; Liu and Klaassen 1996; Liu, LeCluyse et al. 1996; Klaassen and Boles 1997; Boles and Klaassen 1998; Boles and Klaassen 1998; Dunn and Klaassen 1998; Klaassen, Liu et al. 1998; Boles and Klaassen 1999; Dunn, Gleason et al. 1999; Dunn, Kolaja et al. 1999; Dunn and Klaassen 2000). A total of six isoforms were examined. They found that rSULT1A1 expression is not regulated by growth hormone; rSULT1C1 expression in male rats is controlled by male growth hormone secretory pattern; and rSULT1E2 expression is suppressed by female growth hormone secretory pattern. The hydroxysteroid SULTs are primarily expressed in adult female rats. Synthetic glucocorticoid hormones, such as dexamethasone (DEX) and pregnenolone-16-alpha-carbonitrile (PCN), are able to induce some rat hepatic SULT isoforms (Liu and Klaassen 1996; Liu, LeCluyse et al. 1996).

Studies on SULT regulation mechanism indicate some of the SULT isoforms through nuclear receptor mechanisms similar to cytochrome P450s (Runge-Morris, Rose et al. 1996; Runge-Morris 1998; Runge-Morris, Wu et al. 1999; Duanmu, Kocarek et al. 2001; Wu, Kocarek et al. 2001; Duanmu, Locke et al. 2002). A *cis*-acting inverted repeat with three intervening bases (IR3) was identified in the 5'-flanking region of rat SULT1A1 which mediates transactivation by both the glucocorticoid receptor (GR) and the androgen receptor (AR) (Fang, Shenoy et al. 2003). The bile acid, chenodeoxycholic acid (CDCA), is a potent inducer of rSULT2A1, and its inducing effect is mediated through the bile acid-activated farnesoid X receptor (FXR) (Qian, Sun et al. 2001). The

ligand-activated FXR forms a heterodimer with the RXR and regulates rSULT2A1 by binding to an upstream region (an inverted repeat-0 nuclear receptor motif (IR0). An earlier report from the same research group demonstrated that androgens inhibit the rSULT2A1 promoter function (Chan, Song et al. 1998). PXR, which mediates CYP3A induction, mediated the induction of mSULT2A1 and PAPS synthetase 2 (PAPSS2) in mice (Sonoda, Xie et al. 2002). The results indicated that co-transfection of PXR, RXRα, and the IR0 element in the promoter region is necessary for the gene stimulation. This work demonstrated a binding site for PXR/RXR heterodimer within the mSULT2A1 promoter (IR0), indicating that mSULT2A1 is the direct transcriptional target of PXR. It was proposed that PXR serves as a master regulator of phase I and II responses to facilitate rapid and efficient detoxification and elimination of xenobiotics. A study on the suppression of SULT2A1 during the acute phase response also suggested PXR and FXR are responsible for the induction of SULT2A1 (Ashikari-Hada, Habuchi et al. 2004).

Recently, it was reported that the SULT2A1 in human, mouse and rat is a target for transcriptional activation by the vitamin D receptor (VDR) (Echchgadda, Song et al. 2004). FXR and PXR inhibited vitamin D_3 induction of SULT2A1. Another report also suggested a repressive role of PXR and FXR on basal mSULT2A1 expression (Kitada, Miyata et al. 2003). Using knockout mice and human cell lines, it was reported that multidrug resistance protein (MRP4), SULT2A1, CYP3A4, and CYP2B6 are induced by TCPOBOP and phenobarbital (PB) through CAR (CAR) (Assem, Schuetz et al. 2004). This suggests that MRP4 and SULT2A1 participate in an integrated pathway mediating elimination of sulfated steroid and bile acid metabolites from the liver. A study using transgenic mice demonstrated that CAR regulates mouse SULT2A9 expression by

binding to the CAR response elements found within the SULT promoters (Saini, Sonoda et al. 2004). It was concluded that this increased sulfation is responsible for bile acid detoxification (Falany and Wilborn 1994; Banoglu 2000; Zheng, Wang et al. 2003).

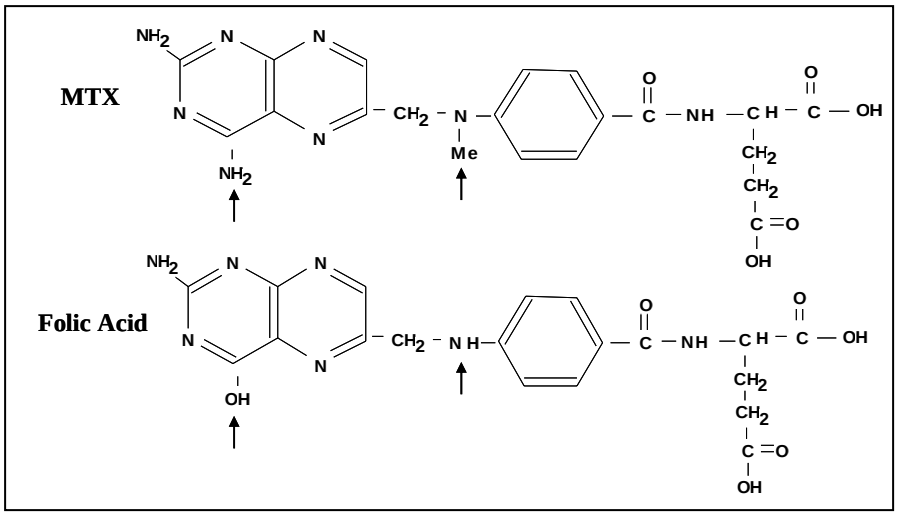
The researches on the transcriptional regulation of hSULT2A1 have made impressive progress in recent years. Both steridogenic factor 1 (SF1) and estrogen-related receptor alpha (ERRα) were reported to regulate hSULT2A1 activity through the same DNA *cis*- element located in the proximal promoter region of hSULT2A1 (Saner, Suzuki et al. 2005; Seely, Amigh et al. 2005). Peroxisome proliferator-activated receptor alpha (PPARα) was also found to involve in the transcriptional regulation of hSULT2A1 through the DNA response element located in - 5949 to -5929 upstream of the promoter region (Fang, Strom et al. 2005). The vitamin D receptor (VDR) was reported to target hSULT2A1 promoter through interaction with CAAT/Enhancer Binding Protein-alpha (C/EBPα) (Song, Echchgadda et al. 2005).

Compared with endogenous growth and sex hormone, SULT induction by xenobiotics is not well studied. Recent data suggest that SULT can be induced by xenobiotics (Runge-Morris 1998; Runge-Morris, Rose et al. 1998; Maiti and Chen 2003a; Maiti and Chen 2003b; Gaworecki, Rice et al. 2004), although the mechanisms for induction are basically unknown. We previously reported that the antifolate and apoptosis-inducing drug, MTX, induces SULTs in rat liver/intestine (Maiti and Chen 2003). In this study, MTX can induce hSULT in a time- and concentration-dependent manner. MTT assay results show the induction was not caused by the cytotoxicity. To investigate the mechanism involved in MTX induction of hSULT2A1, the promoter sequence was cloned. Cotransfection assay, and RNA intereference experiment found

CAR and RXRα were involved in the up-regulation of hSULT2A1. Promoter deletion, mutation and gel shift assay indicate that the IR2 element located in the promoter region of hSULT2A1 mediates the nuclear receptor induction of hSULT2A1. Further studies have showed that PXR and VDR also regulate hSULT2A1 activity. There are cross-talk between different nuclear receptors and DNA-*cis* element.

1.2 Methotrexate (MTX)

Derivatives of the vitamin folic acid are crucial for the biosynthesis of thymidylate, purines and amino acids. They are necessary for normal metabolism and growth. Antifolates are the first antimetabolite class of anticancer agents and were one of the first modern anticancer drugs. The first clinically useful antifolate was 2,4-diamino-pteroylglutamate (4-amino-folic acid; aminopterin; AMT) which was described in 1947 (McGuire 2003). AMT was soon superseded by its 10-methyl congener, methotrexate (MTX). The structure of MTX is very similar to folic acid. MTX can inhibit the physiological functions of folic acid through tight-binding inhibition of dihydrofolate reductase (DHFR).



Detailed examination of the mechanisms of cytotoxicity and selectivity of MTX showed that inhibition of both dTMP synthesis and de novo purine synthesis, secondary to DHFR inhibition, led to DNA synthesis inhibition and subsequent cell death. For this reason, MTX is widely used as clinic drug against cancer and other diseases (Hine, Everson et al. 1990; Sakoda, Saitoh et al. 2003; Iikuni, Iwami et al. 2004).

The transmembrane transport of MTX occurs by two different mechanisms in an energy dependent manner (Genestier, Paillot et al. 2000). The first route involves a transmembrane carrier protein; the affinity of this protein for MTX is very similar to reduced folates and is in the micromolar range. The second route involves a membrane associated folate binding protein. This transporter has a much lower affinity for MTX compared with reduced folates which is in the nanomolar range. The expression of both transporters increases when the concentration of extracellular folate is very low. At high concentration, MTX can passively diffuse into cells.

MTX is converted to various forms of polyglutamate by linking up to five polyglutamate groups *in vivo*. This polyglutamation makes MTX readily transport across

the cell membrane. MTX and its polyglutamate metabolites inhibit activities of many enzymes in the metabolic pathway of folic acid. Long-term MTX therapy inhibits production of thymidylate, purine, and methionine and leads to accumulation of adenosine (Egan and Sandborn 1996). These actions inhibit cellular proliferation and induce apoptosis. In the treatment of cancer, the rationale for use of high dose MTX is that malignant cells become starved of the purine and pyrimidine precursors of DNA and RNA required for proliferation. The rationale for low-dose treatment of other diseases such as psoriasis, rheumatoid arthritis and inflammatory bowel disease is less clear (Fraser 2003).

Most study of MTX is clinically related. Reports on MTX induction of drug-metabolizing enzymes are limited. Induction of cytochrome P450 (CYP) by MTX was not significant. Compared with other antineoplastic drugs, MTX possesses a lesser capacity for regulation of rat hepatic CYP enzymes. When the rat was administrated with MTX for 1, 2, 7, or 14 days, the mRNA level of CYP3A2 mRNA has almost no change and the mRNA level of CYP2C11 even marginally decreased (Cheung, Lee et al. 1996). Also the expression of CYP3A4 was not influenced by MTX in both reporter gene assays and endogenous induction studies (Luo, Cunningham et al. 2002). Microarray and real-time RT-PCR analysis performed in drosophila indicated that many genes including those involved in signal transduction, transcription, and cell cycle regulation, were regulated by MTX (Affleck, Neumann et al. 2006). Here, we have investigated MTX induction mechanisms of hSULTs.

Through inhibiting several folate-dependent enzymes, the key enzyme for DNA and several amino acid synthesis, polyglutamated MTX can decrease the proliferation of

normal human bone marrow granulocyte progenitor cells and cause cytotoxicity (Koizumi, Ueno et al. 1990). The cytotoxicity of MTX to these cells can be completely reversed by equimolar concentration of 1-leucovorin. MTX was also reported to inhibit proliferation of human epidermal keratinocytes and induce differentiation of the cells in vitro. The inhibition effect of MTX is time- and dose- dependent and becomes irreversible after 24 hours. Thymidine can completely prevent these effects caused by MTX, suggesting all these effects are caused by a depletion of thymine deoxyribonucleotides (Omata, Abraham et al. 1992). MTX also induces the differentiation of monocytic tumor cells, which may explain, in part, its therapeutic effects in the treatment of some disorders (Zimecki and Artym 2004).

1.3 Nuclear Receptors

1.3.1 General

Nuclear receptors (NRs) are a large family of transcriptional factors that bind to specific regulatory sequences in DNA, and regulate the transcription of many proteins. Ligands of nuclear receptors are normally small and lipophilic, properties that similar to those of endogenous inducer compounds such as steroids, bile acids, or fatty acids. The activities of nuclear receptors are regulated by their ligands, phosphorylation, and by interaction with other proteins (Astapova, Smirnov et al. 2002). Nuclear receptors play an important role in normal physiological development and metabolism and represent therapeutic targets for a wide range of human diseases including cancer, endocrine and heart disease (Nettles and Greene 2005). A typical nuclear receptor contains a variable NH2-terminal region, a conserved DNA-binding domain (DBD), and a conserved

carboxy-terminal ligand binding domain (Aranda and Pascual 2001). Some nuclear receptors also contain an amino-terminal activation function (AF1), which can activate transcription in a ligand-independent fashion. The nuclear receptor ligand binding domain (LBD) contains a second activation domain, termed AF-2, which is located in the COOH terminus of the LBD, unlike the AF-1 domain, the AF-2 domain is strictly ligand dependent and conserved among the members of the nuclear receptor superfamily. Coactivators bind to the AF2 surface via the amino acid motif LxxLL. The mechanism of transcriptional activation by NRs is via recruitment of these coactivators, which mediate chromatin remodeling and also recruit the basal transcription apparatus (Nettles and Greene 2005).

Evolutionary analysis of the nuclear receptors has led to a subdivision in six different subfamilies (Laudet 1997). The first subfamily includes CAR (CAR/MB67), pregnane X receptor (PXR), Farnesoid X receptor (FXR), liver X receptor (LXR), thyroid hormone receptors (TRs), retinoic acid receptors (RARs), vitamin D receptors (VDRs) and peroxisome proliferators-activated receptors (PPARs). The second subfamily includes retinoid X receptors (RXRs) together with HNF4 orphan nuclear receptors. Almost all nuclear receptors in the first subfamily form heterodimer with RXRα and regulate transcription of their target genes. RXRα is a common hetorodimer partner of nuclear receptor. The third subfamily contains steroid receptors and the estrogen-related receptors (ERRs). The fourth, fifth, and sixth subfamilies contain the nerve growth factor increased protein B (NGFI-B), Fushi Tarazu-factor 1 (FTZ-1), and germ cells nuclear factor (GCNF), respectively.

Although most nuclear receptors transactivate their targets after binding to their recognition sequence, nuclear receptors can also inhibit gene expression. In some cases, inhibition may come from nuclear receptors competition for DNA binding sequences with other nuclear receptors or transcription factors. However, there are also so-called negative DNA response elements (Drouin, Trifiro et al. 1989). The negative DNA response element represses the expression of the target gene by binding with specific transcription factors. Negative DNA response element is usually located very close to the transcriptional start site (Saatcioglu, Perry et al. 1990).

1.3.2 Cross-talk between Nuclear Receptors

Nuclear receptors are part of a complex network of transcription work in vivo. It is not surprising that different nuclear receptors can interact with a variety of other proteins as well as one another in different signaling pathways (Pascussi, Gerbal-Chaloin et al. 2003). Between CAR and PXR, a considerable redundancy exists with regard to overlapping ligands and the binding of both receptors to the DNA-response elements with overlapping affinity (Moore, Parks et al. 2000; Maglich, Stoltz et al. 2002; Zhang, Huang et al. 2004). PXR and CAR might thus compensate for the loss or malfunction of one another to a certain degree, which might explain the lack of an obvious phenotype in the PXR or CAR knockout animals (Zhang, Huang et al. 2004). In the case of heterodimeric receptors, competition for limiting concentrations of RXR presents a mechanism of transcriptional regulation for several nuclear receptors.

Nuclear receptors can also regulate gene expression by mechanisms independent of binding to DNA response element. This means they can alter the expression of genes

that do not contain any DNA response element through positive or negative interfere with the activity of other transcription factors. This mechanism is generally referred to as transcriptional cross-talk (Gottlicher, Heck et al. 1998). It was reportered that several nuclear receptors, such as retinoic acid receptor, glucocorticoid receptor, etc. can act as ligand-dependent transrepressors of AP1 activity. AP1 can also inhibit transactivation by nuclear receptors (Pfahl 1993). The cross-talk between nuclear receptors and other signaling pathways is not restricted to the transcriptional antagonism described above. Phosphorylation of nuclear receptors provides an important link between signaling pathways (Shao and Lazar 1999). Also, nuclear receptors can affect the rate of RNA polymerase II-directed transcription. This interaction may occur directly or indirectly through the action of coactivators or corepressors. Nuclear receptors to interact with several components of the general transcriptional machinery (Schulman, Chakravarti et al. 1995). It is likely that the nuclear receptors can cause the recruitment of basal components of the promoter and the enhancement of transcription.

1.3.3 CAR, PXR and VDR

Several mechanisms of how CAR (Constitutive Androstane/Active Receptor) regulate drug mechanism have been proposed so far.However, none of them explain the whole process of signal transduction (Kawamoto, Sueyoshi et al. 1999). CAR normally resides in the cytoplasm of hepatocytes and undergoes a cytosolic-nuclear translocation upon phenobarbital treatment (Kawamoto, Sueyoshi et al. 1999). As a result, CAR can induce the gene expression of CYP2B6. The androstanol can reverse the effect of CAR. However, it is unknown whether this reversal of inhibition is due to a direct interaction

with CAR. In addition to derepression, direct activation of CAR by a few chemicals has been reported. The chemical TCPOBOP is one of the strongest inducers in mouse but hardly affects CYP2B levels in human (Tzameli, Pissios et al. 2000). Interestingly, CITCO has strong effect on human CYP2B6 but has little effects on mouse CYP2B (Maglich, Parks et al. 2003). Differences in activation of CAR in mouse and human are most likely due to the divergent ligand binding domain of the CAR orthologs from these species (Moore, Parks et al. 2000). Furthermore, CAR activity in the nucleus seems to be under the regulation of protein phosphorylation events (Zelko, Sueyoshi et al. 2001).

In contrast to the classical nuclear receptors, CAR shows both ligand dependent and ligand independent activities. Phenobarbital activates both human and mouse CAR through translocating CAR from the cytoplasm to the nucleus (Honkakoski and Negishi 1998). Because CAR exhibits an intrinsically high transcriptional activity, nuclear localization of the receptor results in the activation of target gene expression in the absence of ligand binding (Forman, Tzameli et al. 1998; Honkakoski and Negishi 1998). The nuclear receptor pregnane X-receptor (PXR) can bind to a wide variety of structurally diverse exogenous and endogenous chemicals and regulate the expression of genes important to drug disposition process such as drug transporters, phase I and II metabolic enzymes (Watkins, Wisely et al. 2001; Carnahan and Redinbo 2005). Nuclear vitamin D receptor (VDR) is known to mediate the biological actions of 1α,25-dihydroxyvitamin D3 through its ability to modulate the expression of target genes (Nezbedova and Brtko 2004). All the three nuclear receptors CAR, PXR and VDR share a common heterodimerization partner, retinoid X-receptor (RXR), and are subject to

cross-talk with other nuclear receptors and with a broad range of other intracellular signaling pathways.

Our laboratory has found that MTX can induce hSULT2A1 by both endogenous induction (Chen, Baker et al. 2005) studies and reporter gene assay. The molecular mechanism involved this induction remains to be elucidated. The present project investigated the nuclear receptors involved in MTX induction of hSULT2A1. We found that several nuclear receptors including CAR, PXR, and VDR can regulate the expression of hSULT2A1. CAR has synergistic effect with MTX; CAR and MTX together can significantly up-regulate the promoter activity of hSULT2A1. PXR represses the promoter activity of hSULT2A1 and can reverse the induction effect of MTX and CAR on hSULT2A1. VDR can also transactivate hSULT2A1 and compete with CAR for the promoter activity of hSULT2A1.

1.3.4 Coactivators and Corepressors

Different cloning strategies have led to the identification of numerous receptor-coactivator proteins which include estrogen receptor associated proteins (ERAPS) (Halachmi, Marden et al. 1994), receptor-interacting proteins (RIP) (Cavailles, Dauvois et al. 1994), or thyroid receptor-associated proteins (TRAPs) (Fondell, Ge et al. 1996). These coactivator proteins interact with nuclear receptors in a ligand dependent manner. A possible application derived from the ligand-dependent recruitment of coactivators by the nuclear receptors is the identification of new ligands. An assay termed coactivator-dependent receptor ligand assay (CARLA) using SRC-1 has allowed for the identification of naturally occurring ligands for different nuclear receptors (Krey, Braissant et al. 1997;

Walfish, Yoganathan et al. 1997; Devchand, Hihi et al. 1999). Although most coactivators interact with the nuclear receptors in a ligand-dependent manner and require the AF-2 domain, some coregulators, including p68 and PGC-1 interact with the AF-1 domain. Other coactivators could be involved in protein degradation, RNA stability, or nuclear transport (Aranda and Pascual 2001).

In addition to the ligand-dependent gene activation, nuclear receptors including TR and RAR repress basal transcription in the absence of ligand. Binding of hormonal ligand to the receptor releases the transcriptional silencing and leads to gene activation. Biochemical studies of cellular proteins associated with free thyroid receptor and RAR led to the identification of a 270-kDa cellular protein named nuclear corepressor (NCoR) (Horlein, Naar et al. 1995), or RIP-13 (Seol, Mahon et al. 1996). In parallel, silencing mediator for retinoic and thyroid hormone receptors (SMRT) was isolated by a yeast two-hybrid screening of a human lymphocyte cDNA library (Chen and Evans 1995). Free TR and RAR interact strongly in vitro with NCoR and SMRT, and addition of ligand induces dissociation from the corepressors. It appears that the AF-2 region serves to trigger the release of corepressors from the nuclear receptors upon the binding of ligand.

1.4 Hormone Response Elements

Nuclear receptors regulate transcription by binding to specific DNA sequences in target genes known as hormone response elements (HREs). These elements are usually located in the proximal promoter region of the target genes. In rare cases, they can present in the enhancer region which is located several thousand base pairs upstream of the transcriptional start site (Fang, Strom et al. 2005). The analysis of both naturally

occurring as well as synthetic HREs revealed that a sequence of 6 bp constitutes the core recognition motif. Two consensus motifs have been identified. The sequence AGAACA is recognized by steroid class III receptors, and AGGTCA serves as recognition motif for all other receptors (Beato, Herrlich et al. 1995). The two half-site motifs can be separated by various numbers of base pairs. Most nuclear receptors bind to their HREs as homo- or heterodimers composed typically of two core hexameric motifs. The two half-sites of the HREs can be configured as directed repeats (DR), inverted repeats (IR), or everted repeats (ER). In some cases, nuclear receptor can bind to HREs as monomers (Seely, Amigh et al. 2005).

Except for the core recognition motif, the small differences in the half-site sequence and the sequence of the flanking extension of the response elements also play important role in determining receptor binding specificity (Mader, Leroy et al. 1993)

1.5 RNA Interference

RNA interference (RNAi) is the suppression of gene expression at the transcription level by nucleotide sequence-specific interactions that are mediated by RNA. In a natural context, dsRNA may be produced from rearranged loci, by transcription from converting promoters or by host or viral-encoded RNA-dependent RNA polymerases (Voinnet 2002). A key component of RNA silencing is a 21-23 nucleotide RNA known as small interfering RNA (siRNA). In Drosophila, the siRNA is derived from dsRNA by the action of an RNaseIII-like enzyme named DICER (Bernstein, Caudy et al. 2001). The siRNA guides the formation of a multi-subunit endonuclease, referred to as the RNA-induced silencing complex (RISC), and RISC

directs the degradation of target mRNA. The regulation of siRNA is very specific and the regulated mRNA often shares sequence similarity with the inducing dsRNA (Hammond, Bernstein et al. 2000; Zamore, Tuschl et al. 2000).

For in vitro study, RNA intereference can be induced in mammalian cells by the introduction of synthetic double-stranded small interfering RNA, which is 21-23 base pairs (bp) in length or by plasmid and viral vector systems that express double-stranded short hairpin RNAs that are subsequently processed to small intereference RNA by the cellular machinery.

Besides its extensive application in basic research, RNAi technology was also used to analyze quickly the function of a number of genes in a variety of organisms. For instance, in *D. melanogaster*, RNAi technology has been successfully used to identify genes with essential roles in signaling transduction cascades, embryonic development, and other basic cellular process (Li, Clemens et al. 2000). Given the gene-specific features of RNAi, this technology will play an important role in disease therapeutic applications. Since siRNA targets cellular mRNA, they are potential therapeutic reagents because of their power to down-regulate the expression pattern of mutant genes in diseased cells. Recently, the therapeutic potential of the siRNA technique has been demonstrated in vivo in mouse models. The hepatitis C virus and the fas gene can be effectively knocked down by RNA interference in mouse liver (McCaffrey, Meuse et al. 2002; Song, Lee et al. 2003). Undoubtedly, the RNA interference technology will become a useful tool in drug metabolism enzyme study. In recent years, RNA interference was widely used in nuclear receptor transcriptional regulation studies (Marquez, Chen et al. 2005; Rigamonti, Helin et al. 2005; Sola, Amaral et al. 2005). In

our experiment we transfected chemically sythesised CAR specific siRNA, we found that after the CAR activity was knocked down by siRNA, the expression of hSULT2A1 in Caco-2 cells signicantly decreased. This indicates that CAR is involved in the transcriptional regulation of hSULT2A1. Our results indicate that siRNA is a useful tool in investigating the transcriptional regulation of human sulfotransferase.

1.6 Significance of the Research

SULTs are important in the regulation of biological signaling molecules. They also play important roles in xenobiotic detoxification and carcinogen bioactivation. Knowledge of hSULTs induction mechanisms could have a significant impact on cancer prevention, toxicology, drug design and development, drug-drug interaction, food safety, and general human health. Induction of hSULTs during cancer treatment can lead to drug resistance as well as increase the bioactivation of procarcinogens and promutagens. An understanding of xenobiotic induction of hSULTs may provide clinicians with a measure to evaluate a patient's ability to tolerate drugs that are metabolized by SULTs. The research results will lead to a better understanding in SULTs' biological functions and their potential roles in cancer prevention and causation.

Chapter II

Hypothesis and Objectives

2.1 Hypothesis

Drug metabolizing enzymes can be induced by endogenous and xenobiotic compounds. The induction mechanism involves different nuclear receptors interacing with DNA *cis*-elements located in the proximal promoter region of the drug metabolizing enzymes. In this project, we want to test if the widely used anti-folate drug MTX can induce the expression of hSULTs. Through endougenous induction study, we found MTX can induce the four major families of hSULTs in both Caco-2 and Hep G2 cells, and then we start to investigate the MTX induction mechanism. Because hSULTs belong to phase II drug metabolizing enzymes, we hypothesize the MTX induction mechanim of hSULTs is similar to the induction mechanism of other drug metabolizing enzymes. We propose some nuclear receptors are involved in the transcriptional regulation of hSULTs. Among these nuclear receptors, RXRα function as a common heterodimer parterner, it interacts with other nuclear receptors and mediates the transcriptional regulation of hSULTs. We hypothesize that some DNA *cis*-elements located in the proximal promoter region of hSULTs also play very important function in hSULT regulation. These DNA *cis*-elements interact with nuclear receptors and regulate the gene expression of hSULTs. So the overall goal of our research is to find out the nuclear receptors and DNA *cis*-elements involved in the transcriptional regulation of hSULTs.

The following diagram describes the system we are investigating. hSULTs are the target gene in our study. We want to investigate the transcriptional regulation of these genes. We use human cell line Hep G2 and Caco-2 cells as models, we first directly treated Hep G2 and Caco-2 cells with MTX, we found MTX can induce the expression of hSULTs in both Hep G2 and Caco-2 cells, and then we start to investigate the MTX induction mechsniam of hSULTs. We hyphthesize the regulation of hSULTs are mediated through the interation of nuclear receptors with DNA-*cis* element located in the proximal promoter region of hSULTs. So the major task of this study is to find out the nuclear receptors and the DNA *cis*-element involved in hSULT regulation.

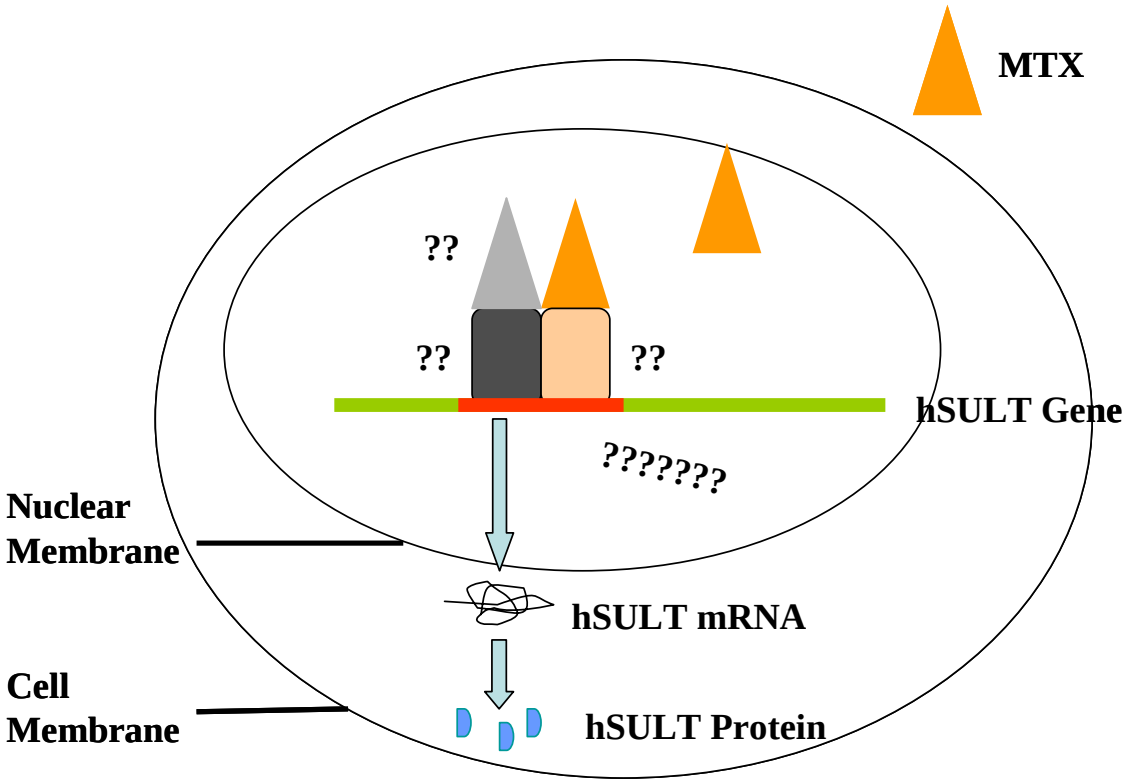


Figure 2.1.1 System may involved in MTX induction of hSULTs.

The model system in this diagram is the human Hep G2 cells and human Caco-2 cells. The ourter circle in the figure represents the cell membrane; the inner circle in the figure represents the nuclear membrane. The target gene in this system is the hSULT. When the cells were treated with MTX, the expression of hSULT, including both the mRNA expression and protein expression, was up-regulated by MTX. We hyphothesized the MTX induction is mediated through the interaction of nuclear receptors with the DNA *cis*-element located in the proximal promoter region of hSULT gene.

2.2 Experimental Objectives

In our studies, we use human Hep G2 and human Caco-2 cell lines as model to investigate the hSULT induction mechanism because human cell lines are easy to manipulate and the experiment is easy to repeat. Hep G2 cells are derived from human liver and Caco-2 cells are derived from human colon. It was believed that human liver and human intestine are the two major organs that involved in drug metabolism. The aboundance of hSULTs in both Hep G2 and Caco-2 cells are very high, it is easy to detect the expression of hSULTs through RT-PCR and western blot methods. To investigate the effect of MTX to hSULTs, we directly treated Hep G2 and Caco-2 cells with different concentrations of MTX for different time, and then we harvested the MTX treated cells and detected the mRNA and protein level of hSULTs through RT-PCR and western blot. With hSULTs catalyzed substrate specific sulfation reaction, we can also detect the enzymatic activities of hSULTs. Interestingly, MTX can significantly induce most tested hSULTs in both Hep G2 and Caco-2 cells. Then we start to investigate the MTX induction mechanism of hSULTs.

To investigate the MTX induction mechanism of hSULTs, we cloned the promoter sequences of hSULTs through PCR with human genomic DNA extracted from Hep G2 cells. The cloned promoter sequences were ligated to the pGL3-Basic luciferase reporter vector so that the expression of the luciferase was regulated by the promoter sequences of hSULTs. The constructed reporter vectors were used in reporter gene assay and the cloned promter sequence was tested for their promoter activities. The biological functional reporter vectors were used in dual luciferase assay for MTX time- and concentration-dependent induction study. So the MTX induction of hSULTs was

confirmed by both endogenous study and reporter gene assay. Further experiments were designed to figure out the MTX induction mechanism of hSULTs.

It is well know that high concentration of MTX can cause cytotoxicity to human cells. To test if the MTX induction of hSULTs was caused by MTX cytotoxicity, we did MTT assay to test the MTX cytotoxicity to Hep G2 and Caco-2 cells.The concentrations of MTX used in MTT assay are the same as dual luciferase assay. Through comparing the MTX cytotoxicity pattern with the MTX induction pattern, we know the MTX induction of hSULTs was not caused by it cytotoxicity. So there must be other induction mechanisms involved in MTX induction of hSULTs, we hypothesize the induction was mediated through the interaction of nuclear receptors with DNA *cis*-elements located in the proximal promoter region of hSULTs.

To find the nuclear receptors involved in the transcriptional regulation of hSULTs, we cotransfected different nuclear receptors together with the reporter vector which contain the 5' flanking region of hSULT promoters. If some nuclear receptors are involved in the regulation of hSULTs, the cotransfection of these nuclear receptors will be able to change the activity of the reporter gene which is regulated by the promoter sequence of hSULT. After we find some nuclear receptors can regulate the promoter activities of hSULTs, we can design other independent experiments to further prove the role of these nuclear receptors involved in hSULT regulation. For example, we can transfect siRNA specific to these nuclear receptors and then detect the expression of hSULTs. Most nuclear receptors are ligand dependent, so we can also use specific ligand of nuclear receptors to test the function of nuclear receptors.

The promoter sequences we cloned is at least more than 800bp upstream of the transcription start site of hSULTs, while the DNA *cis*-element that can interact with nuclear receptors are less than 20bp, so we need to narrow down the promoter sequences to a small range. For this purpose, we can use the step wise promoter deletion to make the promoter sequences become short. After we locate the DNA *cis*-element to a relative small range of promoter sequence, we can do DNA sequence alignment, by comparing our promoter sequences with other characterized DNA *cis*-element, we can find some candidates. With the DNA mutation experiment, we can test the function of the selected DNA *cis*-element. If some DNA *cis*-element is very important in the MTX induction of hSULT, the mutation of this sequence will be able to abolish the MTX and nuclear receptors mediated hSULT induction.

After we find both the nuclear receptors and the DNA *cis*-elements involved in hSULT regulation, we can use the electrophoretic mobility shift assay (EMSA) to test the interaction between nuclear receptors and DNA *cis*-elements. The nuclear extract from Caco-2 and Hep G2 cells can be used as protein sourse in the EMSA assay. Throug the EMSA assay, the specificity of the DNA *cis*-element can be confirmed. To verify the specificity of the nuclear receptors, super shift assay can be carried out and find out the specific nuclear receptors that interact with the DNA *cis*-elements.

It was reported that nuclear receptors can interact with each other and cross-talk with different transcription factors and DNA *cis*-elements. If we find several nuclear receptors can regulate the expression of hSULTs, we want to see if there is cross-talk between these nuclear receptors. To test the cross-talk between different nuclear receptors, we can cotransfect different nuclear receptors together with reporter vectors

into Hep G2 or Caco-2 cells. If several nuclear receptors can all bind to the verified DNA *cis*-elements, the cotransfections of these nuclear receptors will interefere with each other. These transfected nuclear receptors can compete with the same DNA *cis*-element; they can also compete for the limited amount of common heterodimer parterner like RXRα.

Chapter III

Materials and Methods

3.1 Materials

MTX was obtained from ICN Pharmaceuticals (Aurora, Ohio). [1,2,6,7-^{3}H(*N*)] Dehydroepiandrosterone ([^{3}H] DHEA, 60 Ci/mmol) and [2,4,6,7-^{3}H(*N*)]estradiol ([^{3}H]E_2, 72 Ci/mmol) were purchased from NEN (Boston, MA). β-naphthol, [^{14}C]2-naphthol (4.7 mCi/mmol), *p*-nitro-phenyl sulfate (PNPS), 3-(4 to 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 9-*cis*-Retinoic acid, and 3′-phosphoadenosine-5'-phosphosulfate (PAPS) were purchased from Sigma-Aldrich (St. Louis, MO). SDS-polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Western blot chemiluminescence reagent kits (Super Signal West Pico Stable Peroxide and Super Signal West Pico Luminol/Enhancer solutions) were purchased from Pierce Chemical (Rockford, IL). Nitrocellulose membrane (Immobilon-P; Millipore Corporation, Bedford, MA) used in Western blot procedure was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Antibodies against hSULT1A3, SULT2A1 and 1E1 were supplied by Panvera. Antibody against hSULT1A1 was a gift from Dr. Charles N. Falany's laboratory of the University of Alabama at Birmingham. All other reagents and chemicals were of the highest analytical grade available.

DNA restriction enzymes, Wizard® SV Genomic DNA Purification System and Access RT-PCR System were purchased from Promega (Madison, WI). One Shot® Top 10 competent cells and Lipofectamine™ 2000 were from Invitrogen (Carlsbad, CA). Cells culture medium and 0.25% trpsin-EDTA was from Sigma. The characterized FBS and charcoal stripped FBS were purchased from Hyclone. The plasmid extraction kit and total RNA extraction kit were from QIAGEN (Valencia, CA). The DNA gel purification kit was from Q-Biogene (Carlsbad, CA). Protein assay reagent was from Bio-Rad (Hercules, CA). The DIG Gel Shift Kit, 2nd Generation (Cat. No. 03353591910) was from Roche Applied Science. Retinoid X receptor (RXRα) plasmid was from Dr. Ronald M. Evans's laboratory (Howard Hughes Medical Institute, La Jolla, CA). CAR plasmid was from Dr. Steven A Kliewer's laboratory (University of Texas Southwestern Medical Center, Dallas, TX).

3.2 Cell Culture and Drug Treatment

Both Hep G2 and Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). Hep G2 Cells were grown and maintained in Dulbecco's modified eagles's medium nutrient mixture F-12 ham (Sigma) supplemented with 10% fetal bovine serum (FBS). Caco-2 cells were grown and maintained in Dulbecco's modified eagles's medium (Sigma) supplemented with 20% fetal bovine serum (FBS). The cultures were incubated at 37°C in a humidified incubator containing 5% CO_2, 95% air. MTX dissolved in 10 mM sodium hydroxide was added to the cells with a final concentration as required and incubated for different time as indicated. The vehicle control group received only 10 mM NaOH so as the final concentration of NaOH

is 0.2 mM. Folic acid was dissolved in the culture medium directly. Retinoic acid and vitamin D3 was dissolved in ethanol. CITCO was dissolved in dimethyl sulfoxide (DMSO). To avoid the ethanol and DMSO cause significant effect to human sulfotransferase, all vehicle concentration added to cells was kept at 0.1% (V/V) with final concentration. Cells were collected after induction. Total RNA was extracted for RT-PCR and cytosol was prepared for enzymatic assay and Western blot.

3.3 Cytosol Preparation

Both Hep G2 and Caco-2 cells were detached from their culture dishes using 0.25% trypsin- EDTA solution (Sigma) and washed with phosphate buffered saline. The cells pallet was dissolved and homogenized in 300 µl buffer [NaCl: 150 mM, Tris: 20 mM, EDTA: 1 mM, DTT: 1 mM, Trypsin Inhibitor: 0.1 mg/ml, Tween-20: 0.3% (V/V), PMSF: 1 mM]. The debris was removed by centrifugation at 13,000 RPM for 10 min and the supernatant was used in the following enzymatic assay and western blot.

3. 4 Enzyme Assays

3.4.1 PNPS Assay

2-Naphthol sulfation activity from Hep G2 cells was determined as previously described (Chen, Battaglia et al. 1999; Chen, Zhang et al. 2003). This assay determines the activities of different isoforms of phenol sulfating SULTs. Briefly, sulfation activity was determined in a reaction mixture containing 50 mM Tris buffer, pH 6.2, 5 mM PNPS, 20 µM PAPS, and 0.1 mM 2-naphthol. Hep G2 cell cytosols (50 µg protein) were used as the enzyme source in a total reaction volume of 250 µl. After 30 min incubation

at 37 ° in a shaking water bath, the reaction was stopped by adding 250 µl of 0.25 M Tris, pH 8.7. The reaction mixtures were read at 401 nm in a spectrophotometer. Specific activity was expressed as nanomoles per minute per milligram of protein. The data shown in the figures are the average of three independent experiments.

3.4.2 Radioactive Assay

enzymatic activities of hSULT2A1, hSULT1E1, hSULT1A1 in the cytosol was determined using the radioactive assay method as previously described (Chen, Zhang et al. 2003; Maiti and Chen 2003; Maiti and Chen 2003). Two hundred micro-grams for Caco-2 cytosol, 50 µg for Hep G2 cytosol were used for each assay. Other ingredients and reaction conditions were the same as the PNPS assay mentioned above. Radioactive [^{3}H] estradiol (0.15 µM, 0.4 Ci/mmol for store concentration) and [^{3}H] DHEA (2 µM, 1.0 mCi/mmol for store concentration) were used as specific substrate for SULT1E1 and SULT2A1 assay, respectively. [^{14}C] 2-naphthol (0.1 mM, 4.7 mCi/mmol) was used for hSULT1A1 activity in Caco-2 cells. For all assays, 20 µM PAPS was used. All enzymatic reactions were performed in a total reaction volume of 250 µl. After 30 min incubation at 37 ° in a shaking water bath, the reaction was stopped by adding 250 µl of 0.25 M Tris, pH 8.7. Extraction was performed twice by addition of 0.5 ml of water-saturated chloroform. After the final extraction, 100 µl of aqueous phase was used for scintillation counting. Data from radioactive assay represent the average of three independent experiments. PAPS was eliminated from the controls of all assay methods. Assays were run in duplicate and the average of the results was used for enzyme activity calculations.

3.5 Western Blot Analysis

Cytosol protein from Hep G2 cells (10 μg) and Caco-2 cells (15 μg) was used in a 12% polyacrylamide gel in an electrophoresis system (Novex, San Diego, CA, USA). After running at 120 V, the protein bands were transferred overnight at 150 mA onto a PVDF membrane. All membranes were blocked in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20) containing 5% (w/v) dry milk for 1 hr on a shaker at room temperature. The membranes with proteins from Hep G2 and Caco-2 cells were incubated separately with anti-hSULT1A1, anti-hSULT1A3, anti-hSULT2A1, and anti-hSULT1E1 (1:2000) in TBST containing 5% (w/v) dry milk overnight at 4 °C. After incubation, all membranes were washed with TBST for 4×15 min and incubated in secondary antibody (horseradish peroxidase-conjugated Immuno-Pure goat anti-rabbit IgG; H+L) at 1:5000 dilutions in the same buffer for 2 hours. The membranes were washed with TBST for 4×15 min. Fluorescent bands were developed with 4 ml of substrate containing same volume of each Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico stable Peroxidase solution at room temperature for 5 min. The X-ray films were exposed to the membrane and then developed. Films were scanned and the densitometric analysis was performed in a Gel Documentation and Analysis System from Advanced American Biotechnology (AAB) and with AAB software (Fullerton, CA, USA).

3.6 Extraction of Total RNA and RT-PCR

RNeasy Mini protection kit from Qiagen was used for total RNA extraction. One Step RT-PCR kit from Promega was used for RT-PCR. The primer pairs were designed

using the software primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers were synthesized by IDT. Specific primers for different human SULTs are as follows: hSULT1A1, forward: 5'- GAT TCC CTC AGG GAT GGA -3', reverse: 5'- GTG TGC TGA ACC ACG AA -3'. hSULT1A3, forward: 5' -TGA GCC AGA TAC TGG ACA -3', reverse: 5'- CCG TAG GAC ACT TCT CCA -3'. hSULT2A1, forward: 5'- CCC CAA ATC AGG AAC AAA C -3', reverse: 5'- CCA GAA AAA ATA ACC AGA CAC C -3'. Human beta actin (for control), forward: 5'- GGC GGC AAC ACC ATG TAC CCT -3', reverse: 5'- AGG GGA GGG ACT CGT CAT ACT -3'. The specificity of all primers was tested using the BLAST of the National Center for Biotechnology Information Open Reading Frame software. cDNA synthesis from total 0.1 μg of Hep G2 cells total RNA and 0.2 μg of Caco-2 cells total RNA was performed in a 25 μL reaction mixture. The concentrations of the different ingredients in the PCR reaction were used following supplier's protocol. For the control, 200-bp cDNA of human β-actin was synthesized from the same amount of RNA from respective cells.

3.7 MTT Assay

Cell viability was tested using MTT (3-(4 to 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich) based on the capability of the mitochondrial succinate-tetrazolium reductase system to convert the yellow dye (MTT) to a blue formazan (Mosmann 1983). Following treatment with MTX, the cell culture medium was replaced with serum free medium and the cells were treated with MTT stock solution (5 mg/ml, 10 ul) and incubated at 37^{o}C. After 4 hours, 100 μL of dimethyl sulfoxide (Sigma-Aldrich) was added to lyse the cells and solubilize the formazan

reaction product. Thirty minutes later, the 96 well plates were read in a microplate reader (Wallace 1420 Victor 2, Perkin Elmer Inc, Boston, MA, USA) at 550 nm.

3.8 Construction and Mutagenesis of hSULT2A1 Promoter Reporter in Caco-2 Cells

Luciferase reporter constructs were used in the transfection studies. Primer designed for the hSULT2A1 promoter sequence was based on previously described (Otterness, Her et al. 1995; Duanmu, Locke et al. 2002). Briefly, a fragment containing the 5'-flanking region (-1463 to +48) of hSULT2A1 was generated by PCR using genomic DNA extracted from Hep G2 cells. The fragment was inserted into the luciferase reporter vector pGL3-Basic (Promega, Madison, WI) at the *MluI* and *XhoI* sites to drive the promoterless firefly luciferase gene.

Reporter plasmids containing nested deletions of the SULT2A1 5'- flanking region were all generated by PCR reactions. Specifically, constructs –713, -414, -354, -235, -188, -130 and –65 were generated by using a –1463 to +48 fragment of the hSULT2A1 gene as template. A series of 5' primers were designed to incorporate a *SacI* site for sub-cloning (5'- TTACATACACGTCAGCCATCAA - 3' for construct-713, 5' – TGTGGTCTTTTGGATTTGGAG - 3' for construct-414, 5'- GCACGATTGCAGGATTATTT - 3' for construct –354, 5'- TTGTCCTCGTGTTTGTTATTCG - 3' for construct –235, 5'- CAAGCTCAGATGACCCCTAAA - 3' for construct –188, 5'- CAATCTTTTGAGTATGG GTCACA - 3' for construct –130, and 5'- GTGACATGCTGGGACAAGG - 3' for construct -65). The 3' primers were designed with a *SmaI* site that was identical for all of the constructs (5'-

GCGTGGTGTGAGGGTTTC - 3'). These amplified fragments were initially ligated into the pUC19 vector and then cloned into the *SacI* and *SmaI* sites of the pGL3-Basic.

A site-directed mutagenesis construct (construct IR2-Mut) was prepared by using overlap PCR. In initial step of overlap PCR, the left arm of the PCR product was generated from the wild type template, using the same sense primer as deleted construct -414 and the antisense primer (5'-GCAAGCTCAGA**ACT**CCCCTAAAATGG-3') containing desired base changes corresponding to the CAR binding site of the hSULT2A1 promoter. Similarly, the right arm of the PCR product was generated using the sense primer (5'-CCATTTTAGGGG**AGT**TCTGAGCTT GC-3') containing the mutant oligo sequence. The antisense primer was the same as deleted construct -414. Amplified DNAs were gel-purified, and construct -414 sense and antisense primers were used to splice the left arm and right arm DNA products by overlap PCR. The PCR product was initially ligated to pUC19 vector and then subcloned to the upstream of the luciferase gene in pGL3-Basic vector at *SacI* and *SmaI* sites. DNA sequencing at the Oklahoma State University core facility verified all constructs.

3.9 Transfections and Reporter Gene Assays in Hep G2 and Caco-2 Cells

Hep G2 and Caco-2 cells (ATCC, Manassas, VA) were seeded to 24 well plates one day before transfection. Cell culture medium was replaced with 5% charcoal stripped FBS medium just before transfection when the cells reach 70% confluence. Both Hep G2 and Caco-2 cells were seeded onto a 24-well plate at 2 x 10^5 cells/well and transfected after 16 h with 100 ng of reporter plasmid, 50 ng of nuclear receptor expression vectors, and 10 ng (50 ng for Hep G2 cells) of the pRL-TK plasmid (Promega, Madison, WI),

with 5% charcoal stripped FBS. The transfection agents were added to a total volume containing 98 μl of Opti-MEM and 2 μl of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). In the CAR and VDR competition experiment, the nuclear receptor VDR was fixed as 50 ng and the CAR was gradually increased to 50 ng. The pRL-TK plasmid, which expresses *Renilla* luciferase, was used as an internal standard for transfection assay. The pUC19 vector DNA was used as an empty vector to keep the total transfected DNA at a fixed value. CAR, VDR and RXRα nuclear receptor agonists were added with fresh 5% charcoal stripped FBS 6 hours after transfection. The final concentration of these agonists is: MTX, 0.1 μM; CITCO, 50 nM; VD3, 1 μM; 9 *cis*-retinoic acid, 1 μM; and 0.1% (V/V) ethanol. Cells were collected 48 h after transfection and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Each experiment was repeated three times and each test was performed in duplicate. Results are given as means $\pm$ S.E.

3.10 CAR RNA Interference in Caco-2 Cells

Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 20% fetal bovine serum (FBS) to about 40% confluence. Just before transfection, the cell culture medium was replaced with 5% charcoal stripped FBS which contain reduced level of hormone that might cause induction effect of target gene. The siRNA targeting hCAR (siRNA ID: 5535 and the siRNA negative control was chemically synthesized by Ambion (Austin, US). The transfection agents with a total volume of 300 μl of Opti-MEM and 3 μl of Lipofectamine™ 2000 were added to Caco-2 cells according to the manufacturer's instructions in 6-well plates containing 125 nM siRNA per well.

Forty eight hours after transfection, cells were harvested for RNA analysis. For dual luciferase assay, plasmid DNA was first transfected into Caco-2 cells and siRNA was transfected 6 hours later with refreshed 5% charcoal stripped FBS medium. Cells were collected 48 h after transfection and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase® Reporter Assay System.

3.11 Quantitative Real-Time PCR

Total RNA was prepared from MTX, CAR, or CAR interference RNA treated Caco-2 cells. Superscript II (Invitrogen) reverse transcriptase with 50 to 100 ng of total RNA was used to synthesize cDNA, and 1 μl of reverse-transcribed product served as the template in polymerase chain reactions. Real-time PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) following the manufacturer's instruction. Primers were designed with Primer Express as follow: ACTBF321: 5'-AGAAAATCTGGCACCACACC -3', ACTBR462: 5'-GGGGTGTTGAAGGTCTCAAA -3', GI,L5016088; hSULT2A1F163: 5'- TGAGTTCGTGATAAGGGATGAA -3', hSULT2A1R294: 5'- CAGATGGGCACAGATTGGAT -3', GI, 29540544; hCARF959: 5'- CTTCTCTCCTGACCGACCTG -3', hCARR1089: 5'-TCGCATACAGAAACCGATCC -3', GI: 32189358. Real-time PCR was performed on ABI PRISM 7500 (Applied Biosystems, Foster City, CA). Initially, regular PCR products were purified with GENECLEAN Turbo (Qbiogene, Carlsbad, CA) for constructing standard curves (10–10^8 copies). A standard curve was plotted with the threshold cycle (C_T) vs the logarithmic value of the gene copy number. The gene copy number of

unknown samples was generated directly from the standard curve by the software Sequence Detector 1.7. At least two repeats were run for each sample; each experiment was repeated 3 times. All gene copy numbers were normalized to human beta actin mRNA.

3.12 Electrophoretic Mobility Shift Assay (EMSA) and Super Shift Assay

EMSA was performed using digoxigenin-11-ddUTP labeled oligonucleotides which contain the IR2 element DF-191: 5'-GGAACGCAAGCTCAGATGACCCCTAAAATGG -3' or DF-191m: 5' – GGAACGCAAGCTCAGAACTCCCCTAAAATGG -3' (mutated bases underlined). A standard gel shift binding reaction (20 μl) contained 20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM $(NH4)_2$ $S0_4$, 1 mM DTT, 0.2 % (v/v)Tween 20, , 30 mM KCl, lμg poly [d(I-C)], 1 μg poly L-lysine and 5 μg Caco-2 nuclear extract. Reactions were incubated at room temperature for 20 min after the addition of double stranded oligonucleotide probe (0.4 ng). Competitions were performed with 125-fold molar excess of unlabeled oligonucleotides. The nuclear extract was pre-incubated with the antibody at room temperature for 20 min before addition of the DNA probe in the supershfit assay. Antibodies to MB67 (C-20): sc-8541 and RXRα (D-20): sc-553 X were from Santa Cruz Biotechnology (Santa Cruz, CA). The protein-DNA complexes were resolved on a pre-electrophoresed 5% native polyacrylamide gel in 0.5× TBE (45 mM Tris borate, 1 mM EDTA) at room temperature and then blotted to a positively charged nylon membrane. The DIG labeled oligonucleotides were visualized by an enzyme immunoassay using

anti-Digoxigenin-AP, Fab-fragments and the chemiluminescent substrate CSPD. The generated chemilunimescent signals are recorded on X-ray film.

3.13 Statistical Analysis

Sulfotransferase activity and luciferase activity were expressed as median ± SE (standard error). MTX and nuclear receptors effects on hSULT2A1 in reporter gene assay were analyzed for significance by one-way ANOVA (Dunnett's analysis for all treatments with control). MTX time- and concentration-dependent cytotoxicity to Hep G2 and Caco-2 cells and MTX time- and concentration-dependent inducing ability to hSULT2A1 promoter activity in Caco-2 cells were analyzed by two-way ANOVA. All statistical analyses were done with the SAS statistical computer software (SAS 9.1). In all cases, *, $P< 0.05$ was considered significant; **, $P< 0.01$ was considered very significant.

Chapter IV

Results

4.1 MTX Induction of hSULTs in Hep G2 and Caco-2 Cells

4.1.1 MTX Induction of hSULT1A1 in Hep G2 and Caco-2 Cells

The effect of MTX on hSULT1A1 was evaluated by enzyme assay, western blot, and RT-PCR. Figure 4.1.1 demonstrates that MTX can induce hSULT1A1 at both the protein and mRNA level in Hep G2 cells. MTX induction of hSULT1A1 was concentration-dependent. 2-Naphthol sulfation activity significantly increased in response to low doses of MTX treatment (Figure 4.1.1A). The hSULT1A1 induction reached the maximum level when MTX concentration was 0.2 μM, followed by a decreased induction with increasing MTX concentrtion. Treatment of Hep G2 cells for 10 days with 0.2 μM MTX increased the sulfation activity up to 2.5 fold. In Hep G2 cells, the Western blot (Fig 4.1.1B) and RT-PCR (Figure 4.1.1C) results agree with enzyme activity assays. In contrast, MTX did not alter hSULT1A1 expression in Caco-2 cells as indicated by enzyme assay, Western blot, and RT-PCR (Figure 4.1.1D, 4.1.1E and 4.1.1F).

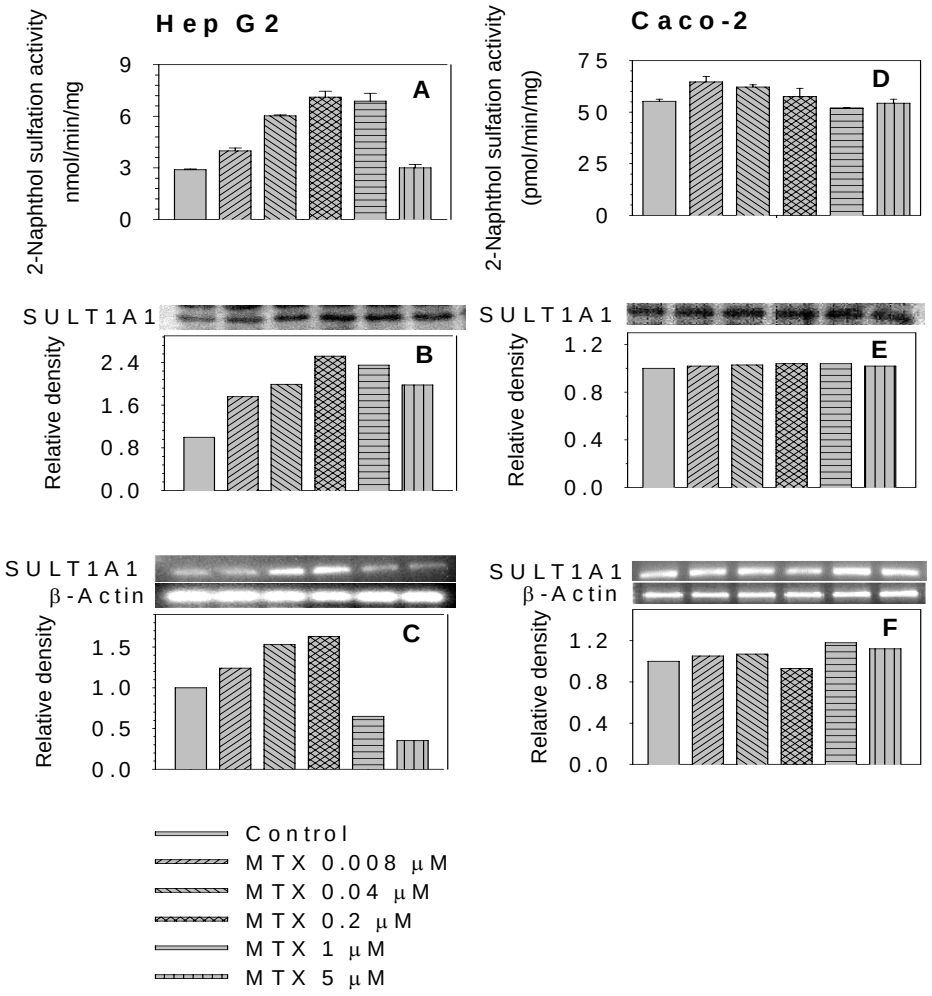


Figure 4.1.1 Effects of MTX treatment on hSULT1A1 activity in Hep G2 cells and Caco-2 cells

For A and D: Specific activity (SA) was expressed as nanomoles or picomoles per minute per milligram of protein. For B and E: each column of the densitometry corresponds to each lane of Western blot. For C and F: each column of the densitometry corresponds to each lane of RT-PCR, human β-actin was used as the internal control for RT-PCR. Values of densitometry analysis (for both Western blot and RT-PCR) were divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.1.2 MTX Induction of hSULT1A3 in Hep G2 and Caco-2 Cells

hSULT1A3 was induced in both Hep G2 cells and Caco-2 cells after treatment with MTX. Western blot results of Hep G2 cells showed that MTX induction of hSULT1A3 increased with MTX concentrations up to 0.2 μM followed by a decreased induction with increasing MTX concentration (Figure 4.1.2A). The RT-PCR (Figure 4.1.2B) results agree with Western blot in Hep G2 cells. Different from Hep G2 cells, MTX induction of hSULT1A3 in Caco-2 cells increased with increasing concentration of MTX up to 5 μM. Western blot results agree with RT-PCR results, indicating that the induction is at the transcriptional level (Figure 4.1.2C and 4.1.2D).

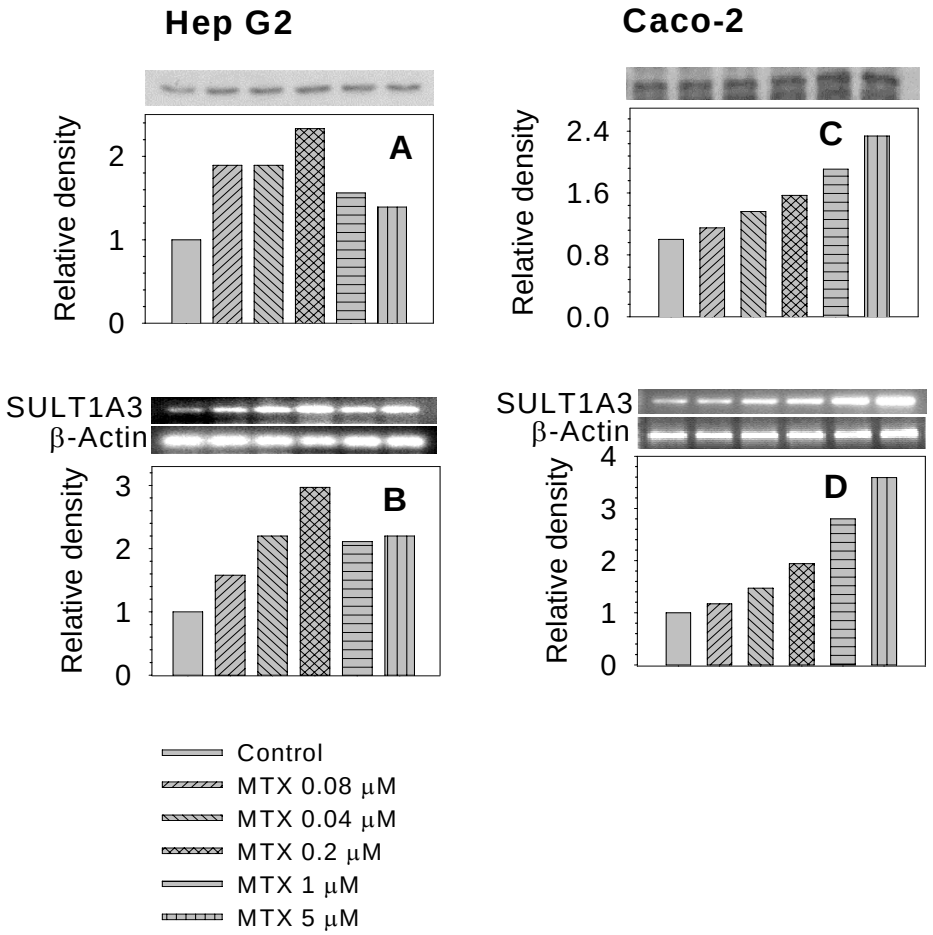


Figure 4.1.2 Effects of MTX treatment on hSULT1A3 activity in Hep G2 cells and Caco-2 cells.

For A and C: each column of the densitometry corresponds to each lane of Western blot.

For B and D: each column of the densitometry corresponds to each lane of RT-PCR, human β-actin was used as the internal control for RT-PCR.

Values of densitometry analysis (for both Western blot and RT-PCR) were divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.1.3 MTX Induction of hSULT2A1 in Hep G2 and Caco-2 Cells

hSULT2A1 was also induced in both Hep G2 and Caco-2 cells after treatment with different concentration of MTX. MTX induces hSULT2A1 in both Hep G2 and Caco-2 cells in a concentration-dependent manner (Figure 4 A, B, D, and E). DHEA sulfation activity in Hep G2 cells increased by 1.7-fold and hSULT2A1 mRNA content increased by 3.6-fold after treatment with 0.2 μM MTX. In Caco-2 cells, DHEA sulfation activity increased 2-fold and hSULT2A1 mRNA increased 1.9-fold after treatment with 1 μM MTX. For both Hep G2 and Caco-2 cells, enzyme assay, Western blot, and RT-PCR results are all in agreement. MTX induction of hSULT2A1 is at the transcriptional level (Figure 4 C and F).

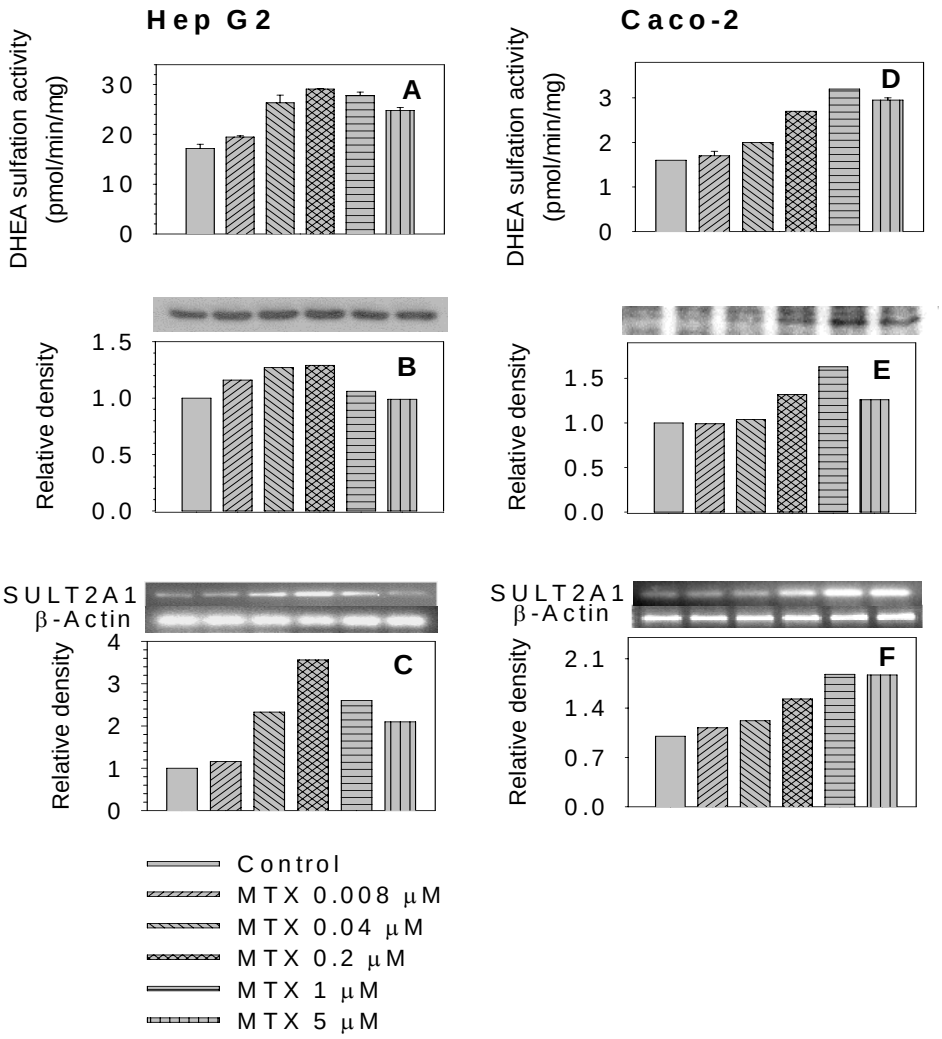


Figure 4.1.3 Effects of MTX treatment on hSULT2A1 activity in Hep G2 cells and Caco-2 cells

For A and D: Specific activity (SA) was expressed as picomoles per minute per milligram of protein. For B and E: each column of the densitometry corresponds to each lane of Western blot. For C and F: each column of the densitometry corresponds to each lane of RT-PCR, human β-actin was used as control for RT-PCR.

Values of densitometry analysis (for both Western blot and RT-PCR) were divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.1.4 MTX Induction of hSULT1E1 in Hep G2 and Caco-2 Cells

MTX effect to hSULT1E1 was evaluated in both Hep G2 and Caco-2 cells. Enzyme assay (Figure 4.1.4A) and Western blot (Figure 4.1.4B) results demonstrated that MTX induce hSULT1E1 in Hep G2 cells with maximum induction at 0.04 μM MTX. hSULT1E1 in Caco-2 cells was not induced by MTX (Figure 4.1.4C).

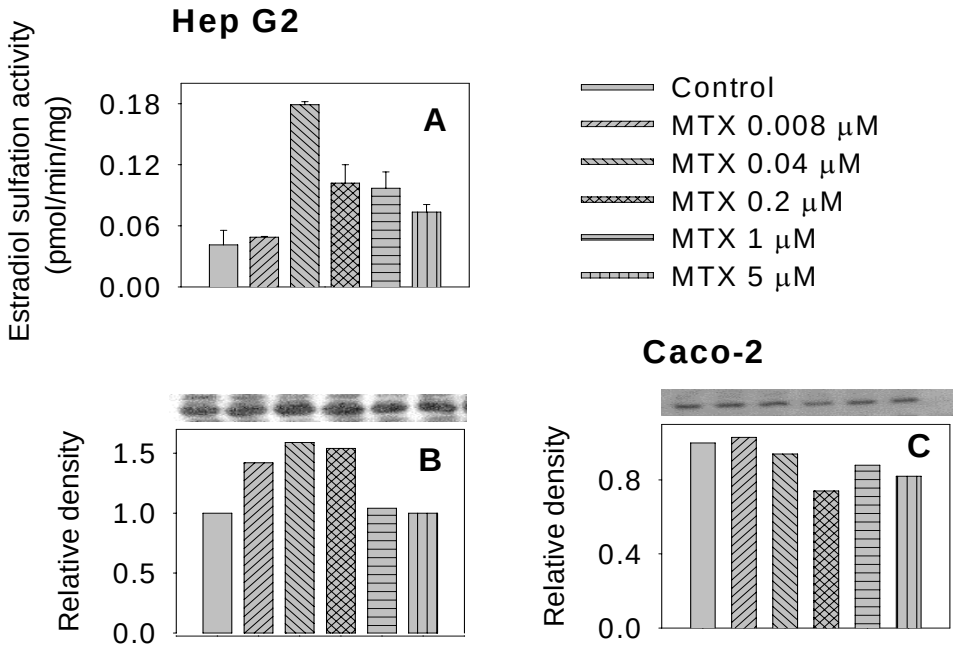


Figure 4.1.4 Effects of MTX treatment on hSULT1E1 activity in Hep G2 cells and Caco-2 cells

For A: Specific activity (SA) was expressed as picomoles per minute per milligram of protein. For B and C: each column of the densitometry corresponds to each lane of Western blot.

Values of densitometry analysis for both western blots were divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.1.5 Folic Acid Inhibition of MTX Induction of hSULT1A1 in Hep G2 Cells

MTX is a folate antagonist. In this investigation, folic acid was used to determine its effect on MTX induction of hSULTs. Our results suggested that MTX induction of hSULTs could be inhibited by high concentrations of folic acid. Figure 4.1.5 demonstrates that the induction of hSULT1A1 by MTX in Hep G2 cells can be inhibited by high concentrations of folic acid. Folic acid induction of hSULTs in Hep G2 is not significant. When incubated together with MTX, folic acid can suppress hSULT1A1 induction. The suppression was concentration-dependent. The MTX induction activity was only partially inhibited when the folic acid concentration was 100 μM. When the folic acid concentration reached 1000 μM, the induction of SULT1A1 was almost totally inhibited (Figure 4.1.5A). The results were supported by Western blot (Figure 4.1.5B) and RT-PCR (Figure 4.1.5C).

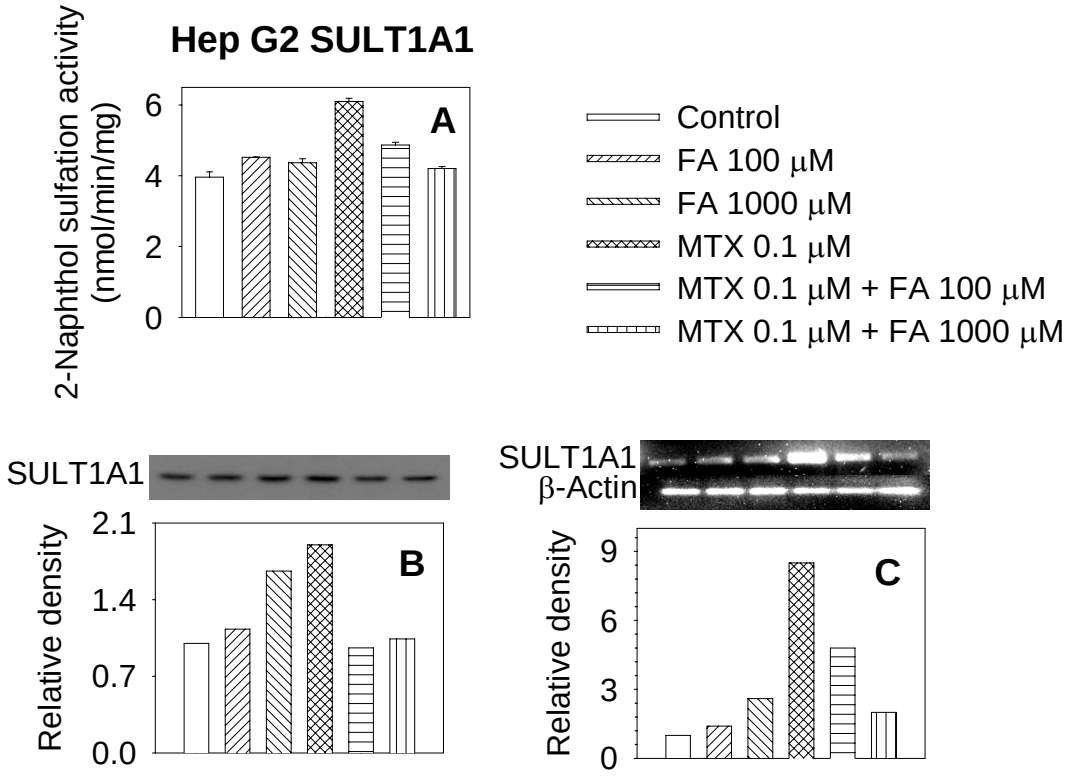


Figure 4.1.5 hSULT1A1 protein and mRNA analysis in folic acid and MTX treated Hep G2 cells

For A: Specific activity (SA) was expressed as nanomoles per minute per milligram of protein. For B: each column of the densitometry corresponds to each lane of Western blot. For C: each column of the densitometry corresponds to each lane of RT-PCR; human β-actin was used as the internal control for RT-PCR.

Values of densitometry analysis (for both Western blot and RT-PCR) were divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.1.6 Folic Acid Inhibition of MTX Induction of hSULT1A3 in Hep G2 Cells

MTX induction of hSULT1A3 in Hep G2 cells was inhibited by the addition of folic acid (Figure 4.1.6). Western blot results (Figure4.1.6A) showed that both 100 and 1000 μM folic acid completely suppressed hSULT1A3 induction in the presence of 0.1 μM MTX. RT-PCR results showed that high levels of folic acid increased hSULT1A3 mRNA expression (Figure 4.1.6B). When added together with MTX, folic acid always inhibited hSULT induction mediated by MTX.

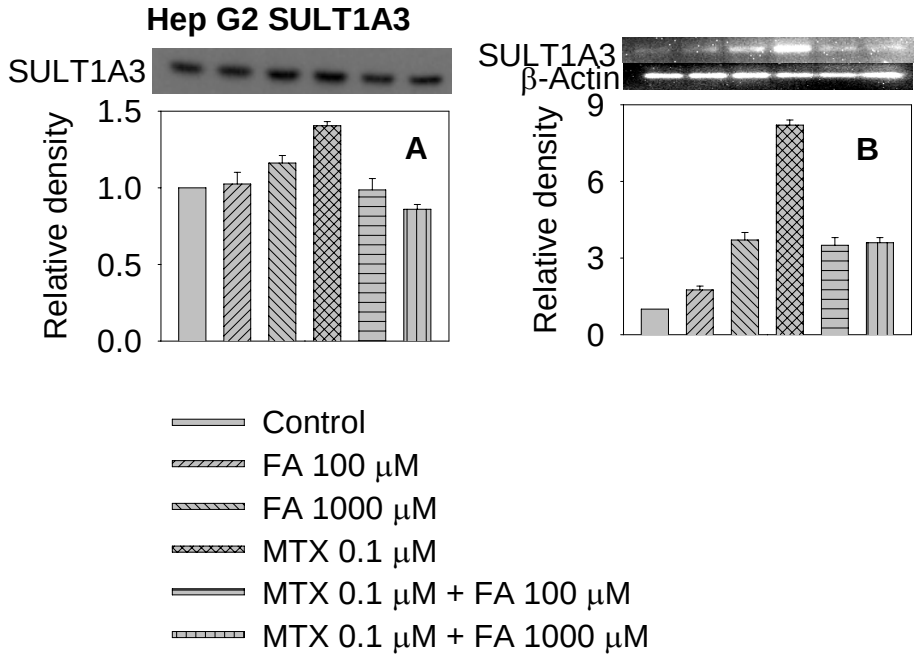


Figure 4.1.6 hSULT1A3 protein and mRNA analysis in folic acid and MTX treated Hep G2 cells

For A: each column of the densitometry corresponds to each lane of Western blot. For B: each column of the densitometry corresponds to each lane of RT-PCR; human β-actin was used as the control for RT-PCR.

Values of densitometry analysis (for both Western blot and RT-PCR) were divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.1.7 Folic Acid Inhibition of MTX Induction of hSULT2A1 in Hep G2 Cells

MTX induction of SULT2A1 was inhibited by 100 μM folic acid (Figure 4.1.7), whereas 1000 μM folic acid almost completely inhibited hSULT2A1 induction. Enzymatic assay results (Figure4.1. 7A) agree with Western blot results (Figure 4.1.7B). hSULT2A1 mRNA expression demonstrated by RT-PCR agrees with protein expression levels demonstrated by enzymatic assay and Western blot results (Figure 4.1.7C). This further demonstrates that MTX induction of hSULTs is at the transcriptional level.

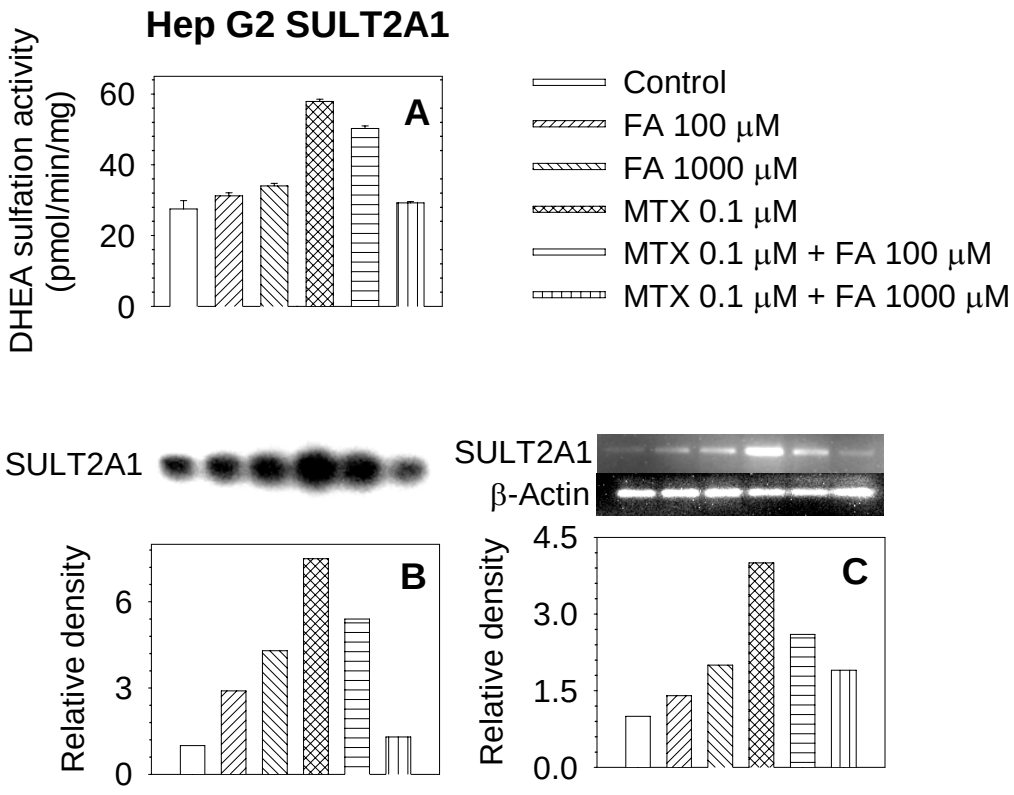


Figure 4.1.7 hSULT2A1 protein and mRNA analysis in folic acid and MTX treated Hep G2 cells.

For A: Specific activity was expressed as picomoles per minute per milligram of protein. For B: Each column of the densitometry corresponds to each lane of Western blot. For C: each column of the densitometry corresponds to each lane of RT-PCR; human β-actin was used as control for RT-PCR.

Values of densitometry analysis (for both Western blot and RT-PCR) were divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.1.8 Folic Acid Inhibition of MTX Induction of hSULT1E1 in Hep G2 Cells

MTX induction of hSULT1E1 was inhibited by folic acid (Figure 4.1.8). The inhibition of hSULT1E1 was also folic acid concentration-dependent. The Western blot results of hSULT1E1 in Hep G2 cells (Figure4.1.8B) agree with enzyme assay results (Figure4.1. 8A).

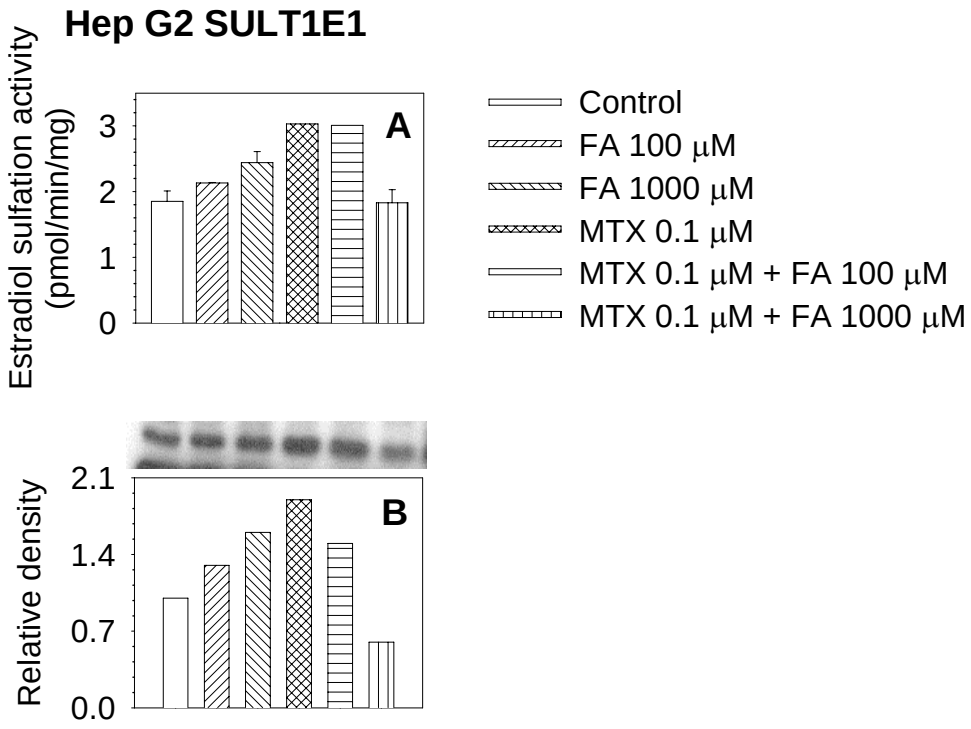


Figure 4.1.8 hSULT1E1 sulfation activity and western blot analysis in folic acid and MTX treated Hep G2 cells

For A: Specific activity (SA) was expressed as picomoles per minute per milligram of protein. For B: each column of the densitometry corresponds to each lane of Western blot.

Densitometry value of western blot was divided by the control value and the division factors were plotted. The control value was calculated as 1.

4.2 MTX Cytotoxicity

4.2.1 The Cytotoxicity of MTX to Caco-2 Cells

MTT assay was used to analyze the metabolic activity of drug treated cells based on the ability of mitochondrial succinate-tetrazolium reductase system to convert the yellow substrate to a blue formazan dye. Here, we use MTT assay to determine the cytotoxicity of MTX to Caco-2 and Hep G2 cells. Figure 4.2.1 shows the cytotoxicity of MTX to Caco-2 cells in a time- and concentration-dependent manner. At low concentration such as 0.02 μM, MTX did not show any significant cytotoxicity even when the Caco-2 cells were exposed to MTX for 8 days. At the highest concentration of 12.5 μM, MTX showed significant toxicity in as early as two days. MTX cytotoxicity increased with both concentration and time. In general, our data suggests that MTX is not obviously cytotoxic to Caco-2 cells below 0.1 μM within one week. The estimated IC50s to MTX for Caco-2 cells are: 2 days (34 μM), 4 days (17 μM), 6 days (11 μM), and 8 days (9 μM).

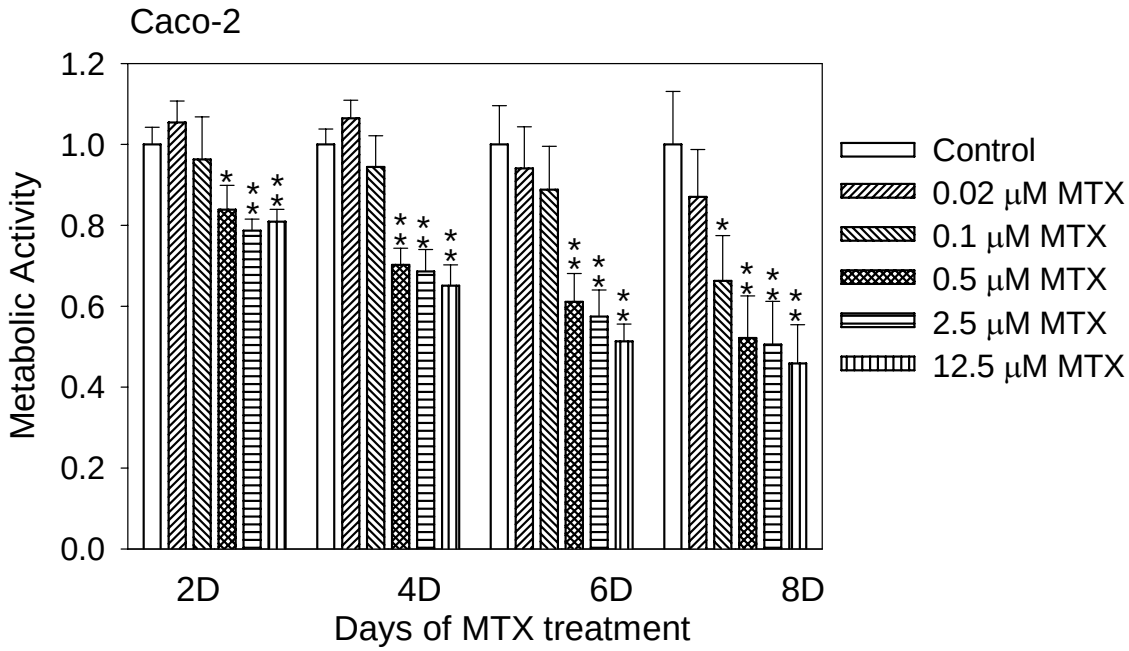


Figure 4.2.1 Time- and concentration-dependent cytotoxicity of MTX to Caco-2 cells Caco-2 cells were treated with different concentrations of MTX for 2, 4, 6 or 8 days. MTT assay was conducted to test the metabolic activity of the treated cells. The *y*-axs represents the metabolic activity of the cells. The OD value of the control cells (cells incubated with physiological saline solution) was taken as 1.0 and the ratio of the treated cells divided by the control represent the metabolic activity of the treated cells. Values less than 1 reflect reduced cell viability. The histograms with standard error are averaged values from four independent experiments; each treatment was done in quadruplicate. *, $p < 0.05$ and **, $p < 0.01$ compared with control in each defined time.

4.2.2 The Cytotoxicity of MTX to Hep G2 Cells

MTX cytotoxicity to Hep G2 cells (figure 4.2.2) is very similar to that of Caco-2 cells and is time- and concentration- dependent. The only difference is that Hep G2 cells are less sensitive to MTX. When exposed to MTX for just 2 days, the treated Hep G2 cells did not show significant decreased cell viability even with the highest concentration of 12.5 μM. In contrast, the Caco-2 cells showed significant cytotoxity when the MTX concentration reached 0.5 μM. Compared with Caco-2 cells, Hep G2 cells are more resistant to MTX. The estimated IC50s to MTX for Hep G2 cells at various exposure times are: 2 days (54 μM), 4 days (29 μM), 6 days (18.5 μM), and 8 days (16.5 μM). In our studies, MTX induction of hSULT at both mRNA and protein level in both Hep G2 and Caco-2 cells, and the induction reaches its maximum around 0.2 μM as described in part 3.1. MTX induction activity decreased when its concentration reached above 0.2 μM. These results suggest that MTX induction to hSULT was not caused by its cytotoxicity.

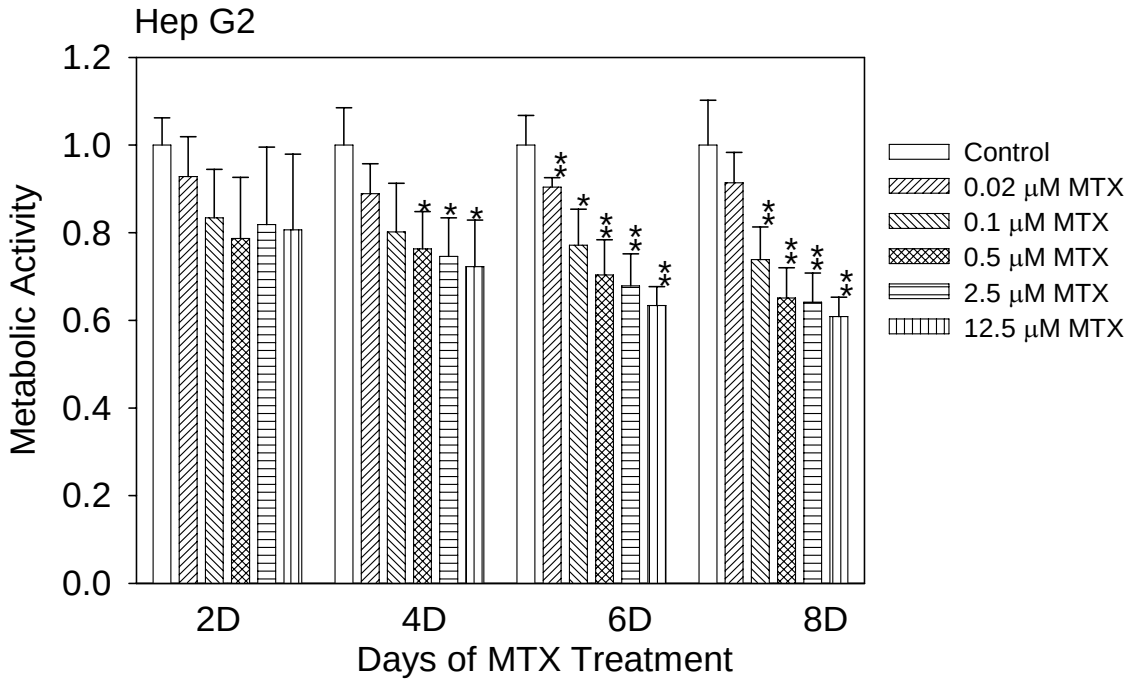


Figure 4.2.2 Time- and concentration-dependent cytotoxicity of MTX to Hep G2 cells Hep G2 cells were treated with different concentrations of MTX for 2, 4, 6, or 8 days. MTT assay was conducted to test the metabolic activity of the treated cells. The *y*-axes represents the metabolic activity of the cells. The OD value of the control cells (cells incubated with physiological saline solution) was taken as 1.0, and the ratio of the treated cells divided by the control represents the metabolic activity of the treated cells. Values less than 1 reflect reduced cell viability. The histograms with standard error are averaged values from four independent experiments. Each treatment group was done in quadruplicate, *, $p < 0.05$ and **, $p < 0.01$ compared with control in each defined time.

4.2.3 MTX Transactivation of hSULT2A1 Promoter Regulated Luciferase Expression

4.2.3.1 Construction of hSULT2A1 Promoter Reporter Vector

To explore the molecular mechanism of hSULT2A1 expression, hSULT2A1 5'-flanking region (-1463 to +48) was generated by PCR with gene specific primers (Otterness, Her et al. 1995; Duanmu, Locke et al. 2002) using genomic DNA extracted from Hep G2 cells. Our sequence of the PCR product was essentially the same as the published sequence (**L36191** and **U13056**) (Otterness, Her et al. 1995). The generated DNA sequence was inserted into pGL3-Basic vector (Promega) at the *MluI* and *XhoI* sites.

4.2.3.2 MTX Transactivation of hSULT2A1 Promoter Regulated Luciferase Expression in Caco-2 cells

To investigate the effect of MTX to the promoter activity of hSULT2A1, the constructed luciferase reporter vector containing hSULT2A1 promoter sequence was transfected into Caco-2 cells; the transfected Caco-2 cells were treated with different concentrations of MTX for different times. The data in figure 4.2.3 shows that MTX induce hSULT2A1 promoter activity in a time- and concentration-dependent manner. At a fixed concentration of MTX, the promoter activity increased with incubation time. At any fixed time (from 12 hours to 48 hours), the promoter activity increased when MTX concentration drop below 2.5 μM. The promoter activity starts to decrease when the MTX concentration reached between 2.5 and 12.5 μM. These results agree with our endogenous induction results as presented in section 4.1.

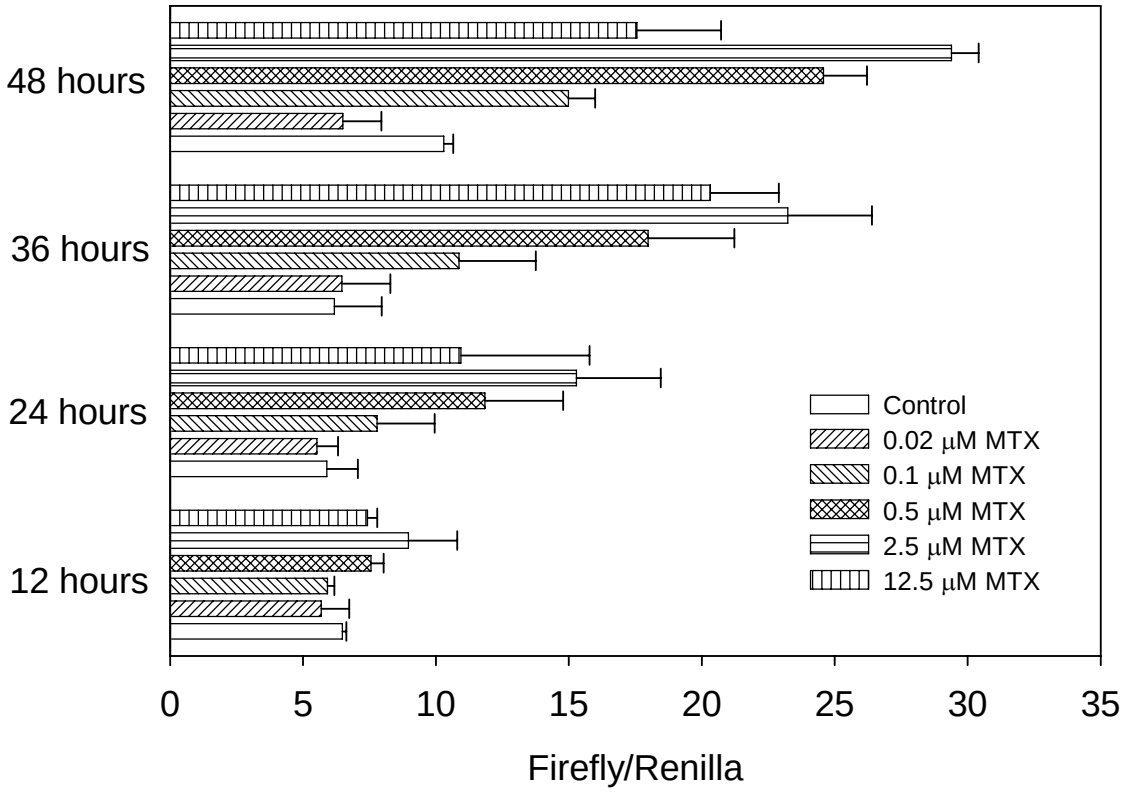


Figure 4.2.3 Time- and concentration-dependent induction of hSULT2A1 promoter activity by MTX in Caco-2 cells

Luciferase reporter gene regulated by 5'-flanking sequence of hSULT2A1 was transfected into caco-2 cells. 6 hours later, the transfection medium was replaced by serum free medium and treated with different concentration of MTX. pRL-TK (Promega) was used as the internal control for transfection assay. Dual-luciferase activities were measured after 12, 24, 36 or 48 hours of MTX treatment. The histograms with standard error are averaged values from three independent experiments; each test was done in duplicate. *, $p < 0.05$ and **, $p < 0.01$ compared with control in each defined time.

4.3 hSULT2A1 is transcriptionally Regulated by CAR

4.3.1 CAR Transactivation of hSULT2A1 Promoter in Caco-2 Cells

To explore the molecular mechanism involved in hSULT2A1 regulation, we cloned the promoter sequence of hSULT2A1 and inserted it into the pGL3-Basic vector. The constructed reporter vector was transfected into Caco-2 cells. CITCO (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime) is a well-known agonist of hCAR reported to induce human CYP2B6 in human primary cells (Maglich, Parks et al. 2003). Results shown in Figure 4.3.1 demonstrate that CITCO can also induce hSULT2A1 in Caco-2 cells. The induction activity of CITCO to hSULT2A1 is similar to that of MTX. Transfection of CAR or RXRα alone caused 2 fold activation of hSULT2A1 promoter. For both CITCO and MTX, when the drug treated cells were cotransfected with CAR, the luciferase activity increased 4 fold. Cotransfection of CAR and RXRα together with 9-*cis*-retinoic acid (9-*cis*-RA) and CITCO caused the activation of hSULT2A1 more than 8 fold. This strongly suggests that the CAR transactivation occurs via formation of a heterodimer with RXRα. This agrees with P-450s and other drug metabolizing enzymes induction mechanisms: most nuclear receptors, which can induce drug-metabolizing enzymes, form heterodimers with RXRα. When CITCO was replace by MTX, the promoter activity of hSULT2A1 was also significantly induced. This suggests that MTX can function as an agonist of CAR and induce the expression of hSULT2A1. All these results suggest a functional DNA *cis*-element is present in the cloned promoter sequence of hSULT2A1, and it can be activated by CAR in both ligand-dependent and ligand-independent manner.

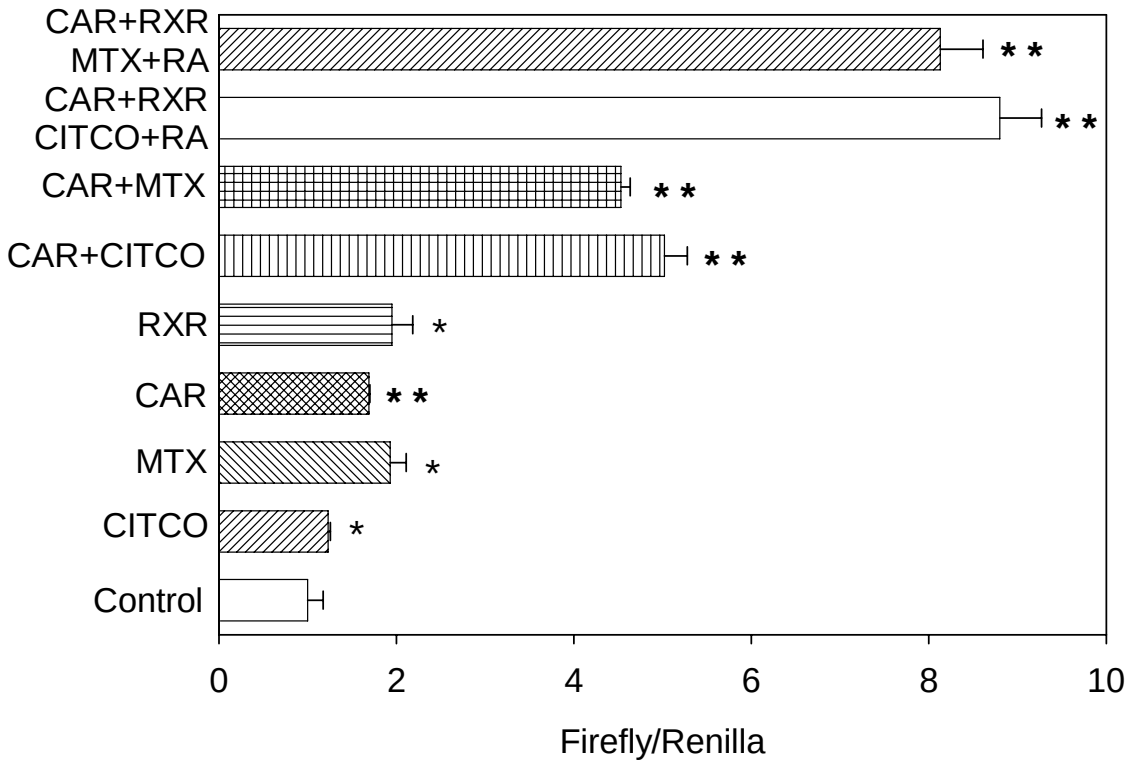


Figure 4.3.1 Effect of CAR and RXRα on CITCO and MTX Induction of hSULT2A1 promoter activity in Caco-2 cells

Luciferase construct containing 5'-flanking sequence of hSULT2A1 were transfected into Caco-2 cells together with hCAR and hRXRα. The transfected cells were treated with MTX, CITCO, retinoic acid and/or alcohol. pRL-TK was used as the internal control for the transfection assay. Fold inductions were calculated relative to the promoter activity in vehicle-treated cells. Concentrations used: CAR (25 ng), RXRα (25 ng), MTX (100 nM), CITCO (100 nM) and 9-*cis*-RA (1 μM). Dual-luciferase activities were measured according to the manufacturer's recommendations. The histograms with standard error are averaged values from three independent transfections; each independent transfection was performed in duplicate. *, $p < 0.05$; and **, $p < 0.01$ compared with vehicle (alcohol) control samples

4.3.2 The Expression of hSULT2A1 is Closely Related to the Expression of CAR

We designed the RNA intereference experiment to confirm the positive role of CAR in hSULT2A1 regulation. We employed CAR specific siRNA (provided by Ambion Inc.) in both reporter gene assay and endogenous study. The results showed in figure 4.3.2 demonstrate that CAR was a transcription factor involved in the up-regulation of hSULT2A1.

In the reporter gene assay experiment, we transfected the hSULT2A1 promoter regulated luciferase reporter vector into Caco-2 cells; the luciferase expression of control cells reflected the basal promoter activity of hSULT2A1. Upon the transfection of CAR specific siRNA, the luciferase expression reduced to approximately 50% (Figure 2A) compared with the control cells. This strongly suggests that CAR was involved in the transcriptional regulation of hSULT2A1. As previously discussed, the cotransfection of CAR can induce hSULT2A1 promoter activity 2 fold; this induction can also be partly knocked down by the CAR specific siRNA.

We use real-time PCR method to check the mRNA copy number of CAR and hSULT2A1 in Caco-2 cells. The results showed in figure 2B and figure 2C indicate that both the transfection and the RNA interference work well. The control cells show in figure 2B contain very low copy number of CAR. When the CAR expression vector was transfected, the mRNA copy number of CAR dramatically increased. The mRNA copy number of CAR decreased about 70% when the CAR transfected cells were treated with CAR specific siRNA. This indicates that the luciferase expression in figure 2A is closely related to the mRNA copy numbers of CAR. When the mRNA amount of CAR increased, the promoter activity of hSULT2A1 increased; when the mRNA amount of

CAR decreased, the promoter activity of hSULT2A1 decreased. Figure 2C shows the mRNA copy number of hSULT2A1 is closely related to the expression of CAR. When the mRNA amount of CAR increased through the transfection of CAR, the mRNA copy number of hSULT2A1 also increased. When the CAR mRNA level was knocked down by the siRNA, the hSULT21 expression level also decreased. These results strongly support that CAR is a transcription factor that can up-regulate the expression of hSULT2A1.

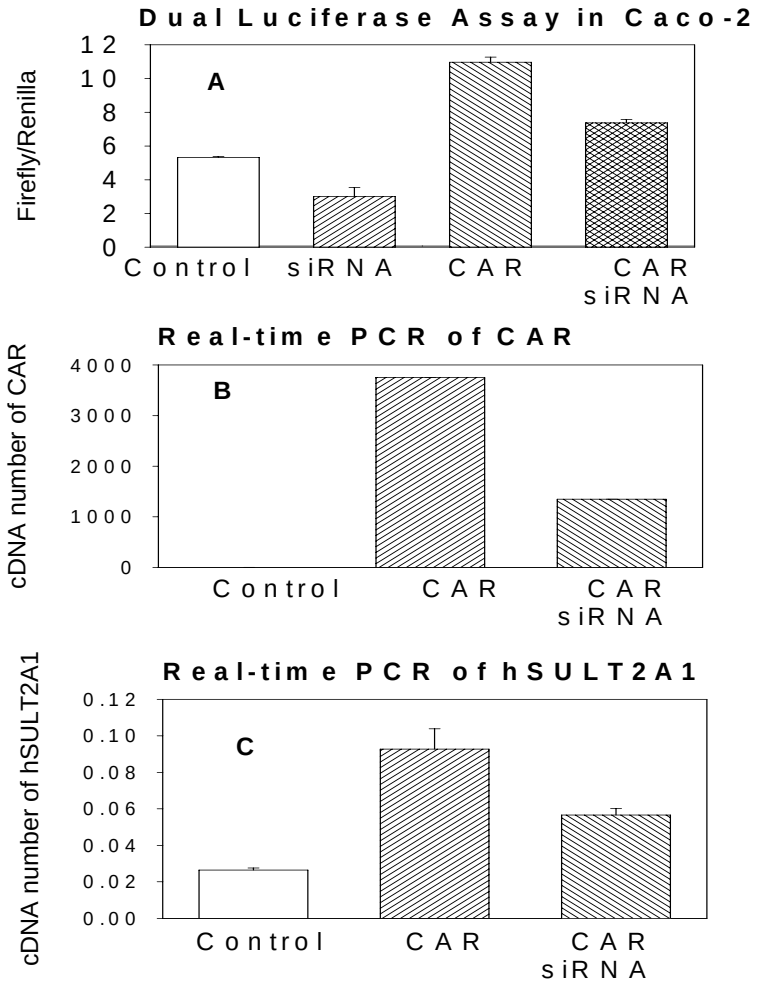


Figure 4.3.2 Effect of RNA interference of CAR on hSULT2A1 transcriptional activity. Figure A. The luciferase reporter vector regulated by the hSULT2A1 promoter was transfected into Caco-2 cells, and dual luciferase activity was measured according to manufacture's recommendation. Figure 4.3.2B and figure 4.3.2C, Caco-2 cells were transfected with CAR expression vector and CAR specific siRNA was transfected 6 hours later. The transfected cells were harvested after 48 hours and analyzed for real-time PCR with gene specific primers for human β-actin, CAR and hSULT2A1 as described in "Materials and Methods." Each treatment group was analyzed in triplicate and the data shown were averaged of three independent experiments.

4.3.3 DNA Response Element Responsible for CAR/RXRα Heterodimer Mediated Induction of hSULT2A1

To identify the DNA response element involved in hSULT2A1 regulation, functional promoter assay with progressively deleted 5' flanking region of hSULT2A1 promoter sequence was performed. Deletion analysis results are shown in Figure 4.4.3. The results suggest that the deletion between -1463 and -713 did not significantly change the promoter activity. Deletion from -731 to -414 decreased the basal promoter activity, but the promoter activity was still fairly high. Deletion between -414 and -188 (C-F) did not significantly change the promoter inducing ability caused by CAR and MTX. When the promoter sequence was deleted to 130 bp upstream of the transcription start site, the reporter vector almost completely lost its induction mediated by CAR and MTX. This suggests that the key element responsible for the promoter activity is located between -188 and -130, although the DNA *cis*-acting regulatory elements located between -1463 and -713 may also be necessary for the full responsiveness of CAR-mediated MTX induction of hSULT2A1.

Careful analysis of the DNA sequence (Figure 4.4. 4) between -188 and -130 revealed a hexameric core sequence AGCTCA between -186 and -181. Two base pairs downstream from this half-site show the imperfect inverted repeat sequence TGACCC (-178 to –173). This forms an imperfect inverted repeat motif IR2 (-186AGCTCAGATGACCC-173). This IR2 sequence may be the element binding site of the CAR/RXRα heterodimer.

Mutation results shown in Figure 4.4.5 demonstrate that this IR2 element is the sequence responsible for induction of hSULT2A1 via CAR/RXRα. Compared with the

control (Figure 4.3.5), the mutated 5'-flanking region of hSULT2A1 (-414 to +48) lost about 50% of its basal luciferase activity. When treated with MTX or CAR, the mutated promoter can not be activated by CAR or MTX. Interestingly even, the transfected CAR can cause the inhibition of the basal luciferase activity. This suggests that there may be other signal transduction pathways involved in hSUST2A1 induction.

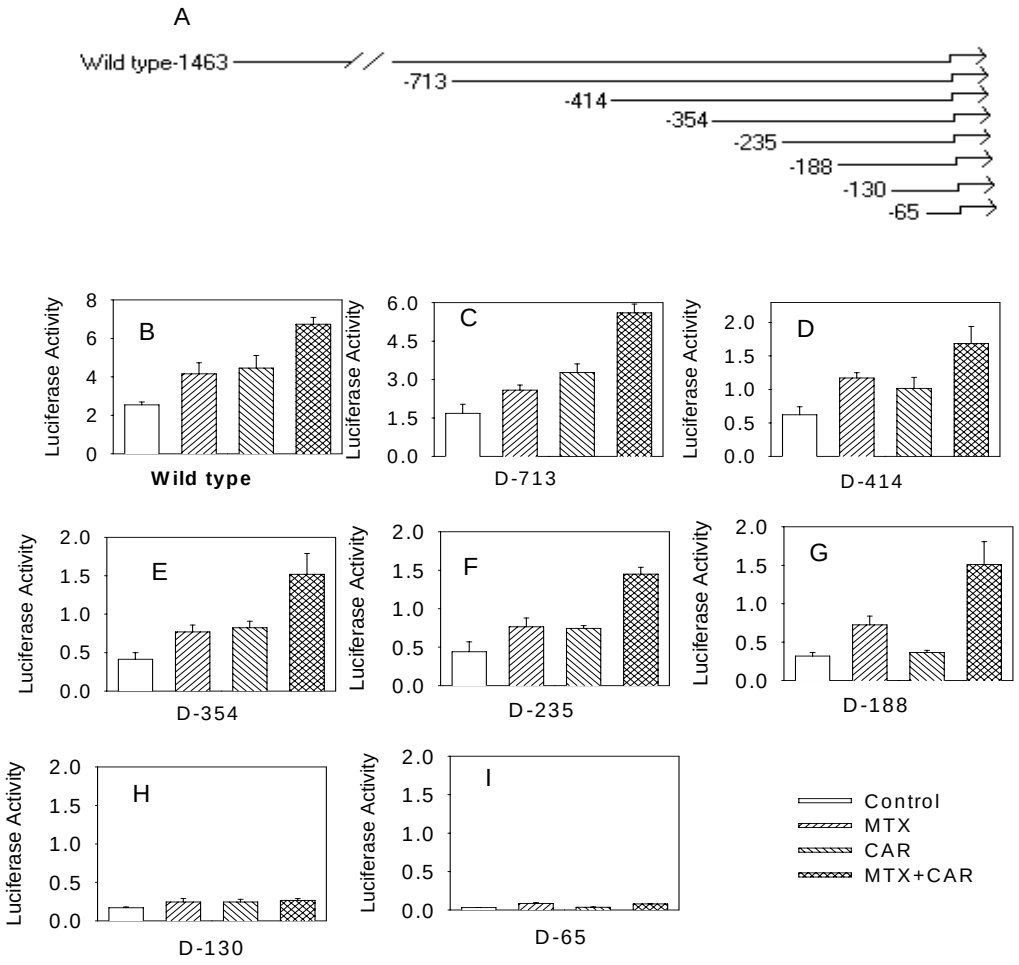


Figure 4.3.3 Deletion analysis of hSULT2A1 promoter

Figure A, schematic diagram of deleted hSULT2A1 promoter constructs. Figure B-I, transactivation of deleted promoter constructs by CAR and MTX. The constructed pGL3-Basic vectors include hSULT2A1 5'-flanking region from the number indicated in the figure A to +48. Transfected Caco-2 cells were treated with MTX for 48 h as indicated. 50 ng CAR was used for transfection. 100 nM MTX was used for treatment. Promoter activity is expressed as normalized luciferase activity. The histograms with standard error are averaged values from three independent transfections; each independent transfection was performed in duplicate.

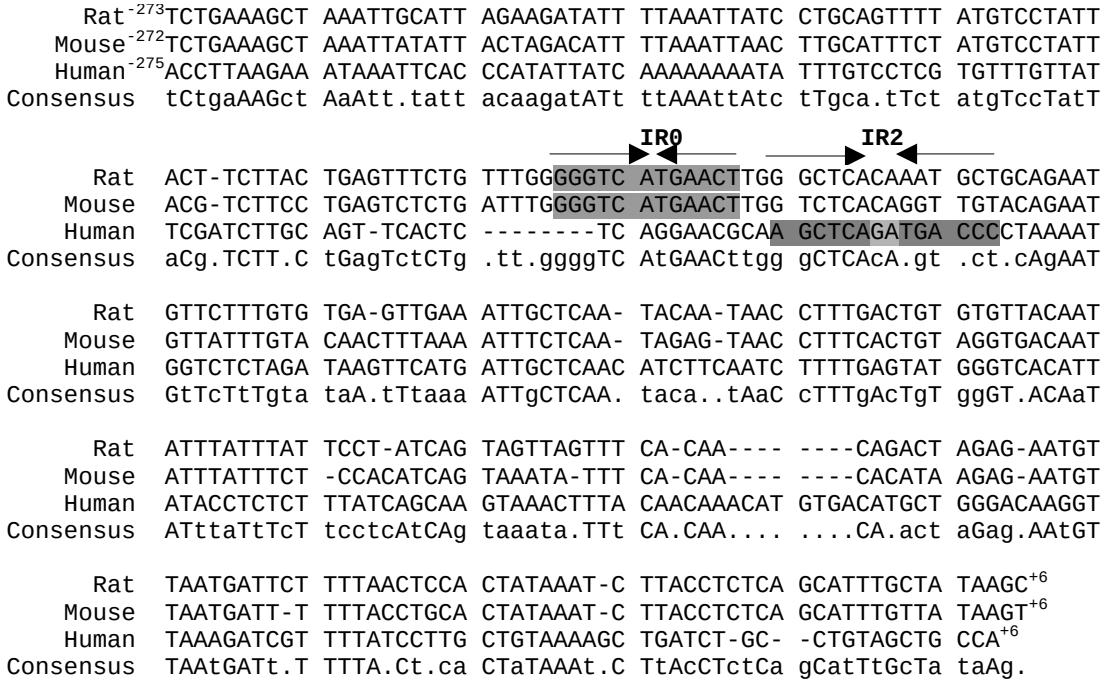


Figure 4.3.4 Sequence alignment of rat, mouse, and human SULT2A1 promoter regions. MultAlin program was used for the alignment of rat (**GenBank accession no. M29301**), mouse (Echchgadda, Song et al. 2004), and human (**GenBank accession no. U54701**) SULT2A1 promoter regions.

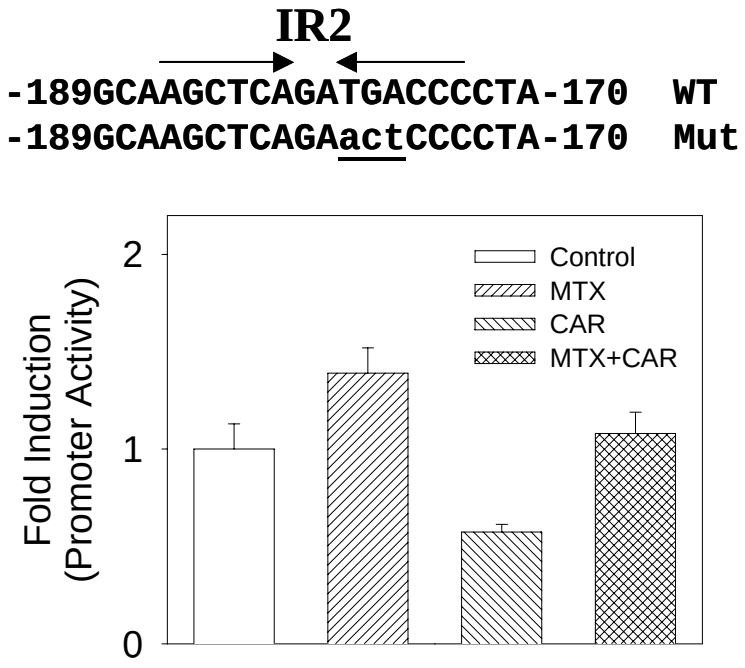


Figure 4.3.5 Point mutation analysis of the IR2 DNA *cis*-element

Mutated 5'-flanking region of hSULT2A1 (-414 to +48) as shown in the figure was inserted into pGL3-Basic vector. The constructed reporter vector and CAR were transfected into Caco-2 cells and treated with MTX. pRL-TK was used as internal control for transfection assay. Dual-luciferase activities were measured according to the manufacturer's recommendations. The histograms are averaged values from three independent experiments (each performed in duplicate).

4.3.4 Electrophoretic Mobility Shift Assay (EMSA) and Super Shift Assay for the Interaction of CAR and RXRα with IR2 Element

Electrophoretic mobility shift assay (EMSA) and super shift assay were used to prove the interaction of CAR and RXRα with the identified IR2 element in the promoter region of hSULT2A1. The 31 bp oligonucleotide (5' -GGA ACG CAA GCT CAG ATG ACC CCT AAA ATG G - 3') which include the IR2 element and its mutant (5' - GGA ACG CAA GCT CAG AAC TCC CCT AAA ATG G - 3') were ordered from IDT. Both the IR2 sequence and IR2 mutant sequence were labeled with digoxigenin (DIG) molecule. The DIG molecule labeled oligonucleotides was visualized by an enzyme immunoassay using anti-digoxigenin-AP, Fab-fragments and the chemiluninescent substrate CSPD. Experimental results showed that nuclear extract from Caco-2 cells caused the IR2 probe shift (Figure 3.3.3 lane 2). 125 fold excess of cold IR2 sequence inhibited this shift (lane 3) and mutated cold IR2 probe (125 fold) did not inhibit this shift (lane 4). Also, mutated IR2 labeled probe was not shifted by the nuclear extract (lane 5). These results strongly suggest that certain nuclear receptors can bind to the IR2 element. Super shift assay (lane 6 and 7) results showed that hCAR and hRXRα antibodies caused the nuclear extract shift to disappear (or tremendously decrease). The super shift band was not found. There are two possible causes of the disappearance of the shift band. The antibody may cause the corresponding receptor-oligonucleotide complex precipitate; therefore not appearing in the gel or running into the gel. A second possibility is that the interaction between antibody and nuclear receptor disables the receptor from binding to the IR2 element. The super shift assay results suggest that CAR and RXRα (or their heterodimers) can bind to the IR2 sequence.

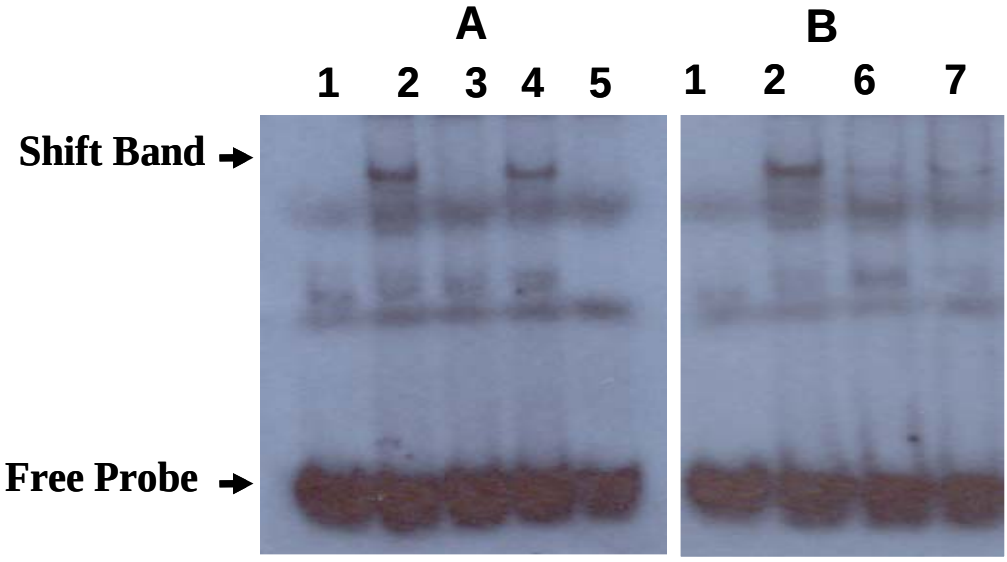


Figure 4.3.6 EMSA of Caco-2 nuclear extract and super shift of CAR and RXRα.

Figure A, Gel shift assay of IR2 sequence. The labeled IR2 wild type sequence (lane 1) or mutated IR2 sequence (lane 5) were incubated with Caco-2 nuclear extract (lane 2) for 20 minutes at room temperature to form possible DNA protein complexes. 125-fold excess of cold competitors was added to test the specificity of the complexes (lane 3, 4). Figure B, super shift assay of Caco-2 nuclear extract. Antibody specific to CAR (lane 6) and RXRα (lane 7) was added to the nuclear extract and incubated for 20 minutes at room temperature before the shift reaction.

4.4 Interaction among CAR, PXR, and VDR for the Regulation of hSULT2A1

4.4.1 Interaction of CAR, PXR, and VDR with the IR2 DNA *cis*-element

As discussed in section 4.3, the IR2 element located at -186 /-173 of hSULT2A1 promoter region plays a very important role in MTX induction of hSULT2A1. We found several nuclear receptors that can interact with the IR2 element and regulate the activity of hSULT2A1. When we transfected the Caco-2 cells with reporter vector which was regulated by the wild type hSULT2A1 promoter vector, we found the cotransfection of CAR and VDR can significantly increase the promoter activity of hSULT2A1 compared with the control cells which was treated with transfection reagent only. The cotransfection of PXR significantly inhibits the promoter activity of hSULT2A1. Then we mutated the IR2 sequence through overlap PCR as described in materials and methods. The mutated promoter sequence regulated luciferase reporter vector was transfected into Caco-2 cells. The basal luciferase activity regulated by this mutated promoter sequence decreased about 1 fold compared with wild type promter sequence (figure 4.4.1). Most interestingly, all three nuclear receptors inhibit the luciferase activity when they cotransfected with the reporter vector regulated by the mutated IR2 element. This mean that CAR and VDR reverse their up-regulation effect and start to repress the mutated promoter activity of hSULT2A1, and PXR further represses this mutated promoter activity. These data indicate all the three nuclear receptors can interact with the IR2 element. CAR and VDR induce the activity of hSULT2A1, and PXR inhibits the activity of hSULT2A1.

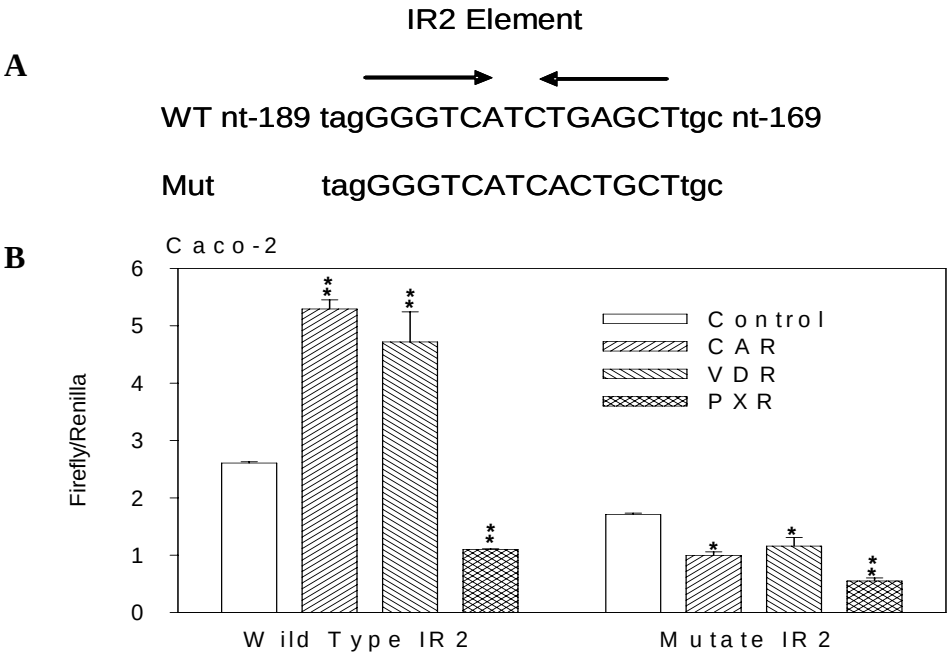


Figure 4.4.1 CAR, VDR, and PXR involved in the transcriptional regulation of hSULT2A1 promoter activity.

A, DNA sequence of wild type IR2 DNA response element and mutated IR2 DNA response element. B, Wide type or mutated 5'-flanking region of hSULT2A1 (-414 to +48) was inserted into pGL3-Basic vector as described under "materials and methods." Dual luciferase activities were determined according to the manufacturer's recommendations. pRL-TK was used as the internal control for transfection assay. CAR, VDR or PXR were cotransfected with a final concentration of 50 ng per well in a 24 well plate. MTX (100 nM and VD3 (50 nM) were added to VDR cotransfected cells. The histograms with standard error are averaged values from three independent experiments; each independent transfection was performed in duplicate. *$p<0.05$ and **$p<0.01$ compared with control within the same group (either wild type group or mutated group).

4.4.2 PXR Inhibition of MTX and CAR Mediated up-regulation of hSULT2A1

Since PXR inhibits the activity of hSULT2A1 and CAR transactivates the MTX mediated induction of hSULT2A1, we want to see if PXR can inhibit the MTX and CAR induction of hSULT2A1. Without any treatment of MTX or CAR, PXR can decrease the basal luciferase activity 50% compared with the control which received only transfection reagent (figure 4.4.2). In the presence of CAR or MTX, PXR can decrease the promoter activity of hSULT2A1 induced by MTX, CAR, or MTX together with CAR. Without PXR, either MTX or CAR can induce the luciferase activity 2 fold. The luciferase activity came down to the control level when PXR was cotransfected. This means PXR can repress the induction of hSULT2A1 mediated by MTX or CAR. As previous discussed, MTX and CAR together can induce the promoter activity of hSULT2A1 4 fold, When PXR was cotransfected, the induction come down to only 2 fold. All these results indicate that PXR can inhibit the MTX and CAR induction of hSULT2A1. We also performed the endogenous induction study for PXR and found that the transfection of PXR can repress the expression of hSULT2A1.The endogenous study supported our reporter gene assay results.

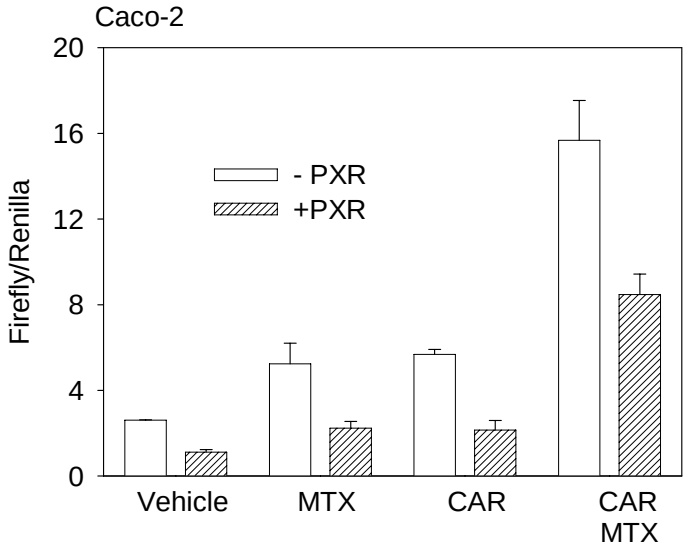


Figure 4.4.2 PXR inhibition of MTX and CAR induction of hSULT2A1 promoter activity.

Luciferase reporter vector regulated by wild type hSULT2A1 promoter sequence was transfected into Caco-2 cells and treated with MTX. CAR or PXR nuclear receptors were cotransfected with a final concentration of 50 ng per well in a 24 well plate. pRL-TK was used as internal control for transfection assay. Luciferase expression was determined according to the manufacturer's recommendations. The histograms with standard error are averaged values from three independent transfections; each independent transfection was performed in duplicate.

4.4.3 Competition between VDR and CAR for the Promoter Activity of hSULT2A1

Both VDR and CAR can up-regulate the promoter activity of hSULT2A1, therefore, we wanted to see if there is a competition relationship between VDR and CAR. Caco-2 cells were transfected with a fixed amount of VDR (50 ng), increasing amounts of CAR (from 0 to 50 ng), and the luciferase reporter vector regulated by hSULT2A1 promoter. The transfected cells were subsequently treated with VD3, MTX, or ethanol (vehicle). Cotransfection of increasing amounts of CAR caused a progressive decline in the vitamin D responsiveness of the hSULT2A1 promoter and a concomitant enhancement in the MTX-mediated induction of this promoter (figure 4.4.3). Because CAR shows ligand independent transcriptional activity, the luciferase activity in the control cells increasd with the increasing amounts of CAR. In the competition of VDR and CAR, hSULT2A1 is more sensitive to CAR because when the CAR concentration had just reach 5 ng (the ratio of CAR to VDR is 1:10), VDR started to lose its induction ability. These results suggest that VDR and CAR compete for the same DNA response element (the IR2 element), but CAR has a much higher binding affinity to the IR2 element.

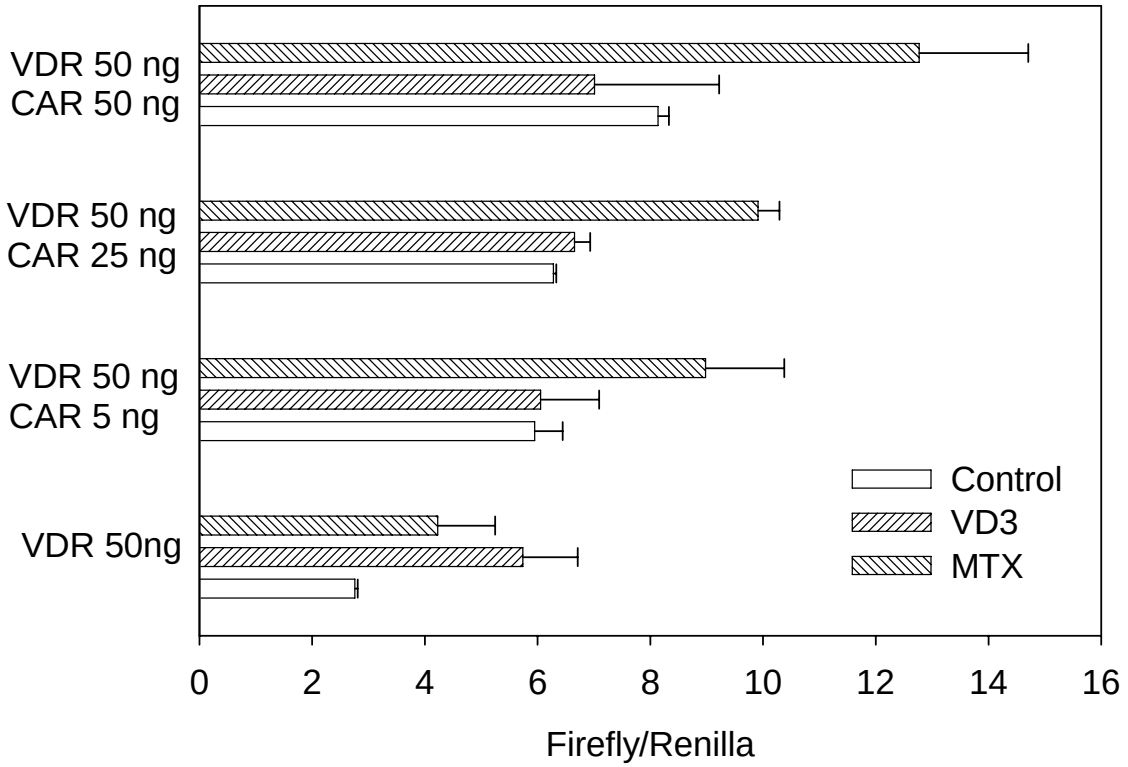


Figure 4.4.3 VDR and CAR compete for the promoter activity of hSULT2A1 Luciferase reporter vector regulated by hSULT2A1 promoter was transfected into Caco-2 cells and treated with VD3 or MTX as described. VDR was cotransfected with a fixed amount of 50 ng, and increasing amount of CAR. pRL-TK was used as the internal control for the transfection assay. Luciferase expression was determined according to the manufacturer's recommendations. The histograms with standard error are averaged values from three independent experiments; each independent transfection was performed in duplicate.

4.5 MTX Time-dependent Endogenous Induction of hSULT2A1 and Synergistic Effect with CAR

4.5.1 MTX Induction of hSULT2A1 in a Time-dependent Manner in Both Caco-2 and Hep G2 Cells

As we discussed in part 4.1, MTX can induce the four main families of hSULTs in a concentration-dependent manner in both Caco-2 and Hep G2 cells. To further investigate the effect of the time involved in MTX induction, we performed the MTX time-dependent experiment and found that MTX induces SULT2A1 in a time-dependent manner in both Caco-2 and Hep G2 cells (Figure 4.5.1). When the Caco-2 cells was treated with 0.1 μM MTX for 2 days, the mRNA level of hSULT2A1 increased 4 fold compared with the control cells. When the MTX exposed time increased to 6 days, the mRNA of hSULT2A1 in the treated cells showed 7 fold inductions. The MTX induction pattern of hSULT2A1 in Hep G2 cells is similar to Caco-2 cells, the mRNA level of hSULT2A1 increased 2 or 4 fold after being treated with MTX for 2 or 6 days. Western blot results agree with real-time PCR. hSULT2A1 protein level was induced by MTX in a time-dependent manner in both Caco-2 and Hep G2 cells.

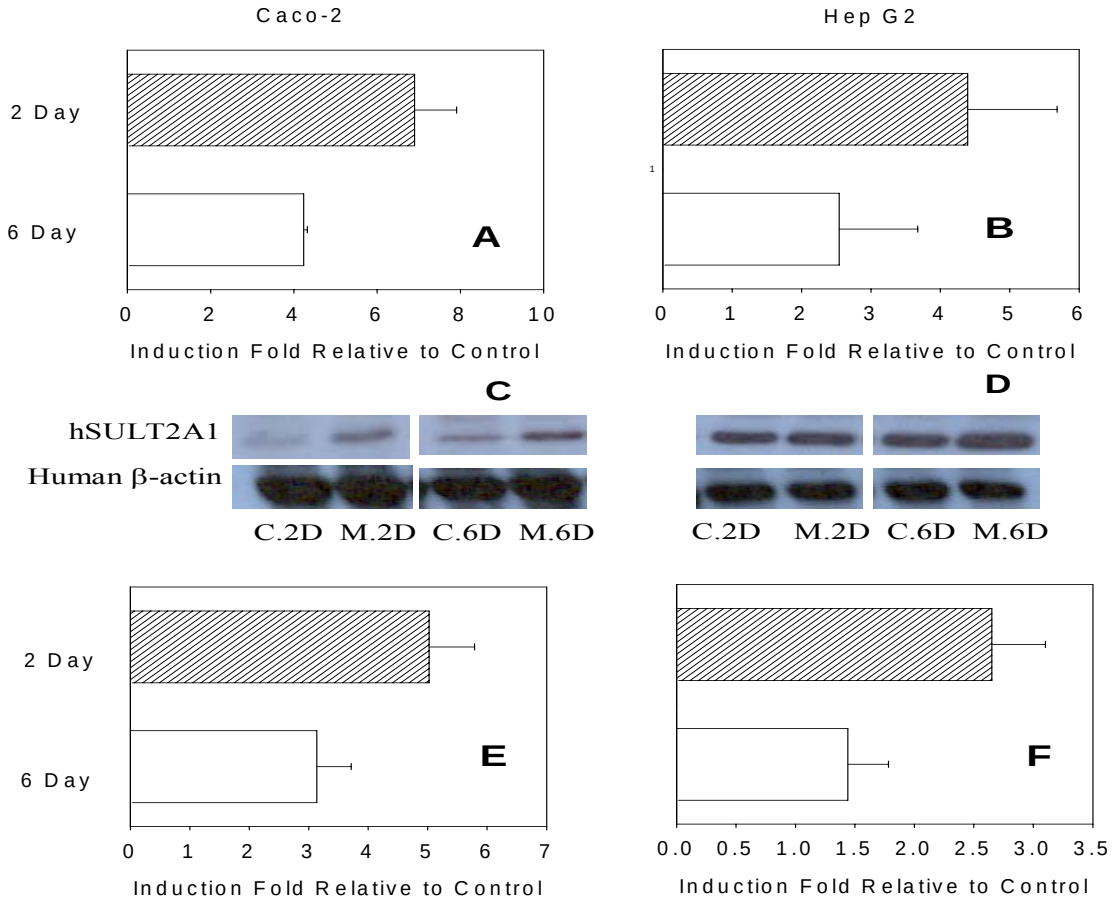


Figure 4.5.1 Time-dependent induction of hSULT2A1 by MTX in Caco-2 and Hep G2 cells

Caco-2 and Hep G2 cells were treated with MTX for 2 days or 6 days. Cells were harvested for real-time PCR and for western blot. For A and B: real-time PCR. For C and D: a representative of western blot. For E and F: each column of the densitometry corresponds to each lane of western blot. Human β-acitin was used as the control for both RT-PCR and western blot. The copy number of MTX treated cells divided by the copy number of control cells gives the induction fold. The control value was calculated as 1. The histograms with standard error are averaged values from three independent experiments.

4.5.2 CAR Transactivation of MTX Induction of hSULT2A1 in Caco-2 Cells

Reporter gene assay was used to evaluate the role of CAR involved in the MTX induction of hSULT2A1 in Caco-2 cells. Caco-2 cells were transfected with luciferase reporter vector regulated by hSULT2A1 promoter sequence. The transfected cells were exposed to different concentration of MTX from 8 nM to 50000 nM for 48 hours with or without cotransfection of CAR. The data shows in Figure 4.4.2 indicates that MTX can induce the promoter activity of hSULT2A1 in a concentration-dependent manner. This agrees well with the endogenous study of MTX induction of hSULT2A1 in Caco-2 cells as described in section 4.1. When the Caco-2 cells were cotransfected with CAR, the luciferase activity further increased in all MTX concentrations used. CAR always has synergistic effect with MTX in hSULT2A1 regulation. This strongly indicates CAR can transactivate MTX induction of hSULT2A1 in Caco-2 cells.

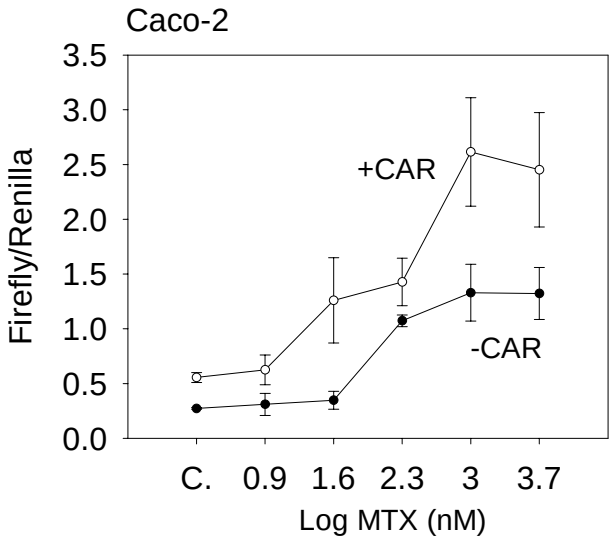


Figure 4.5.2 MTX and CAR induction of hSULT2A1 in Caco-2 cells

Luciferase reporter vector regulated by hSULT2A1 promoter sequence (from -1463 to +48) was transfected into Caco-2 cells and treated with different concentrations of MTX (8 nM, 40 nM, 200 nM, 1000 nM and 5000 nM) or CAR. pRL-TK was used as the internal control for the transfection assay. Luciferase activity was measured according to the manufacturer's recommendations. Values are averaged from three independent experiments; each independent transfection was performed in duplicate.

Chapter V

Discussion

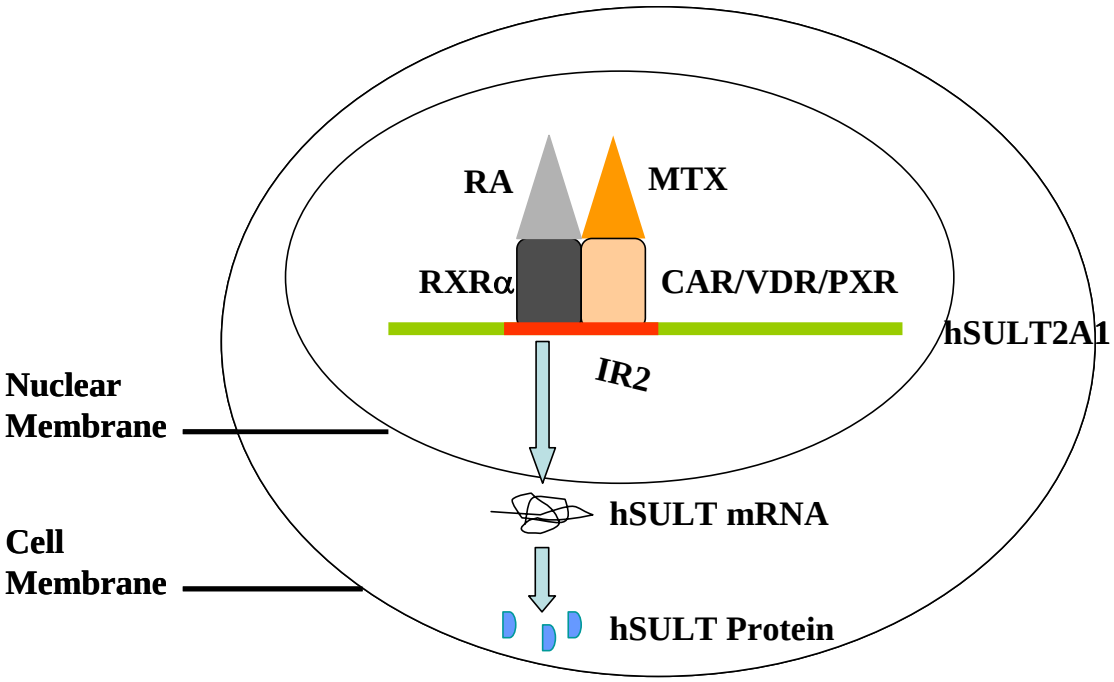


Figure 5.1.1 System involved in MTX induction of hSULT2A1.

This figure is the answer of figure 2.1 on page 24. The question marks in figure 2.1.1 were answered in this figure. The nuclear receptors involved in hSULT2A1 regulation are CAR, VDR, PXR, and RXRα. The RXRα functions as a common heterodimer parterner and interacts with CAR, VDR, and PXR. The 9-*cis*- retinoic acid (RA) acts as ligand for RXRα. MTX acts as agnist for CAR. The DNA *cis*-element that interacts with nuclear receptor heterodimers located in -183/-173 of hSULT2A1 promoter region, it is an IR2 element.

5.1 System Involved in MTX Induction of hSULT2A1

Back to our hypothesis in chaper 2, we proposed that the MTX induction of hSULTs are mediated through the interaction of nuclear receptors with the DNA *cis*-elements located in the proximal promoter region of hSULT genes. As indicated in figure 5.1.1, we approved our hypothesis through a series experiments. With the endogenous induction study, we found MTX can induce hSULTs in both Hep G2 cells and Caco-2 cells, these inductions were further confirmed by dual luciferase reporter gene assay. Then we chose Caco-2 cells as model to investigate the MTX induction mechanism. With the cotransfection assay, we found CAR, VDR, and RXRα can up-regulate the expression of hSULT2A1, PXR can down-regulate the expression of hSULT2A1. The positive role of CAR was further confirmed by the RNA intereference experiment. With the promoter deletion, DNA sequence alignment and promoter mutation, we found the IR2 element located at -186 to -173 of hSULT2A1 promoter region was the DNA *cis*-element responsible for the up-regulation of hSULT2A1. Through EMSA assay and super shift assay, we confirmed the interaction of the IR2 element with CAR and RXRα. Since we found several nuclear reptors all involved in the regulation of hSULT2A1, we tested the cross-talk of these nuclear receptors and we found that CAR, VDR, and PXR can all interact with the IR2 element. CAR competed with VDR for the promoter activity of hSULT2A1. PXR repressed the up-regulation of CAR in hSULT2A1 regulation.

5.2 MTX Induction of hSULTs in Hep G2 and Caco-2 Cells

Human liver and human intestine are the two major organs that are involved in drug metabolism. Hep G2 cells derived from a human hepatoblastoma that is free of

known hepatotropic viral agents. A wide variety of drug metabolizing enzymes have been found to expressed in Hep G2 cells (Javitt 1990; Schmiedlin-Ren, Thummel et al. 2001). Caco-2 cells originally derived from a human colon adenocarcinoma and have been extensively used over the last twenty years as model for human intestinal cells (Sambuy, De Angelis et al. 2005). Since differentiated Caco-2 cells express various cytochrome P450 isoforms and phase II enzymes such as UDP-glucuronosyltransferases, SULTs and glutathione-S-transferases, this model is also used in the study of drug metabolizing enzymes (Meunier, Bourrie et al. 1995). In our study, we found the abundance of hSULT2A1 and hSULT1A1 in both Hep G2 and Caco-2 cells are very high, so here we use Hep G2 and Caco-2 cell lines as model to investigate the effect of MTX on the transcriptional regulation of hSULTs.

MTX is a drug that is widely used cilnic against cancer and other diseases. MTX and its polyglutamate metabolites are folic acid analogues with inhibitory activity against many enzymes in the metabolic pathway of folic acid. MTX inhibits the production of thymidylate, purine, and methionine and leads to accumulation of adenosine (Jandik, Kruep et al. 1996). These actions inhibit cellular proliferation and induce apoptosis. Inducers of cytochrome P-450 (CYP) have been reported to enhance MTX-induced hepatocytotoxicity (Tsuda, Yamada et al. 1999). MTX is also reported to slightly down-regulate some of the CYP genes (Cheung, Lee et al. 1996). MTX inductions any drug metabolizing enzymes are basically unknown.

Regulations of SULT expression by endogenous molecules (i.e. hormones and bile acids) are relatively well documented (Fang, Abdolalipour et al. 2005; Janer, Lavado et al. 2005). The biochemical consequences of these regulations are of importance in

relation to their physiological processes. There are some reports studying drug or xenobiotic induction of SULTs in either animal tissues or cultured human cells (Runge-Morris 1998; Maiti and Chen 2003; Maiti and Chen 2003). In the present investigation, we report that MTX can increase hSULT expression and activity in cultured human cells. MTX induced hSULTs in Hep G2 cells and Caco-2 cells differently. After being treatment with MTX, the protein and mRNA level of hSULT2A1 (P-PST), hSULT1A3 (M-PST), hSULT2A1 (DHEA-ST), and hSULT1E1 (EST) in Hep G2 cells were induced; hSULT1A3 and hSULT2A1 in Caco-2 cells were induced. Our enzyme assay and Western blot (protein level) are in good agreement with RT-PCR result. This clearly indicates that MTX induction of hSULTs is at the transcriptional level. We have also shown that MTX can induce rat SULTs (Maiti and Chen 2003). Combined with known results from publications of other drug metabolizing enzymes, our results support a nuclear receptors mediated mechanism for MTX induction of hSULTs.

5.3 Cytotoxicity and Transcriptional Activation of hSULT2A1 in Hep G2 and Caco-2 Cells Exposed to MTX

Through endogenous induction study, we found MTX can induce hSULTs in a concentration-dependent manner in both Caco-2 and Hep G2 cells. It is well known that high concentrations of MTX can cause cytotoxicity to human cells. We wanted to see if MTX induction of hSULTs was caused by the MTX cytotoxicity. We cloned the promoter sequence of hSULT2A1 and ligated it into the basic luciferase reporter vector so the expression of luciferase was regulated by the hSULT2A1 promoter. We used this

reporter vector to test the transcriptional activation of hSULT2A1 by MTX, and we used the MTT assay to test the cytotoxicity of MTX to Caco-2 and Hep G2 cells.

MTX elicited time- and concentration-dependent cytotoxicity to both Hep G2 cells and Caco-2 cells (figure 4.2.1 and figure 4.2.2), but Caco-2 was more sensitive to MTX. Caco-2 cells showed significant cytotoxicity when tread with 0.5 μM MTX for only 2 days. In contrast, Hep G2 cells did not show any cytotoxicity until treated with 0.5 μM MTX for 4 days. This may be because Hep G2 cells express more enzymes involved in drug metabolism. Because the human intestine is mainly involved in nutrient absorption, the intestine cells have less ability to detoxify exogenous molecules; so even when CAco-2 cells are are exposed to low concentrations of MTX for a short time, their viability of Caco-2 cells is significantly decreased.

In our reporter gene assay, Caco-2 cells were more sensitive to MTX treatment compared with Hep G2 cells. When the transfected Caco-2 cells were treated with MTX, the promoter activity of hSULT2A1 in Caco-2 cells showed time- and concentration-dependent induction (figure 3.2.3). In a fixed time (we tested from 12 hours to 48 hours), the promoter activity of hSULT2A1 increased when the MTX concentration increased from 0.04 μM to 5 μM. After that, when the MTX concentration increased to 12.5 μM, the promoter activity decreased. For a fixed concentration of MTX (from 0.04 μM to 12.5 μM), the promoter activity of hSULT2A1 increased when the exposure time increased. Compared with Caco-2 cells, the basic promoter activity of hSULT2A1 increased when the MTX incubation time increased. The concentration-dependent induction in Hep G2 cells was not so obvious. The different induction patterns in Hep G2 and Caco-2 cells suggest different cells have different transcription factor expression when applied in

different signal transduction pathways, these cells will respond in different ways. The MTX concentrations used in reporter gene assay are much higher than the MTX concentrations used in the endogenous study. The induction of hSULTs reaches the highest level when the MTX concentration is 0.1 μM in the endogenous induction study; while in reporter gene assay, the induction reaches the highest level when MTX concentration is 5 μM. The reason for this difference may come from the different methods. In the reporter gene assay, we transfected as many as $1X10^{10}$ copies of reporter genes into $1X\ 10^5$ cells. Undoubtedly, as a consequence, the full induction of hSULT2A1 promoter activity needs much higher concentration of MTX in the reporter gene assay.

From the MTT assay results, we know low concentration and short time of MTX exposure does not cause any significant cytotoxicity to Caco-2 and Hep G2 cells. When the Caco-2 cells were treated with MTX for 2 days, the estimated IC50 (inhibitory concentration 50%) was 34 μM. In the induction study, however, 2 days exposure of 0.1 μM MTX caused the promoter activity of hSULT2A1 to significantly increase. Obviously, the induction of hSULT2A1 activity was not caused by the cytotoxicity of MTX. In the following MTX induction mechanism studies, we chose the concentration of 0.1 μM MTX for all experiments. With this concentration, we excluded the possibility that MTX induction of hSULT2A1 was caused by its cytotoxicity.

5.4 hSULT2A1 is transcriptionally Regulated by CAR

Phase I and phase II drug metabolizing enzymes, such as cytochrome P-450 and UDP-glucuronosyltransfersase (UGT) are well known to be regulated by endogenous hormones as well as by therapeutic drugs and other xenobiotics. SULTs, one of the major

families of phase II drug metabolizing enzymes, are also well known to be regulated by hormones. Xenobiotic induction of SULTs, though, is not well studied (Runge-Morris 1997; Gaworecki, Rice et al. 2004). Nuclear receptors, which mediate the induction of some phase I and phase II enzymes, have recently been shown to mediate the induction of SULTs. Reports on the nuclear receptor mediated induction mechanisms of SULTs focused primarily on SULT2A1 from different species. An IR0 element in rats and mice was proposed to be the DNA binding site for different nuclear receptors in regulation of SULT2A1. The nuclear receptors form heterodimers with RXRα. These nuclear receptors include FXR/RXRα (Aste, Cozzi et al. 2001), PXR/RXRα (Duanmu, Locke et al. 2002), VDR/RXRα (Echchgadda, Song et al. 2004), and CAR/RXRα (Saini, Sonoda et al. 2004). FXR and PXR were also reported to repress basal mSULT2A1 expression using FXR-null or PXR-null mice (Kitada, Miyata et al. 2003) and to inhibit vitamin D_3 induction of mSULT2A1 via VDR (Echchgadda, Song et al. 2004).

In the present study, we have shown that the promoter of hSULT2A1was induced by the highly selective hCAR agonist CITCO in Caco-2 cells. Most importantly, we found that the widely used clinic anti-folate drug MTX has a high potency to induce hSULT2A1 which is similar to CITCO. Our results indicate that hCAR transactivates the induction of hSULT2A1. RXRα enhances CAR transactivation of hSULT2A1. This agrees with earlier reports on nuclear receptor transactivation of various drug-metabolizing enzymes. Almost all known nuclear receptors form heterodimers with RXRα to transactivate drug metabolizing enzymes. The induction caused by MTX has synergistic effect with CAR and RXRα, which suggests MTX can function as an agonist of hCAR. The RNA interference experiment further supports the expression of

hSULT2A1 was closely related to the expression of hCAR. When the hCAR expression vector was transfected into Caco-2 cells, the mRNA levels of both hCAR and hSULT2A1 were significantly increased, which mean hCAR can mediate the induction of hSULT2A1. When the CAR was knocked down by siRNA, the mRNA level of hSULT2A1 was also decreased, and this was further confirmed by reporter gene assay results.

Our deletion analysis suggests that the binding site of CAR/RXRα is located between -188 to -130 in the 5'-flanking region of hSULT2A1. Sequence alignment result (Figure 4.3.4) indicates that the IR0 sequence, which is responsible for transactivation of rat and mouse SULT2A1, does not exist in the hSULT2A1 5'-flanking region. Analysis of -188 to -130 DNA sequence of hSULT 5'-flanking region revealed an IR2 element. Our mutation results (Figure 4.3.5) further show the importance of this IR2 element in the transactivation of hSULT2A1 via the CAR/RXRα heterodimer during MTX induction. The identified IR2 element in our report agrees with a previous prediction of several putative (A/G)G(G/T)TCA nuclear receptor half-site motifs in the 5'-flanking region of hSULT2A1 (Duanmu, Locke et al. 2002).

It is well known that nuclear receptors can bind to a broad range of ligands and regulate genes that are involved in drug clearance and disposition. These nuclear receptors share parterners, ligands, DNA response elements and target genes. Moreover, they influence mutually their relative expression (Chou, Prokova et al. 2003; Kodama, Koike et al. 2004). Our results suggest that there are cross-talk between CAR and PXR. The cotransfection of PXR can significantly decrease the induction caused by CAR and MTX. Our results also show that CAR can enhance the transcriptional activity of

hSULT2A1. When the IR2 sequence was mutated, the cotransfection of CAR repressed the mutated promoter activity. This suggests that there may be other nuclear receptors and DNA binding elements that also involved in transactivation of MTX induction of hSULT2A1. The mutation affects the binding of CAR/RXRα heterodimer to the mutated IR2 sequence and abolishes the induction of CAR. The cotransfection of extra exogenous CAR may compete for limited endogenous RXRα with other nuclear receptors and cause a final inhibition effect of the promoter activity.

In conclusion, our results demonstrate the positive role of hCAR in the regulation of hSULT2A1. We identified a CAR/RXRα heterodimer-binding element, IR2. This IR2 element is different from the reported IR0 element in rat and mouse SULT2A1 gene. The relative position of IR2 in the hSULT2A1 gene and IR0 in rat and mouse is very close (Figure 4.3.4). These results could explain the differences and similarities between hSULT2A1 induction and rat and mouse SULT2A1 induction. Further characterization of the role of MTX involved in hSULT2A1 regulation may be of considerable therapeutic significance. We are currently investigating the role of other nuclear receptors like PXR, and VDR in basal and regulated SULT2A1 expression in Hep G2 and Caco-2 cells. These studies should provide insights into the cross-talk between different nuclear receptors in hSULT2A1 transcriptional regulation.

5.5 Cross-talk among CAR, PXR, and VDR in the Transcriptional Regulation of hSULT2A1

hSULT2A1 is an important cytosolic enzyme in human liver and human intestine that catalyzes the sulfation of steroid hormones such as DHEA and xenobiotic alcohols.

The expression of rSULT2A1 has known to be regulated by several nuclear receptors through the same DNA *cis*-element located in the proximal promoter region of rSULT2A1. Song first reported that the primary bile acid chenodeoxycholic acid (CDCA) acts as a regulator of rSULT2A1 through the farnesoid X receptor (FXR) by binding to an IR0 element located at -193bp to -169bp of the promoter (Song, Echchgadda et al. 2001). Other reports demonstrated that both PXR and VDR can bind to the same DNA response element and up-regulate the expression of rSULT2A1 (Sonoda, Xie et al. 2002; Echchgadda, Song et al. 2004). In this case, PXR and FXR compete with VDR for the same IR0 element. It has also been reported that both steridogenic factor 1 (SF1) and estrogen-related receptor alpha (ERRα) bind to the same DNA response element and regulate hSULT2A1 expression (Saner, Suzuki et al. 2005; Seely, Amigh et al. 2005). Recently, the vitamin D receptor (VDR) was reported to interact with hSULT2A1 promoter sequence through the same DNA *cis*-element as we reported here (Song, Echchgadda et al. 2005). In our experiments, we found both CAR and VDR can up-regulate the expression of hSULT2A1, PXR inhibit the expression of hSULT2A1.

Although all CAR, PXR, and VDR are involved in the transcriptional regulation of hSULT2A1, different nuclear receptors have different effects. CAR and VDR induce the expression of hSULT2A1; PXR represses the expression of hSULT2A1. All three nuclear receptors can bind to the same IR2 element located in the proximal promoter region of hSULT2A1. The IR2 element functions as positive DNA response element for both CAR and VDR; but for PXR, it functions as negative DNA response element. After the IR2 sequence was mutated, the basic promoter activity decreased.This indicates that this IR2 sequence was very important for the maintenance of the full activity of the

promoter. All three nuclear receptors show inhibition effect on the mutated hSULT2A1 promoter activity when cotransfected with the mutated luciferase promoter vector. This suggests the complexity of the signal transduction pathway of hSULT2A1. The inhibition of nuclear receptors may be because they can not bind to the mutated IR2 sequence, they start to compete for the limited amount of common heterodimer parterner such as RXRα. The exhaust of RXRα causes other nuclear receptors or transcription factors can not bind to their regulatory sequence and perform their function. As a result, the promoter activity is further decreased.

CAR activates MTX induction of hSUTL2A1 whereas PXR inhibits this induction. This suggests PXR may compete with CAR for binding to the IR2 element. PXR may also transactivate other drug metabolizing enzymes when appropriate inducers are present. The hormonal and metabolic milieu of the cells determined which of the competing nuclear receptors will assume a dominant role in regulating hSULT2A1 expression. This is supported by our mutated IR2 results (Figure 3.4.5), which suggest that there may be other nuclear receptors and DNA binding elements that can also transactivate MTX induction of hSULT2A1. In the case of MTX induction of hSULT2A1, CAR is at least one nuclear receptor responsible for the stimulation of gene expression through the IR2 element. Our results also agree with recent reports that CAR transactivates hSULT2A1 expression (Assem, Schuetz et al. 2004; Kim, Shigenaga et al. 2004; Saini, Sonoda et al. 2004) and PXR inhibits CAR mediated induction (Kitada, Miyata et al. 2003; Echchgadda, Song et al. 2004)

VDR competes with CAR in the up-regulation of hSULT2A1 (figure 3.4.7). This suggests that the two nuclear receptors may share common DNA response elements and

heterodimer parterners such as RXRα. Because both nuclear receptors can bind to the same DNA *cis*-element, their relative concentrations determine their final effect. In the competition experiments, CAR is more sensitive than VDR, when the CAR amount is only 5 ng, CAR can repress the VD3 induction of hSULT2A1 to the control level. Higher CAR concentration increases the basal hSULT2A1 promoter activity and MTX induction activity, but VD3 induction activity through VDR was inhibited by CAR.

In summary, our results have shown that there is nuclear receptor cross-talks in the regulation of hSULT2A1. Our data show that MTX induces hSULT2A1 in a time-dependent manner in both Caco-2 and Hep G2 cells. All three nuclear receptors: CAR, PXR, and VDR were found to participate in the MTX transcriptional regulation of hSULT2A1. These nuclear receptors compete with each other for common DNA *cis*-element and heterodimer parterners such as RXRα. The final effect of these nuclear receptors to hSULT2A1 depends on their concentrations, cell types and physiological conditions. Understanding the mechanism of MTX induction of hSULT2A1 may contribute to better understanding of drug resistance, drug efficiency, drug-drug interaction, and the development of better drug treatments.

5.6 Summary

hSULT2A1 is a cytosolic sulfotransferase which occurs predominantly in human liver, adrenal and intestine. The activity of this enzyme is not gender-dependent but shows highly dynamic expression throughout the lifespan (Saner, Suzuki et al. 2005). The hSULT2A1 substrates includes physiological hydroxysteroids such as

dehydroepiandrosterone, pregnenolone, estrogen, pregnenolone, bile acid and xenobiotic alcohols (Chatterjee, Song et al. 1994; Song, Echchgadda et al. 2001; Strott 2002).

Besides endogenous molecules, xenobiotics and therapeutic drugs such as tamoxifen were also reported to be sulfated by hSULT2A1 (Chen, Yin et al. 2002; Falany, Pilloff et al. 2005). The regulation of hSULT2A1 has been reported to be mediated by several transcription factors including steroidogenic factor 1 (SF1), GATA-6, peroxisome proliferator-activated receptor alpha (PPARα), estrogen-related receptor alpha (ERRα), essential Role of the CAAT/Enhancer Binding Protein alpha and vitamin D receptor (VDR) (Fang, Strom et al. 2005; Saner, Suzuki et al. 2005; Seely, Amigh et al. 2005; Song, Echchgadda et al. 2005). To investigate the molecular mechanism involved in MTX induction of hSULT2A1, we cloned the 5' flanking region of hSULT2A1 promoter sequence and ligated it to the luciferase reporter vector (pGL3-Basic, Promega). Step wise deletion and mutation studies show the IR2 sequence located in the proximal promoter region of hSULT2A1 is very important in hSULT2A1 regulation. Through cotransfection experiments, we found CAR can significantly induce the promoter activity of hSULT2A1. With the presence of MTX, CAR has synergistic effect with MTX and can further increase the activity of hSULT2A1. Further studies include siRNA and real-time PCR confirmed the positive role of CAR in the up-regulation of hSULT2A1. DNA gel shift assay and super shift assay results suggest CAR can heterodimerize with RXRα and bind to the IR2 DNA *cis*-element. Contrast to the up-regulation of CAR, PXR can suppress the promoter activity of hSUlT2A1. PXR serves as an antagonist of CAR. The cotransfection of PXR can decrease the induction of hSULT2A1 caused by both MTX and CAR. The nuclear receptor VDR, however, shows a different effect on CAR

induction, by binding to its ligand Vitamin D3 (VD3), VDR can compete with CAR for the promoter activity of hSULT2A1. Our super shift data show both CAR and RXRα can bind to the IR2 element. This is very similar to the endogenous hormone regulation of other drug metabolizing enzymes; where the nuclear receptors share parterners, DNA response elements and target gene. The cross-talk between different nuclear receptors determines the net activity of MTX treatment toward hSULT2A1 expression.

5.7 Significance of MTX Induction Mechanism of hSULTs

Our research on the mechanism of MTX induction of hSULT2A1 provides novel information not only on therapeutic drug regulation of hSULT, but also on the nuclear receptors involved in signaling transduction network of drug metabolism enzymes. Because CAR was involved in the gene regulation of a large overlapping set of genes including cytochrome P450s, multidrug resistance proteins, and SULTs (Kast, Goodwin et al. 2002; Ferguson, Chen et al. 2005; Xu, Li et al. 2005), the net therapeutic effect of MTX needs to be fully recognized. Our study provides important insights into the influence of therapeutic drugs on human drug metabolism enzymes and the research is expected to make significant contributions to understanding of carcinogen bioactivation, cancer prevention, drug resistance, drug design and development, drug-drug interactions, food safety, toxicology, and public health.

Chapter VI

Conclusion

1. MTX can induce the main four families of hSULTs (hSULT1A1, hSULT1A3, hSULT2A1 and hSULT1E1) at both mRNA and protein level in Hep G2 cells in a concentration-dependent manner.
2. MTX can induce hSULT1A3 and hSULT2A1 in both mRNA and protein level in Caco-2 cells in a concentraion-dependent manner.
3. MTX induces hSULT2A1 at both mRNA and protein level in both Caco-2 and Hep G2 cells in a time-dependent manner.
4. The cytotoxicity of MTX to Hep G2 and Caco-2 cells were tested, we approved the induction of hSULTs caused by MTX was not come from its cytotoxicity.
5. High dose of folic acid can inhibit the MTX induction of hSULTs at both mRNA and protein level in Hep G2 cells.
6. The promoter sequence of hSULT2A1 was cloned from Hep G2 genomic DNA and has promoter activity.
7. The promoter sequence of hSULT1A1 was cloned from Hep G2 genomic DNA. This promoter has two alternative sequences. We cloned P1, P2 and the full sequence (P). All three promoter sequences have biological function. The promoter activity of P1 is almost 10 fold higher than P2 and P.

8. All three promoter sequences: P1, P2 and P can be up-regulated by MTX.
9. MTX has no influence on the three promoter sequences with the reporter gene assay in Hep G2 cells.
10. MTX induces hSULT2A1 in Caco-2 cells in a time- and concentration-dependent manner with the reporter gene assay, which is consistent with the endogenous study.
11. CAR can induce the promoter activity of hSULT2A1 in both Caco-2 and Hep G2 cells in a concentration-dependent manner
12. RXRα can induce the promoter activity of hSULT2A1 in Caco-2 cells
13. CAR can induce the endogenous mRNA level of hSULT2A1. Knock down the CAR through siRNA, can abolish the up-regulation of hSULT2A1 caused by CAR
14. The IR2 element located in the proximal promoter region of hSULT2A1 is very important in the regulation of hSULT2A1.
15. PXR can inhibit the promoter activity of hSULT2A1 in both Caco-2 and Hep G2 cells in a concentration-dependent manner
16. VDR can induce the promoter activity of hSULT2A1 in both Caco-2 and Hep G2 cells.
17. EMSA and super shift assay show CAR and RXRα can bind to the IR2 element
18. PXR can repress the induction of hSULT2A1 mediated by MTX and CAR in Caco-2 cells
19. CAR can compete with VDR for the promoter activity of hSULT2A1 in Caco-2 cells.

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Scope and Method of Study: We use human Caco-2 and Hep G2 cell lines as model to investigate the MTX effect to human sulfotrasferases through enzymatic assay, western blot, and RT-PCR methods. We used MTT assay to detect the cytotoxicity of MTX to these cell lines. Through the reporter gene assay, RNA interference, step wise promoter deletion, site directed promoter mutation, real-time PCR, DNA gel shift assay, and super shift assay, we found several nuclear receptors involved in the transcriptional regulation of hSULT2A1.

Findings and Conclusions: We found MTX can induce human sulfotransferases in a time - and concentration-dependent manner in both human Caco-2 and Hep G2 cell lines. The induction of human sulfotransferases mediated by MTX can be repressed by folic acid in human Hep G2 cell line. The toxicity study indicates the the induction of hSULTs was not caused by the cytotoxicity of MTX. Through multiple molecular techniques, we found the IR2 sequence located in -186 to -173 of hSULT2A1 promoter region plays very important function in hSULT2A1 regulation. The nuclear receptor CAR and VDR up-regulate the promoter activity of hSULT2A1, and the PXR down-regulate the promoter activity of hSULT2A1. Obviously, there is cross-talk between different nuclear receptors in hSULT2A1 regulation.

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