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**UDP-GLUCURONOSYLTRANSFERASE 2B7 GLUCURONIDATION
OF THE ACTIVE TAMOXIFEN METABOLITES**

A Dissertation in

Integrative Biosciences

by

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ABSTRACT

Tamoxifen (TAM) is a non-steroidal selective estrogen receptor modulator that was approved by the FDA in 1977 for the treatment of breast cancer. Although it is generally well tolerated, significant adverse effects have been reported, including severe hot flashes and an increased risk for venous thromboembolism and endometrial cancer. The phase I metabolism of TAM is primarily performed by CYP2D6 and CYP3A4/5, resulting in the major, active metabolites *N*-desmethyl-4-hydroxy-tamoxifen (endoxifen) and 4-hydroxy-tamoxifen (4-OH-TAM). Interestingly, *CYP2D6* variant genotypes that result in an inactive or less active phenotype results in greater levels of circulating endoxifen and is also associated with clinical outcomes. However, despite adjusting for *CYP2D6* genotype, large variability in the circulating levels of endoxifen are still observed, indicating that additional mechanisms, such as other metabolizing pathways, are involved. The UDP-glucuronosyltransferases (UGT) are a super family of phase II metabolizing enzymes that conjugate a glucuronic acid moiety to a substrate, increasing the polarity and thereby facilitating excretion. The present dissertation identified UGTs 1A8, 1A10, and 2B7 as the most active UGTs against *trans*-endoxifen, *in vitro*. In addition, *UGT2B7* genotype is associated with the glucuronidation phenotype of human liver microsomes (HLM) against both *trans*-endoxifen and *trans*-4-OH-TAM. HLM specimens that were hetero- or homozygous for the polymorphic *UGT2B7*^{268Tyr} allele exhibited a significant decrease in the glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM. A previous study reported the phosphorylation of UGT2B7

by the non-receptor tyrosine kinase, Src, which altered UGT2B7 enzyme activity against the endogenous substrate, 4-hydroxy-estrone. Therefore, the effect of over-expression of Src in UGT2B7 cells on the glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM was examined. Stable over-expression of Src in the wild-type UGT2B7 cells resulted in a significant decrease in the glucuronidation of both TAM metabolites, similar to the level observed in cell lines only stably expressing the polymorphic UGT2B7^{268Tyr}. Interestingly, over-expression of Src in the variant UGT2B7^{268Tyr} cell line did not alter glucuronidation activity. The evidence presented in this dissertation provides additional knowledge of the metabolism of TAM and specifically, how the pharmacogenetics of the UGT family of phase II metabolizing enzymes cause inter-individual differences in TAM metabolism.

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LIST OF ABBREVIATIONS

Å	Angstrom(s)
AhR	arylhydrocarbon receptor
AI	aromatase inhibitor
Ala	alanine
ANOVA	analysis of variance between groups
AR	androgen receptor
ARE	arylhydrocarbon responsive element
ATP	adenosine triphosphate
BH	breast homogenate
BIG 1-98	Breast International Group 1-98 Trial
BM	breast microsome
bp	base pair
BRCA1	breast cancer type 1 susceptibility protein
CAS	Crk- and Src-associated substrate
CHK	Csk homologous kinase
COS-1	CV-1 in Origin, carrying SV-40
Crk	adaptor protein; proto-oncogene
Csk	c-Src kinase
c-Src	cellular-Src
CYP450	cytochrome P450
Cys	cystine
DDT	dichlorodiphenyltrichloroethane
DHEA	dehydroepiandrosterone
DHT	androgen dyhydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOX	doxorubicin
E ₂	17β-estradiol
endoxifen	<i>N</i> -desmethyl-4-hydroxy-tamoxifen
EPR	endoplasmic reticulum
ER	estrogen receptor
ERE	estrogen receptor element
FAK	focal adhesion kinase
FDA	Food and Drug Administration
FXR	farnesoid X-receptor
Gly	glycine
GST	glutathione-S-transferase
GT-1	glycosyltransferase 1
G418	geneticin
HEK293	human embryonic kidney, clone 293
His	histidine
HLM	human liver microsomes
HPLC	high performance liquid chromatography
HRP	horse-radish peroxidase
kDa	kilo Dalton

K _m	Michaelis-Menten equilibrium constant
Leu	leucine
Lys	lysine
MCF-7	Michigan Cancer Foundation-7 cell line
MDRP	multidrug resistant protein
m/z	mass-to-charge ratio
NAT	N-acetyltransferase
NCCTG	North Central Cancer Treatment Group Trial
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
Nrf2	Nuclear factor-like 2
NST	nucleotide sugar transporter
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PM	plasma membrane
PP1	4-Amino-5-(methylphenyl)-7-(t-butyl)pyrazolo-(3,4-d)pyrimidine
PP2	4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine
Pro	proline
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene fluoride
PXR	pregnane X-receptor
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcription-PCR
SAHA	suberoylanilide hydroxamic acid
SAM68	Src-associated in mitosis, 68 kDa
SDM	site-directed mutagenesis
SDS-PAGE	sodium dodecyl sulfate polyacrilamide gel electrophoresis
SEER	Surveillance Epidemiology and End Results
Ser	serine
SERM	selective estrogen receptor modulator
SH	SRC homology domain
SNP	single nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitor
SULT	sulfotransferase
SYF ^{-/-}	mouse cells deficient in Src, Yes, and Fyn
T-47D	Human ductal breast epithelial tumor cell line
TAM	tamoxifen
TBST	tris-buffered saline with Tween-20
Thr	threonine
TK	tyrosine kinase
Tyr	tyrosine
UDP	uridine diphosphate
UDPGA	uridine-5'-diphospho- α -D-glucuronic acid
UGT	UDP-glucuronosyltransferase
UPLC	ultra performance liquid chromatography
Val	valine

$V_{\max}$	maximal velocity
v-Src	viral-Src
4-OH-estrone	4-hydroxy-estrone
4-OH-TAM	4-hydroxy-tamoxifen
4-MU	4-methylumbelliferone

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Chapter 1

Literature Review

A. Abstract

Cancer is a complex disease that affects over 1.5 million individuals each year in the United States. Physiological pathways, such as those involved in cell growth and division, apoptosis, cell motility, angiogenesis, and many other areas are continually investigated as potential targets for anti-cancer agents. This discussion will introduce the basic concepts of cancer and more specifically, the mechanisms involved in breast cancer and the treatment of breast cancer. The main focus will be to introduce the anti-breast cancer agent, tamoxifen, and its mechanism of action, as well as the pathway for its metabolism. Finally, the UDP-glucuronosyltransferases (UGTs) will be introduced. The discussion of the UGT superfamily of phase II drug metabolizing enzymes will include a foundation of their genetic characteristics, tissue expression, structure, function, regulation, and clinical importance.

B. Introduction to cancer biology

i. Cancer epidemiology. Cancer is a complex disease etiology that affects a large portion of the population. Surveillance Epidemiology and End Results (SEER) data estimate that, in 2010, 1,529,560 people were diagnosed with and 569,490 died from cancer in the United States. This translates to an age-adjusted incidence rate for all races of 461.6 for diagnosis and 183.8 for death rate per 100,000 individuals in the United States between 2003 and 2007. For all races, the top five cancer sites were prostate (154/100,000), female breast (121/100,000), lung and bronchus (68/100,000), colon and rectum (49/100,000), and corpus (body of uterus) and uterus (24/100,000).¹

ii. The molecular basis of cancer. Cancer is a result of uncontrolled cellular proliferation caused by multiple mutations in the DNA that result in alteration of normal cell processes, such as cell signaling, cell cycle checkpoints, and apoptosis. Genomic mutations may be due to environmental or dietary exposures to carcinogens, endogenous genotoxic products, and inherited aberrations in normal cellular processes, such as DNA repair, apoptosis or drug metabolism. Hanahan and Weinberg suggest that six essential characteristics can be identified in the over 100 unique types of cancers.² The essential characteristics include self-perpetuating growth signals, desensitization to anti-growth signals, absence of apoptosis, constitutive replication ability, angiogenesis, and invasion of adjacent tissue and subsequent metastasis.²

The development of cancer, or tumorigenesis, appears to be a multi-step process. Cancer begins as a neoplasm, which Pitot defines as a heritably altered and

autonomous growth of tissue.³ The alterations are passed on to the cell's progeny and are relatively autonomous. A neoplasm undergoes initiation, the process whereby an irreversible change in a single cell is caused by exposure to a chemical, physical or biological agent that is mutagenic to DNA.³⁻⁴ Metabolism, DNA repair, and cell proliferation are three important processes for this stage. The effectiveness of an initiating agent appears to be dependent on the metabolism of xenobiotics, or compounds that are not typically found endogenously. The agent has been shown to be most effective when it is present during the DNA synthesis phase of the cell cycle both *in vitro* and *in vivo*. In addition, at least one round of cell division in the presence of the initiation factor is required in order for the cell to become truly initiated.³

An initiated cell must often be promoted in order to progress to a malignancy. Promotion is the reversible stage of progression where an agent that is able to alter gene expression and/or apoptosis supports the growth of initiated cells, but does not directly interact with DNA.³⁻⁴ A promoting agent selectively augments cell replication of pre-neoplastic cells, yet inhibits apoptosis of neoplastic cells.³ In the promotion phase, cells require the constant presence of the promoting agent.³⁻⁴

The clinical disease state of a malignant neoplasm is the progression stage, where a number of initiated and promoted cells transition to rapidly growing, virulent, and malignant cells with the hallmark characteristic of irreversible karyotypic instability.³ In this stage, it is common to observe mutations in proto-oncogenes, such as c-Src, tumor suppressor genes and irreversible changes in gene expression, such as those due to alterations in DNA methylation.³

C. Introduction to breast cancer

i. **Breast cancer epidemiology.** Breast cancer is the most diagnosed cancer in women in the United States. In 2010, approximately 207,090 women were diagnosed with and 39,840 died of breast cancer. Currently, one in every eight women will be diagnosed with breast cancer in their lifetime.¹ About 60 to 65 percent of all diagnosed breast cancer cases consist of estrogen receptor (ER) positive tumors and of these, 60 to 65 percent will respond to hormonal treatment.⁵

ii. **Causes of breast cancer.** Although the exact causes of breast cancer are unknown in many cases, several prominent theories have been suggested. Only about 5 percent of breast cancer cases can be directly attributed to a germ line mutation, such as in the case of a *BRCA1* deletion or mutation.⁶ Clearly, additional factors beyond genetics are involved in breast carcinogenesis.

A major theory attributes steroid hormones, both endogenous and xenobiotic in nature, as the cause of many breast cancers. It has been hypothesized that endogenous or xenobiotic steroid hormones can either directly bind to endogenous hormones or other factors, or alter the levels and ratios of endogenous hormone metabolites. Both routes result in the aberrant proliferation of breast cells.⁷

Several forms of evidence support the theory that hormones can cause breast cancer. The breast cancer cell line, MCF-7, exhibits increased rates of proliferation when treated with 17β -estradiol (E_2).⁸ Treatment of the murine cell line C57/MG with 16α -hydroxyestrone caused DNA damage, an increase in proliferation, and production of soft agar colonies, indicating attachment-free growth.⁹ Increased levels of cell cycle entry markers were observed when MCF-7 and T-47D cells were treated

with E₂ or the xenoestrogens (synthetic estrogens) DDT or Red Number 3.¹⁰ At the *in vivo* level, the genotoxic metabolite, 4-hydroxy-catecholesterol, has been observed at particularly high levels in breast tumors as compared to normal breast tissue.¹¹ In addition, high estrogen levels in post-menopausal women are associated with an increased risk for breast cancer.¹²

D. Drug Metabolism

i. Phase I metabolism. Endogenous compounds, such as hormones, bilirubin, and bile acids, and exogenous compounds, such as carcinogens and drugs, are metabolized by the body to eliminate activity and facilitate excretion. Phase I metabolizing reactions create a more polar, and often more active, compound by adding or unmasking a functional group such as OH-, NH₂-, or SH-.¹³ The CYP450s are responsible for the phase I metabolism of 80 percent of clinical drugs¹⁴ and are localized to the endoplasmic reticulum (EPR) membrane.³

ii. Phase II metabolism. Phase II metabolizing reactions involve the conjugation of a polar moiety to a substrate, resulting in a more polar, and typically inactive, form of the parent compound.^{13, 15} This process is not only considered to be a detoxification pathway,¹⁶ but also facilitates excretion of the compound from the body.^{13, 15} Enzymes involved in this process include the UDP-glucuronosyltransferases (UGT), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione-S-transferases (GSTs), and methyltransferases. Phase II enzymes are typically located in the cytosol of the cell,^{3,15} however, the UGTs are mostly localized to the EPR membrane.¹⁶ The UGTs represent the predominant

enzyme family involved in drug metabolism as 40 to 70 percent of clinical drugs are glucuronidated.^{13, 17}

E. The role of estrogen in breast cancer

In the classic genomic signaling of estrogen, E₂ passively diffuses through the plasma and/or nuclear membrane, where it binds to the ER located in the cytosol and nucleus and causes a conformational change in tertiary and quaternary structure¹⁸ that results in dimerization of the ER.¹⁹ This forms an active ligand-receptor complex that is able to bind to DNA response elements that are located upstream of steroid-responsive genes²⁰ and often causes transcription,^{19, 21} but may also repress transcription.²²

High circulating estrogen levels have been associated with cancers of the breast, ovary, and endometrium.²³ Phase II metabolism via the UGTs (discussed further in section G) and other enzymes has been hypothesized to play an important role in circulating steroid levels. In a small study of 170 healthy, premenopausal women, variant *UGT1A1* and *UGT2B4* genotypes were associated with increased E₂ and the androgen, dehydroepiandrosterone (DHEA) levels, respectively.²³

F. Tamoxifen

i. Introduction to tamoxifen. Tamoxifen (TAM; 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(Z)-ene) is a nonsteroidal triphenylethylene antiestrogen that was approved by the Federal Drug Administration (FDA) in 1977 for the treatment of breast cancer. A 47 percent annual reduction in recurrence rate and a 26 percent annual reduction in death rate are observed in

women taking TAM for 5 years.²⁴ The typical prescribed dose is 20 mg per day, as a higher dose has not been found to provide additional benefit.²⁵ TAM is readily absorbed by the body following oral administration and the serum half-life is 7 to 14 days, with steady state levels reached in 4 weeks.²⁶ The major route of elimination is feces, although a small amount is excreted through the urine.²⁷ The drug is administered to patients in the *trans*-isomer form, due to its higher affinity for the ER.²⁸⁻²⁹ The anti-estrogenic moiety of the compound is thought to be the dimethylaminoethoxy side chain and the *trans* configuration.²⁸⁻²⁹

TAM is generally considered to be a well-tolerated therapy; however, treatment can produce many side effects, some of which may cause compliance issues and discontinuation of treatment. Side effects include menopausal-like symptoms, such as hot flashes (occurs in at least 50 percent of patients), vaginal dryness and discharge, irregular menses, nausea, insomnia, depression, fatigue, as well as retinopathy, increased risk of endometrial cancer, endometrial hyperplasia, endometrial polyps, increased endometrial thickness, ovarian cysts, and thromboembolic events.²⁹ In 1996, the International Agency for Research on Cancer classified TAM as a carcinogen for the endometrium. Studies have found the hazard ratio to be 2.4 for endometrial cancer and the increased risk is associated with duration and dose of TAM usage.^{24, 30-31}

ii. The mechanism of action of tamoxifen. The mechanism of action of TAM is similar to that of estrogen signaling and depending on the tissue, can act as either an agonist or antagonist of E₂. TAM is a selective estrogen receptor modulator (SERM),³² which competes with E₂ for binding at both ER α and ER β . Binding of TAM

to the ER causes a conformational change resulting in the dimerization and subsequent binding to the ER response element (ERE) that results in either the stimulation or inhibition of the expression of estrogen-regulated genes, respectively.³³⁻³⁶ ER α and ER β are two distinct receptors for estrogens generated from unique genes that have differential tissue expression patterns. TAM or active TAM metabolites that bind to ER α act as an estrogen agonist; or binding to ER β results in estrogen antagonism.^{33, 36} In addition, tissue-specific factors appear to play a role in TAM's agonism or antagonism of E₂. For example, TAM elicits an E₂-agonistic effect in uterine tissue, but an antagonistic effect in breast tissue.³⁷ TAM blocks the tumor at the G₁ phase of the cell cycle, which slows proliferation. Interestingly, the *cis* isomer of a metabolite of TAM, 4-hydroxy-tamoxifen (4-OH-TAM), has been shown to accumulate in non-TAM responding breast tumors, despite the administration of TAM in the *trans* form.²⁹

iii. Tamoxifen metabolism. TAM has a complex metabolism pathway that involves multiple Phase I and Phase II metabolizing enzymes (discussed more in depth in section D). A large portion of orally administered *trans*-TAM is demethylated by cytochrome P450 (CYP) 3A4/5 to form *N*-desmethyl-TAM,³⁸ with the remaining either hydroxylated by CYP2D6 to form 4-OH-TAM,³⁸⁻⁴⁰ hydroxylated by CYP3A4/5 to form α -4-OH-TAM,³⁸ oxidated by flavin-containing monooxygenases 1 and 2 to form TAM-*N*-oxide,⁴¹⁻⁴² or *N*-glucuronidated by UGT1A4 to form TAM-*N*-glucuronide.⁴³⁻⁴⁴ The demethylated and hydroxylated metabolite *N*-desmethyl-4-OH-TAM (endoxifen)²⁷ is formed by either demethylation of 4-OH-TAM by CYP3A4/5 or the hydroxylation of *N*-desmethyl-TAM by CYP2D6.³⁸ Both *trans*-4-OH-TAM and *trans*-

endoxifen can be converted to the *cis* isomer either spontaneously⁴⁵ or by CYP1B1.^{39, 46} In addition, TAM, 4-OH-TAM, and endoxifen are found conjugated to glucuronic acid in the urine, bile, and feces of women taking TAM.⁴⁷⁻⁴⁸

iv. The major, active metabolites of tamoxifen. Early studies found 4-OH-TAM to have a greater affinity for the ER than TAM itself⁴⁹ and both 4-OH-TAM and endoxifen have up to 100-fold greater potency than TAM at inhibiting the estrogen-dependent proliferation of cells.⁵⁰ In addition, 4-OH-TAM and endoxifen have been shown to be essentially equal in affinity for ER binding, inhibition of estrogen-dependent cell line proliferation,⁵ antagonism of E₂-induced expression of the progesterone receptor,⁵¹ and induction of estrogen-responsive global gene expression in MCF-7 cell lines.⁵² Importantly, both metabolites are abundant in the plasma of TAM-treated women, although endoxifen is often present at levels 5- to 10-fold higher than 4-OH-TAM.^{27, 47, 53} These data suggest that TAM is a prodrug and that 4-OH-TAM and endoxifen are the main, active metabolites that elicit a response in breast tumors. As mentioned previously, TAM and its metabolites exist in the *trans* or *cis* isomeric configuration and can interconvert spontaneously or due to catalysis by CYP1B1.^{39, 45-46, 54}

In general, the *trans*, but not the *cis*, configuration is considered to have anti-estrogenic activity, although the data are not consistent on this point. For example, an early study utilizing rat uterus observed *trans*-4-OH-TAM to have much greater affinity for the ER than that of *cis*-4-OH-TAM.⁵⁵ Similarly, the *trans* isomer of 4-OH-TAM preferentially accumulates in MCF-7 cells, while the *cis* isomer remains in the

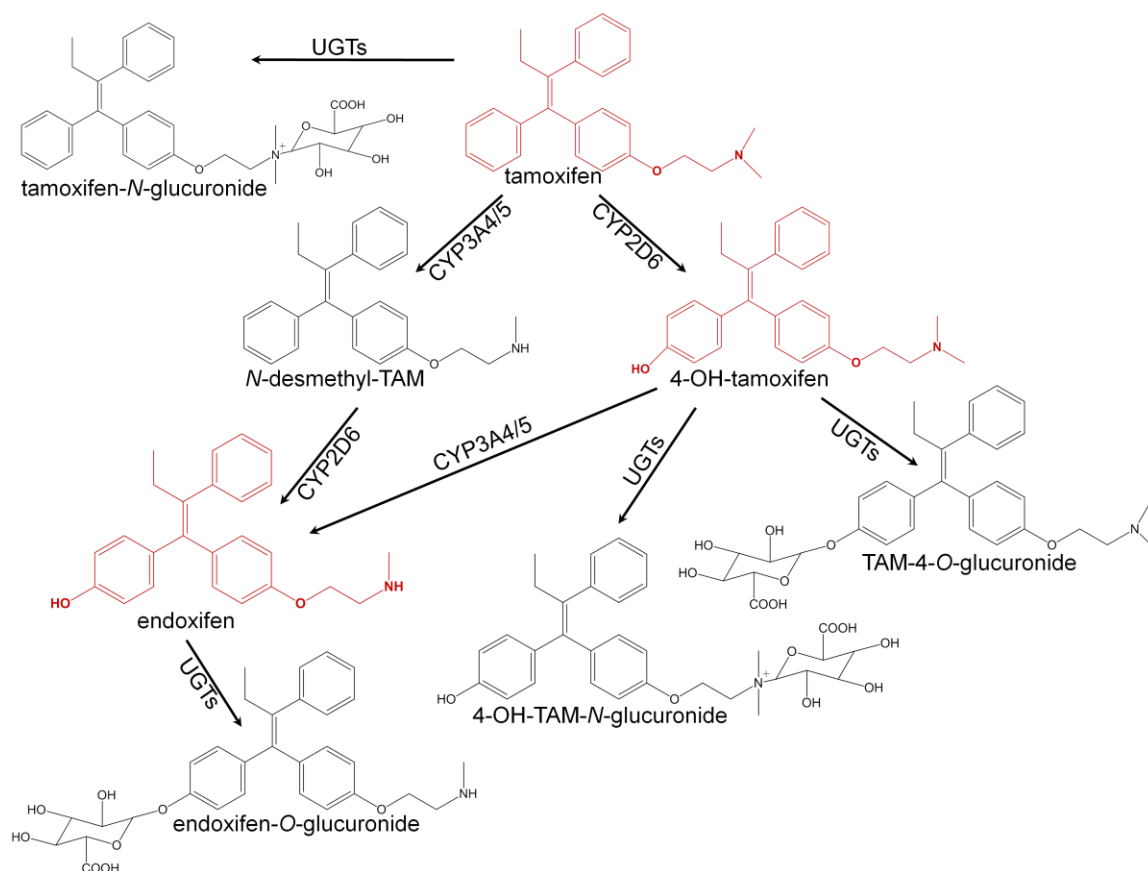


Figure 1-1. A simplified schematic of the tamoxifen metabolism pathway.

Tamoxifen is administered in the *trans* configuration and undergoes extensive metabolism by a variety of enzymes. Cytochrome P450's hydroxylate or demethylate tamoxifen to form the major, more active metabolites 4-hydroxy-*N*-desmethyl-tamoxifen (endoxifen) and 4-hydroxy-tamoxifen (4-OH-TAM) which are subsequently glucuronidated by the UGTs resulting in deactivation and elimination. All species are shown in the *trans* configuration, but are able to convert to the *cis* configuration.

cell media, leading to the almost exclusive association of the *trans* isomer with the ER.⁵⁴ In contrast, or perhaps due to unknown mechanisms, a later study found that *cis*-TAM had estrogenic activity in MCF-7 cells, but *cis*-4-OH-TAM and *trans*-4-OH-TAM had anti-estrogenic activity.⁵⁶ A more recent report illustrated that both the *trans* and *cis* isomers of 4-OH-TAM and endoxifen exerted equal inhibitory effects on progesterone receptor induction by E₂.⁵⁷ Clearly, more investigation will be required to resolve these experimental discrepancies.

v. Tamoxifen resistance. Although up to 65 percent of ER-positive tumors respond to hormonal therapy such as TAM,⁵ many of these tumors will acquire resistance.⁵⁸ Multiple hypotheses exist to explain the mechanism behind resistance to TAM therapy including alterations in the number or responsiveness of the ER, decreased dependency on E₂, reduced cellular accumulation of active TAM species, increased tumor accumulation of E₂, and multidrug resistance.⁵⁸⁻⁵⁹

Acquired TAM resistance does not appear to be related to the loss of the ER, as a majority of resistant tumors maintain ER expression. In this case, it is possible that despite the presence of the ER, other signaling pathways may become dominant. In addition, some breast cancer tumors appear to become dependent on TAM for growth, similar to the concept of tumor dependency on E₂. It is probable that in this case ER signaling occurs, but TAM adopts an agonist role due to unknown mechanisms. However, TAM-driven tumor growth is observed in a limited number of tumors and typically other hormonal therapy is effective.⁵⁸

An early study provided evidence that MCF-7 tumors in athymic mice become TAM resistance after 4 to 6 weeks of TAM treatment due to both decreased

accumulation of *trans*-TAM in the tumor and increased accumulation of *cis*-tamoxifen. Decreased tumor dependency on E₂ was not observed.⁵⁹ In addition, the accumulation of *cis*-4-OH-TAM was observed in human breast cancer patients receiving TAM therapy. Although TAM levels within tumors varied, a general trend of decreased levels was associated with TAM resistance.⁶⁰ Interestingly, when TAM and its metabolites are converted to analogs that cannot be isomerized, tumor dependency can still be acquired in mice.⁶¹ Clearly, other mechanisms instead of, or in addition to, accumulation of the less active and agonistic *cis* isomer, cause TAM resistance.

Multidrug resistance (MDR) is a phenomenon whereby a tumor acquires resistance to multiple anticancer drugs due to increased efficiency of drug transporters, enabling the tumor cells to remove drugs at an increased rate. This mechanism protects the tumor from the toxic effects of the drug. MDR can be accomplished by induction of the efflux transporters by the particular anticancer drug. Alternatively, drug treatment has been observed to result in over-expression⁶² and/or mutations that alter substrate specificity in transporter genes associated with MDR.⁶³⁻
⁶⁴ In addition, MDR-associated transporters, such as Abcg2, can influence methotrexate pharmacokinetics in mice.⁶⁵

Alterations in MDR-associated genes have been associated with acquired TAM resistance. The major mechanism for this is by induction of MDR-associated genes resulting in the increased expression of proteins that provide transport and efflux functions to cancer cells. For example, the multidrug resistance-associated protein (MRP) is expressed at higher levels in TAM resistant MCF-7 cells, as

compared to TAM sensitive MCF-7 cells.⁶⁶ Expression of multidrug resistant protein 8 (MRP8, or more commonly ABCC11) is also increased in TAM resistant MCF-7 cells. Interestingly, treatment of MCF-7 cells with E₂ reduced *ABCC11* mRNA expression, which was reversed when the cells were treated with TAM.⁶⁷ Implications of MDR in TAM resistance have been observed *in vivo*; variant *ABCC11* genotype has been associated with longer recurrence-free survival in breast cancer patients treated with TAM.⁶⁸

vi. Tamoxifen pharmacogenetics. Polymorphisms, such as single nucleotide polymorphisms (SNPs), are heritable changes in the germ-line DNA that can occur in intronic or exonic regions and may alter mRNA expression or protein function. The CYP450 demethylation and hydroxylation of TAM has been found to be an important pathway that can be affected by CYP450 polymorphisms. As previously stated, CYP2D6 hydroxylation of TAM and *N*-desmethylTAM is critical in the formation of 4-OH-TAM and endoxifen.^{38, 69} *CYP2D6* is a highly polymorphic gene, with 19 inactive and 7 reduced activity alleles currently identified in the population.⁶⁹ An inactive or less active enzyme would result in less TAM converted to 4-OH-TAM and endoxifen. Indeed, *in vivo* studies have found that *CYP2D6* status in women treated with TAM was associated with endoxifen plasma concentrations. Namely, *CYP2D6* genotypes that resulted in a reduced activity or an inactive enzyme had lower plasma levels of endoxifen.^{50, 69-70} In addition, TAM treated breast cancer patients that were CYP2D6 poor metabolizers, due to reduced activity or inactive CYP2D6, experienced increased recurrence, mortality rates and fewer side effects as compared to patients that were extensive metabolizers.^{50, 69} In this counterintuitive

situation, individuals who have reduced metabolism produce less endoxifen, the active product that is responsible for therapeutic benefit and side effects. Similarly, breast cancer patients treated with serotonin reuptake inhibitors (SSRIs) for hot flashes (in addition to TAM), have lower plasma levels of the active metabolite, endoxifen, because SSRIs are CYP2D6 inhibitors.⁷¹

Despite adjusting for CYP2D6 status, wide variability of 4-OH-TAM and endoxifen plasma concentrations are still detected in women treated with TAM.⁶⁹⁻⁷⁰ This suggests that additional mechanisms, such as other metabolic pathways, are important in 4-OH-TAM and endoxifen plasma concentrations and, potentially, patient outcome.

Glucuronidation is a major detoxification pathway that inactivates TAM metabolites^{57, 72} and facilitates their excretion from the body. Similar to the CYP450s, the UGTs are known to be highly polymorphic. **Therefore, it is important to characterize which UGTs are responsible for the metabolism of 4-OH-TAM and endoxifen and to subsequently determine how polymorphisms in these specific enzymes may be playing a role in patient response. This is the central focus of the present dissertation research program.**

G. The UDP-glucuronosyltransferases

i. UGT function. The UGTs predominately catalyze the conjugation of uridine 5'-diphospho- α -D-glucuronic acid (UDPGA) to a nucleophilic functional group such as a hydroxyl, amino, sulfuryl, carboxyl, or carbonyl moiety (Figure 1-2).^{13, 16, 73} However, *O*- and *N*-linked glucuronides are the predominant species. All UGT isoforms have been found to perform *O*-linked glucuronidation, which mainly forms

aryl-*O*-(phenolic)-glucuronides, but also acyl-*O*-glucuronides (at a carboxylic acid) and alkyl-*O*-(enolic)-glucuronides (such as in coumarin). *N*-linked glucuronidation is typically performed by UGT1A4 and UGT2B10 and can occur at non-quaternary amines, such as heterocyclic amines and primary and secondary amines. Moreover, tertiary amines, such as cyclic tertiary amines, alicyclic tertiary amines, and aromatic heterocyclic amines, can be conjugated.¹⁶

ii. UGT nomenclature. The first Arabic number in UGT nomenclature represents the family to which the enzyme belongs, the letter represents the subfamily, and the second Arabic number represents the specific gene. The UGT families share at least 40 percent nucleic acid homology and each enzyme within a family shares at least 60 percent.¹³ The UGT enzymes are classified into four families; 1, 2, 3 and 8 (Figure 1-3). The family names were assigned by the UDP Glycosyltransferase Nomenclature Committee in the early 1990's, although only two families had been discovered. UGT8 was the first family name reserved for any non-drug metabolizing UGT family, and families 3-7, which were not yet discovered, were reserved for the anticipated discovery of drug metabolizing UGT families.⁷⁴ Currently, only one additional drug metabolizing UGT family has been identified; the UGT3 family.

The gene clustering that is observed in the UGT families is an indication of gene duplication events. However, the UGT2B family in primates does not cluster with that of rodents, suggesting that separate and independent gene duplication events occurred following speciation.¹⁶

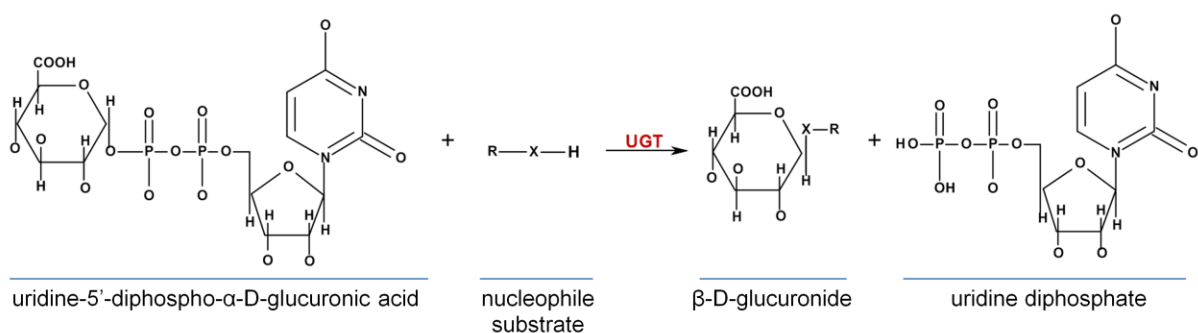


Figure 1-2. Glucuronide conjugation of a nucleophilic substrate by a UGT. The glucuronic acid moiety of uridine-5'-diphospho- α -D-glucuronic acid is conjugated to a nucleophilic substrate by UGT enzymes to produce a glucuronide-conjugate of the parent substrate and uridine diphosphate. The product is generally more easily excreted and generally inactivated.

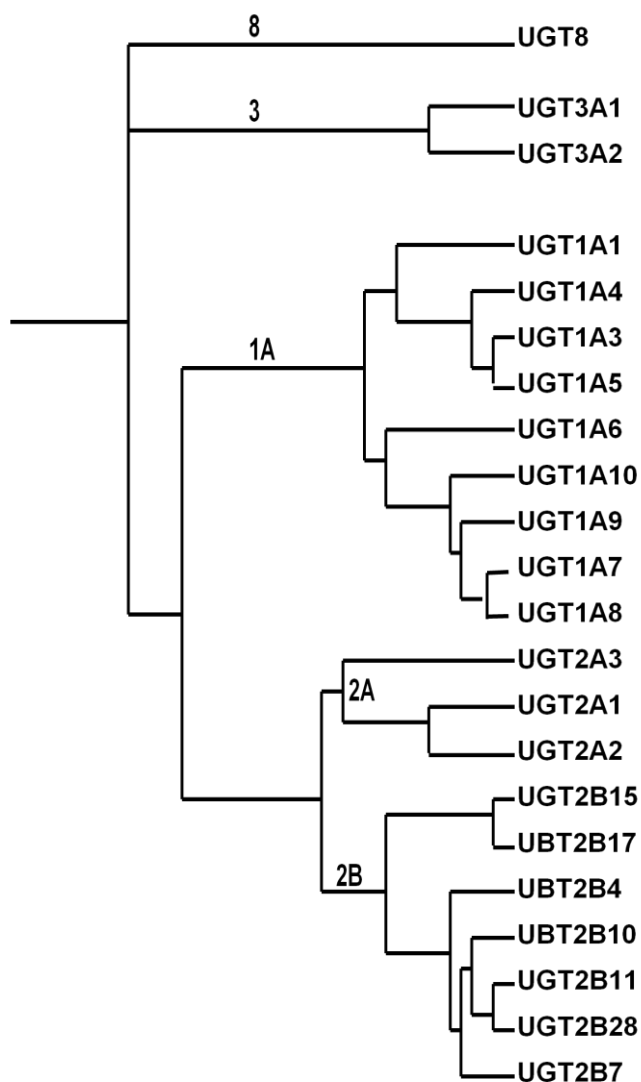


Figure 1-3. A dendrogram illustration of UGT family homology. The dendrogram illustrates the *UGT* gene clusters and relative homology. The *UGT* families all share at least 40 percent homology and share at least 60 percent homology within the individual families. *This figure was adapted from Mackenzie 2005.*

iii. UGT family and gene structure. The *UGT1A* family spans approximately 160 kb and is located on chromosome 2-q37 (Figure 1-4). The family members consist of 5 exons; a unique first exon that is transcribed and spliced to the common 2-5 exons, that results in 245 shared amino acids.^{16, 75} The 5' region of each exon 1 contains the promoter elements necessary for transcription and the 3' boundary region to each exon 1 contains the consensus sequence necessary for splicing via the RNA splicesome. The process of transcribing the individual genes of the *UGT1A* family is often referred to as alternative splicing, but current evidence suggests differently. Each transcript is independently produced following transcription initiation due to regulatory sequences flanking each exon 1 and alternative splicing is not involved. True alternative splicing is considered to occur when exons are spliced differentially after transcription has occurred. The *UGT1A* family encodes the following proteins: *UGT1A1*, *1A3*, *1A4*, *1A5*, *1A6*, *1A7*, *1A8*, *1A9*, and *1A10*. Of these, UGTs *1A7*, *1A8* and *1A10* have been found to have exclusive extra-hepatic expression (Table 1-1).¹⁶

In 2006, Levesque and colleagues discovered an additional exon located at the 3' end of the *UGT1A* gene locus that results in translation of a protein that is inactive.⁷⁶ Subsequently, the new exon was named exon 5b (as 5a is the original 5th exon), that can be spliced to exon 4 in lieu of, or in addition to, exon 5a. Exon 5b encodes a stop codon and therefore both variant genes translate into the same protein isoform, as the open reading frames are identical. Therefore, there are an additional 8 *UGT1A* protein isoforms, for a total of 16 translated *UGT1A* enzymes in humans (Figure 1-5). The variant gene products are referred to as “variants” and

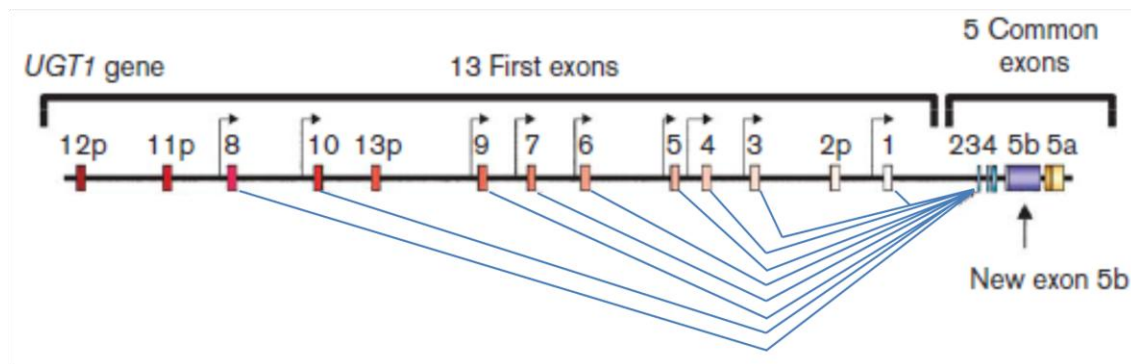


Figure 1-4. The *UGT1A* gene locus on chromosome 2q37. The promoter element of each unique first exon initiates transcription to the common exons 2-5, resulting in 245 shared amino acids. To date, nine translational and four pseudogenes in the *UGT1A* family have been identified at the 2q37 locus. *This figure was modified from Girard 2007.*

Table 1-1. Tissue expression of the human UGTs. The tissue site of UGT expression is listed with the associated literature references. The methods of detection included quantitative PCR and/or real-time PCR and/or immunoblot.

UGT	Tissue	Reference
1A1	liver, stomach, small intestine, colon, bladder	Nakamura 2008; Ohno 2008
1A3	liver, small intestine, colon, bladder	Nakamura 2008; Ohno 2008
1A4	liver, stomach, small intestine, colon, kidney, bladder, ovary	Nakamura 2008; Ohno 2008
1A5	liver, esophagus, stomach, small intestine, colon, brain, kidney, bladder, breast, uterus, prostate, cervix, heart, trachea	Nakamura 2008; Ohno 2008
1A6	liver, stomach, small intestine, colon, kidney, bladder, adrenal gland, trachea	Nakamura 2008; Ohno 2008
1A7	liver, esophagus, small intestine, colon, kidney, bladder, thymus, cervix, trachea, adipose	Nakamura 2008; Ohno 2008
1A8	small intestine, colon, kidney, bladder, adrenal gland, breast, trachea	Nakamura 2008; Ohno 2008
1A9	liver, esophagus, small intestine, colon, kidney, bladder, adrenal gland, testis	Nakamura 2008; Ohno 2008
1A10	esophagus, stomach, small intestine, colon, kidney, bladder, adrenal gland, ovary, uterus, trachea	Nakamura 2008; Ohno 2008
2A1	lung, larynx, trachea, tonsil, colon, floor of mouth	Bushey 2010
2A2	liver	Izukawa 2009
2A3	liver	Izukawa 2009
2B4	liver, esophagus, lung, kidney, bladder, adrenal gland, breast, uterus, testis, thymus, prostate, heart, trachea	Nakamura 2008; Ohno 2008
2B7	liver, lung, stomach, small intestine, colon, kidney, bladder, adrenal gland, breast, ovary, uterus, testis	Nakamura 2008; Ohno 2008
2B10	liver, bladder, ovary, uterus, testis	Nakamura 2008; Ohno 2008
2B11	liver, lung, stomach, small intestine, colon, kidney, bladder, breast, ovary, uterus	Nakamura 2008; Ohno 2008
2B15	liver, stomach, small intestine, colon, kidney, bladder, adrenal gland, breast, ovary, uterus, testis, prostate, trachea	Nakamura 2008; Ohno 2008
2B17	liver, lung, stomach, small intestine, colon, kidney, adrenal gland, breast, ovary, testis, brain, placenta, heart, trachea, spleen, adipose	Nakamura 2008; Ohno 2008
2B28	liver, bladder, breast	Nakamura 2008
3A1	liver, kidney, stomach, duodenum, colon, testes	Meech 2010
3A2	kidney, testes, thymus, trachea, spleen, prostate	Meech 2010; Mackenzie 2010

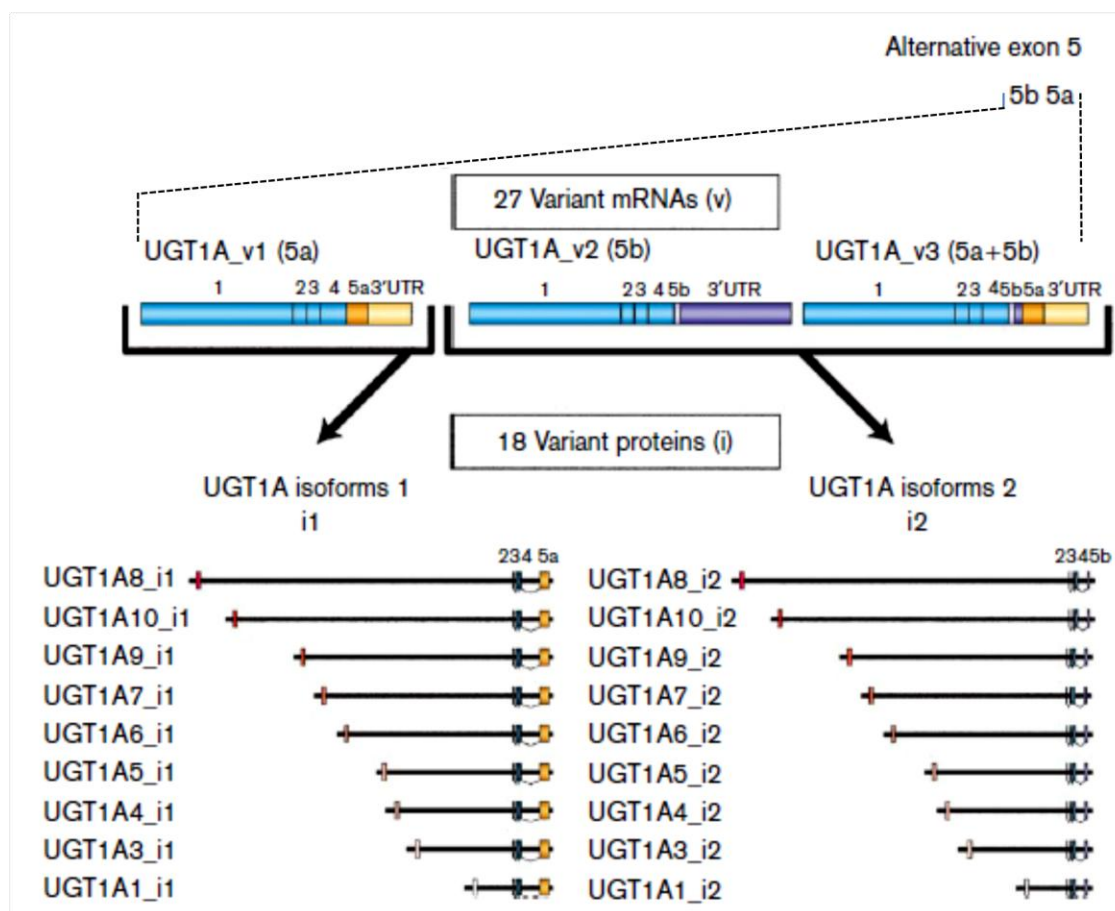


Figure 1-5. Alternative splicing of the *UGT1A* gene locus results in 18 *UGT1A* isoforms. Newly discovered exon 5b can be spliced instead of, or in addition to, exon 5a resulting in two gene variants that produce the same protein isoform. All i2 variant enzymes are inactive against a variety of substrates and can negatively regulate the i1 proteins. *This figure was modified from Girard 2007.*

denoted as *UGT1A_v1* for the wild-type variant containing the original exon 5a and *UGT1A_v2* or *UGT1A_v3* for the exon 5b or exon 5a and 5b containing variants, respectively. The protein is referred to as an “isoform” and denoted as UGT1A_i1 for the wild-type isoform and UGT1A_i2 for the product of *UGT1A_v2* or *UGT1A_v3*. The truncated isoforms have no glucuronidating activity against a variety of substrates and co-expression studies have found that they can negatively regulate the active isoform.⁷⁷ The isoforms were observed to form both homo-oligomeric and hetero-oligomeric complexes, where the i2-i2 complexes are inactive and the i1-i2 complexes have reduced activity, due to a decrease in maximal velocity (V_{max}).⁷⁸⁻⁷⁹

The UGT2A family is not yet well characterized, although initial reports have begun to investigate the expression and substrate targets of UGT2A1 and 2A2.⁸⁰⁻⁸² UGT2A1 and 2A2 are located on chromosome 4 and have 6 exons (Figure 1-6). Similar to the UGT1A family, the first exon is unique and the remaining 5 exons are shared.⁸³ The first transcripts were cloned from nasal mucosa, but recent studies have found UGT2A1 to be well expressed in the lung, larynx, trachea, tonsil, and colon.⁸⁰ Activity assays have demonstrated that UGT2A1 effectively glucuronidated polycyclic aromatic hydrocarbons (PAHs).⁸⁰

The UGT2B family is located on chromosome 4-q13 and consists of 6 unique exons (Figure 1-6).^{16, 75} Interestingly, three additional exons were recently identified in UGT2B4 that form three inactive splice-variant isoforms and are well expressed in human liver, gastrointestinal tract, and other tissues. The three inactive isoforms also decrease the activity of the wild-type, active isoform when co-over-expressed in mammalian cells.⁸⁴

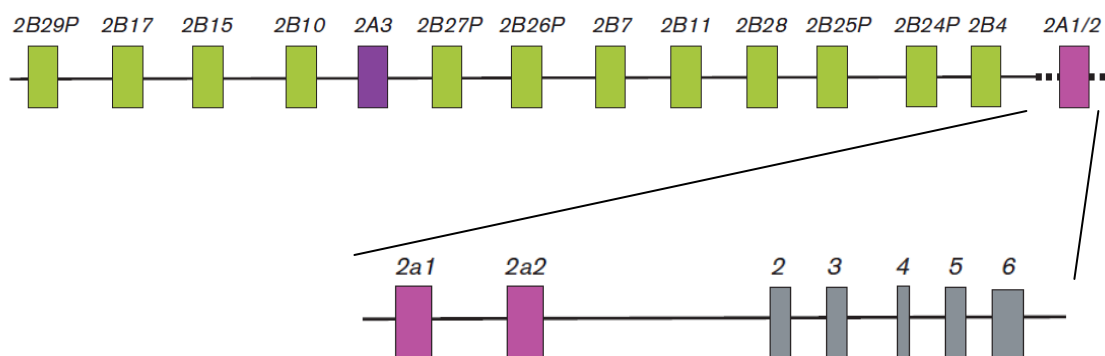


Figure 1-6. Gene map of the UGT2 family. The UGT2B family is located on chromosome 4q13 and each member consists of 6 exons. The UGT2B family includes 7 genes and 5 pseudogenes that are single, unique genes. The UGT2A family includes UGTs 2A1 and 2A2, which are similar to the UGT1A family in that a unique first exon is joined with the common 2-6 exons. Similar to the UGT2B family, UGT2A3 is a single, unique gene. *This figure was modified from Mackenzie 2005.*

The UGT3 family was the last to be identified and its function was recently determined to be that of an N-acetylglucosaminyltransferase. The two genes, UGT3A1 and 3A2, are located on chromosome 5p13.2 and share approximately 80 percent sequence homology, but only 40 percent to UGT1A1, and are comprised of 7 exons that result in a 50-53 kDa protein of 523 amino acids. Interestingly, UGT3A1 utilizes UDP-N- acetylglucosamine⁸⁵ and UGT3A2 utilizes UDP-glucose and UDP-xylose, as opposed to UDPGA, as the sugar donor.⁸⁶ A secondary bile acid and several estrogens are N-acetylglucosaminidated by UGT3A1 and it has been found to be expressed in the liver and some tissues of the gastrointestinal tract.^{85, 87} UGT3A2 conjugates a larger variety of substrates than UGT3A1, such as 4-methylumbelliferone (4-MU), 1-hydroxypyrene, bioflavones, and estrogens and has been found to be well expressed in the kidney, thymus, and testes.⁸⁶

The UGT8 family encodes the UDP-galactose ceramide galactosyltransferase enzyme, which is critical in the biosynthesis of important components of myelin including glycosphingolipids, cerebroside, and sulfatides. As indicated by the name, this enzyme utilizes UDP-galactose as the co-substrate and it is located in the EPR membrane. The gene consists of 5 exons, is located on chromosome 4q26, and is expressed in oligodendrocytes and Schwann cells.⁸⁸

The UGT1 and 2 families are the main UGT families that perform drug metabolism¹³ and are the focus of this dissertation. Therefore, the remainder of this dissertation is written in the context of the UGT1 and 2 families and the use of the abbreviation “UGT” will refer to only these two families.

iv. UGT polymorphisms. The UGT superfamily is known to be highly polymorphic, with both synonymous and non-synonymous single-nucleotide polymorphisms (SNPs) occurring frequently. Gene deletions and microsatellite polymorphisms have also been discovered. Several examples of the clinical importance of UGT polymorphisms are highlighted in section G.vii.

Non-synonymous SNPs cause an amino acid change in the protein, that often lead to alterations in enzyme activity, although, this is not always the case. Many SNPs and other polymorphisms cause a decrease or increase in enzyme activity or expression, which is often dependent on the substrate. For example the UGT2B7^{268Tyr} variant exhibited decreased activity against substrates such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).⁸⁹ Conversely, the UGT2B7^{268Tyr} variant exhibited an increase in activity against some morphine derivatives⁹⁰, 4-hydroxyestrone (4-OH-estrone), and 4-hydroxyestradiol.⁹¹ Some studies have observed the wild-type and variant isoforms of UGT2B7 to exhibit similar activity levels, such as with epirubicin⁹² and mycophenolic acid.⁹³

v. UGT structure. Recently, a partial crystal structure of UGT2B7 was obtained of the C-terminal region (Figure 1-7). Previous predictions classified the UGTs as members of the glycosyltransferase 1 (GT-1) family that adopt a GT-B fold, based on sequence homology and donor ligands. Enzymes with this classification consist of two Rossmann-type folds with a linker region, one domain being the N-terminus and the other being the C-terminus.⁹⁴⁻⁹⁵ The partial crystal structure of amino acids 285-451 confirm this prediction and show a Rossmann-type fold. A β -sheet consisting of six strands is located in the center of the structure and

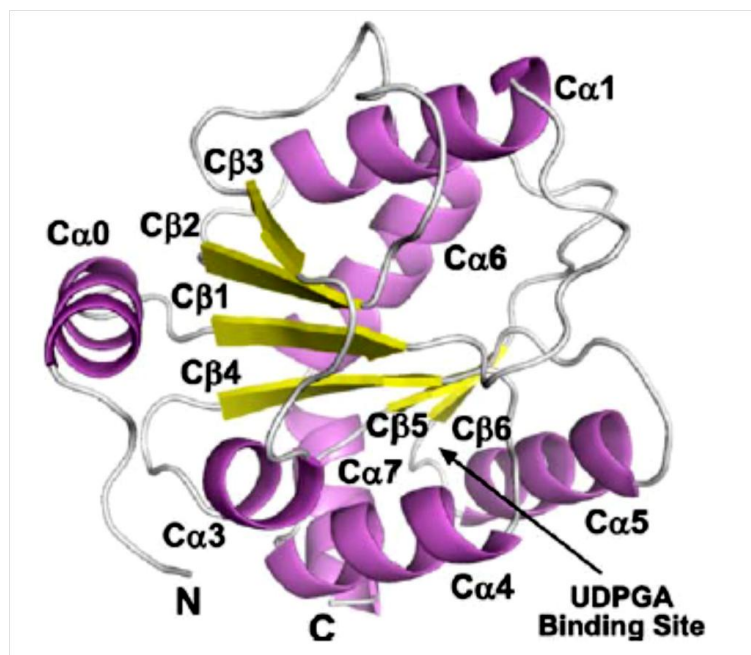


Figure 1-7. A ribbon-diagram of the UGT2B7 partial crystal structure. The C-terminal amino acids 285-481 of UGT2B7 was crystallized to a 1.8 Å resolution and includes the proposed UDPGA binding site. *Figure was modified from Miley 2007.*

surrounded by seven α -helices. Because the crystal structure is of the C-terminal end of the enzyme, the proposed UDPGA binding site is observed and is similar to that of glycosyltransferases in bacteria and plants. Unfortunately, the polypeptide of the crystal structure did not include the amino acid that is altered in the highly prevalent UGT2B7 polymorphism, His268Tyr.⁹⁶

Despite the slightly different genetic organization of the UGTs, only the 280 amino-terminal amino acids are divergent. For example, all UGTs, except for UGT1A10, have the N-terminal EPR-retention signal peptide, that is cleaved off following enzyme insertion into the EPR membrane.¹⁶ However, studies of UGT1A6 localization to the EPR found that the EPR-retention signal is not required for EPR retention, but that amino acids 140-240 are important and that a dilysine motif located at the C-terminal stop-transfer sequence alone is sufficient for retention.⁹⁷⁻⁹⁸

vi. UGT localization. The UGTs are transmembrane proteins generally localized to the EPR membrane with the catalytic site positioned within the EPR lumen with a short C-terminal cytosolic segment.^{13, 75, 87, 97, 99} A study has reported localization to the nuclear membrane as well.⁹⁹ This orientation requires that substrates and UDPGA be transported into the lumen of the EPR, by unknown mechanisms and the nucleotide sugar transporters (NSTs), respectively.¹⁰⁰⁻¹⁰² The NSTs are antiporters that transport UDPGA into the lumen in exchange for UDP-N-acetylglucosamine.¹⁰¹⁻¹⁰² Evidence suggests that newly conjugated glucuronides are rapidly transported out of the EPR lumen and into the cytosol by facilitated diffusion using organic anion transporters.¹⁰⁰

vii. UGT pharmacogenetics. The UGTs are well expressed in a variety of tissues at levels that vary among individuals (Table 1-1). In the liver, a recent study of 25 different human liver samples identified the following UGT mRNA expression by real-time PCR: UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15 and 2B17. Barely detectable levels of expression were found for UGTs 1A5, 1A7, 1A8, 1A10, 2B11, and 2B28, and this low level of expression is probably not physiologically relevant.¹⁰³

Inter-individual variation in UGT expression and activity is common and Bock has suggested that it is driven by three main factors; 1) genetic diversity due to polymorphisms, alternate splicing events, and epigenetics, 2) liver-enriched transcription factors, and 3) ligand-activated transcription factors.⁷⁵ In addition to these factors, recent evidence suggests that post-translational factors such as dimerization¹⁰⁴⁻¹⁰⁷ and phosphorylation¹⁰⁸⁻¹¹¹ may also play an important regulatory role in UGT activity.

UGT expression is inducible and several transcription factor ligands have been linked to this process, such as the aryl hydrocarbon receptor (AhR), the farnesoid X-receptor (FXR), and the pregnane X-receptor (PXR).¹¹²⁻¹¹³ In addition, some UGT-targeted substrates have been shown to regulate the transcription of the active UGT, suggesting UGTs are involved in feedback loops.¹¹⁴ For example, bilirubin, the by-product of heme catabolism, was observed to elicit a time and dose-dependent rise in UGT1A1 mRNA levels when incubated with rat liver microsomes.^{112, 114} In addition, UGT1A1 transcription has been shown to be regulated by the AhR and known ligands for AhR upregulate the transcription of UGT1A1. A recent study has linked

the previous data and illustrated that the response element in the UGT1A1 promoter is activated by bilirubin via an AhR-mediated pathway.¹¹⁴⁻¹¹⁵ Other transcription factor ligands, such as the bile acid and the FXR and the PXR, as well as the androgen dihydrotestosterone (DHT) and the androgen receptor (AR), have also been found to regulate the expression of some UGTs.¹¹⁴ Interestingly, a promoter polymorphism in linkage disequilibrium with the *UGT2B7*2* variant prevented induction by Nrf2, a transcription factor that induces wild-type UGT2B7 via an ARE-like promoter element.¹¹³

Induction of the UGTs by exogenous compounds has also been reported. UGT1A1 has been induced by drugs such as dexamethasone, a synthetic glucocorticoid,¹¹⁶ rifampicin, clotrimazole, carcinogens such as benzo[a]pyrene¹¹⁷ and dietary components such as the red wine polyphenol resveratrol, curcumin,¹¹⁸ the isothiocyanate sulforaphane,¹¹⁹ chrysin and multiple other flavonoids.¹¹⁷⁻¹¹⁸ Interestingly, exogenous compounds selectively induce the UGTs. For example, treatment of Caco-2 cells with chrysin results in the induction of UGT1A1, but not UGTs 1A6, 1A9, and 2B7.¹²⁰ Induction activities have been found to be regulated by enhancer elements,¹¹⁷ DNA response elements often mediated by the aryl hydrocarbon receptor (AhR) pathway,¹²¹ and cell signaling pathways.¹¹⁹

H. UGT2B7 phosphorylation by Src

i. Introduction to Src. Src, a non-receptor tyrosine kinase originally discovered as viral-Src (v-Src), a viral gene of the Rous Sarcoma virus found in chickens, is able to trigger cellular transformation.¹²²⁻¹²³ Cellular Src (c-Src) is a physiological gene that is present in all animals but not yeast, bacteria, or plants, and

is ubiquitously expressed in all tissues and cell-types. c-Src is a proto-oncogene, and can become an oncogene when altered or over-expressed. Aberrant gene expression of c-Src can cause dysregulation of the cell cycle and has been associated with cancer. However, c-Src also plays a critical role in a variety of normal cellular processes, such as differentiation, proliferation, cell division,¹²⁴⁻¹²⁵ survival,¹²⁶ cell adhesion, cell motility,¹²⁶⁻¹²⁸ morphology, and bone remodeling and reabsorption.^{124, 129-130} During most of the cell cycle, c-Src is dormant. c-Src only becomes activated during the G₂/M transition and is required for cell division in fibroblasts.¹²⁵ In fibroblasts, c-Src is found bound to endosomes, perinuclear membranes, secretory vesicles, and the cytoplasmic face of the plasma membrane,¹³¹⁻¹³⁵ as well as in the cytoplasm and perinuclear region of the Golgi apparatus.¹³⁴⁻¹³⁵

ii. Src in cancer. Many studies have reported increased levels of c-Src in human cancers such as breast, colon, gastric, lung, pancreatic, neural, and ovarian.¹²³ Cell lines that express high levels of activated Src become more invasive *in vivo*¹³⁶ and are associated with metastasis in animal models.¹³⁷ In addition, breast cancer exhibits increased Src activity as compared to normal tissue.¹³⁸ Interestingly, cells that acquire TAM resistance exhibit increased levels of Src and there is an association in ER-positive patients of activated Src in the cytoplasm of their breast tumor and reduced survival time with endocrine therapy. MCF-7 cells that over-express active Src develop a greater migratory and invasive behavior and their growth is not inhibited when treated with TAM. When these cells are treated with a

Src specific inhibitor, their morphology changes so that the cells appear to regain their cell-to-cell contacts and both migratory and invasive behavior is decreased.¹³⁹

iii. UGT phosphorylation. Recent studies have suggested that the UGT1A family is phosphorylated by protein kinase C (PKC)¹⁰⁸⁻¹⁰⁹ and UGT2B7 is phosphorylated by Src.¹¹¹ A database search with UGT2B7 identified three putative PKC sites, located at Thr123, Ser132, and Ser437 and two tyrosine kinase (TK) sites, located at Tyr236 and Tyr438. Curcumin, which is both a PKC and a Src inhibitor, decreased UGT2B7 activity without affecting PKC-site phosphorylation. In addition, site-directed mutations of the PKC sites did not alter enzyme activity, suggesting that PKC site phosphorylation is not important for UGT2B7 activity. However, site-directed mutations at either or both TK sites resulted in decreased enzyme activity. Transient co-transfection of UGT2B7 with Src caused a 1.5-fold increase in UGT2B7 activity against 4-OH-estrone, an endogenous substrate for UGT2B7.¹¹¹ These data suggest that UGT2B7 is phosphorylated by Src and that phosphorylation is important for enzyme activity.

In contrast, Abe and colleagues reported that UGT1A protein, but not mRNA, levels were decreased when treated with PKC inhibitors, such as curcumin, and not tyrosine kinase inhibitors. In addition, immunoprecipitation studies did not provide evidence of phosphorylation of the UGT1As. This study found that a decrease in 4-MU glucuronidation was a result of reduced protein and probably not phosphorylation.¹⁴⁰ Clearly, more research is needed to determine if phosphorylation is truly playing a role in UGT activity.

I. UGT pharmacogenetics is clinically significant

i. **Hyperbilirubemia is caused by UGT1A1 polymorphisms.** Several clinically significant syndromes have been observed that are a result of inherited polymorphisms in the UGTs. The most prominent are those characterized by hyperbilirubemia, a disorder where blood levels of bilirubin reach 35 $\mu\text{M/L}$ or greater. If circulating bilirubin levels exceed 300 $\mu\text{M/L}$, it will cross the blood-brain barrier and cause fatal necrosis of neurons and glial tissue. Bilirubin is the natural breakdown product of heme, 80 percent of which is due to the catalysis of hemoglobin and 20 percent is due to free circulating heme and the breakdown of heme-containing proteins such as CYP450s, tryptophan pyrrolase, and catalase.¹⁶

Bilirubin is glucuronidated almost exclusively by UGT1A1 and polymorphisms in the *UGT1A1* gene can lead to serious clinical outcomes.¹⁴¹ The most severe is the autosomal recessive Crigler-Najjar Syndrome¹⁶ which can be caused by up to 50 different mutations in UGT1A1.¹³ Crigler-Najjar Syndrome is further classified into two types, based on the amount of UGT1A1 enzyme activity remaining. Type I is characterized by the absence of UGT1A1 activity¹³ and causes nonhemolytic icterus and kernicterus, the accumulation of bilirubin in glial cells and nerve terminals, within the first several days of life.¹⁶ Homozygotes of the syndrome die in early childhood and the only available treatment is immediate liver transplant.¹⁶ Crigler-Najjar Syndrome Type II is characterized by a severe deficiency in UGT1A1, but at least 10 percent of enzyme activity remains, probably due to impaired transcription,¹⁴² which results in a milder hyperbilirubemia and individuals who are affected live into

adulthood without neurological impairment.^{13, 16} Treatment includes induction therapy (such as with phenobarbital) or phototherapy.¹⁶

The third hyperbilirubemia syndrome is the autosomal dominant Gilbert Syndrome which is primarily caused by a polymorphism in the TATAA box region of the *UGT1A1* promoter that is present in about 10 percent of the population.^{13, 143} It is clinically benign, as only a mild form of hyperbilirubinemia results,¹³ with an onset typically due to physiological stress, infection, fasting or physical activity.¹⁶ Individuals affected by Gilbert Syndrome do not require treatment.¹⁶

ii. The *UGT1A1* TATAA box polymorphism. The *UGT1A1**28, *36 and *37 alleles are characterized by variant TA repeat(s) between nucleotides -23 and -38, upstream of the ATG start-site. The wild-type promoter that results in full transcription of *UGT1A1* contains A(TA)₆TAA. The initial study that reported that an additional TA repeat caused Gilbert's Syndrome determined that the repeat caused an 82 to 67 percent decrease in luciferase activity, most likely due to a decrease in the binding ability of the transcription factor IID. In addition, every Gilbert's Syndrome patient studied had the A(TA)₇TAA element in the *UGT1A1* promoter (*UGT1A1**28).¹⁴¹ Subsequent studies have found 8 TA repeats in some Gilbert's Syndrome patients (*UGT1A1**37), which also resulted in decreased luciferase expression, and 5 TA repeats (*UGT1A1**36), that caused an increase in the luciferase activity as compared to the wild-type 6 TA repeats. Interestingly, the prevalence of this polymorphism varies in different populations. The 7 TA repeats occur in about 39 percent of Caucasians, 43 percent of blacks, and 16 percent of

Asians and 5 and 8 TA repeats are predominately found in blacks at 3.5 percent and 6.9 percent, respectively.¹⁴⁴⁻¹⁴⁵

iii. UGT pharmacogenetics of anti-cancer agents. The UGTs are critical in the metabolism and ultimate excretion of a variety of clinical drugs. A prominent example of this is the drug irinotecan. Irinotecan and its major, active metabolite, SN-38, is an FDA-approved topoisomerase I inhibitor for the treatment of colon cancer as a monotherapy or in combination. The information pamphlet for irinotecan has been updated to include a warning about the association between *UGT1A1*28* genotype and potentially fatal neutropenia. SN-38 is mainly glucuronidated by UGT1A1, but other UGT1A family members have been found to also be involved in the metabolism pathway. Diarrhea and neutropenia are the most common potentially fatal side effects and both have been linked to polymorphisms in the UGTs. The *UGT1A1*28* allele is associated with irinotecan-induced neutropenia and the FDA now recommends that clinicians test patient *UGT1A1*28* status to aid in determining the proper irinotecan dosage.¹⁴⁶

The promising FDA-approved histone deacetylase suberoylanilide hydroxamic acid (SAHA) is glucuronidated in the liver primarily by UGT2B17. The deletion polymorphism of *UGT2B17* was recently observed to significantly reduce SAHA glucuronidation in human liver microsomes (HLM).¹⁴⁷ Interestingly, stratification by sex revealed that males exhibited greater UGT2B17 glucuronidation against SAHA, as well as other compounds.¹⁴⁸

J. Hypothesis and Aims

An overarching goal of Dr. Philip Lazarus' laboratory is to study how the pharmacogenetics of the UGTs effect the metabolism of a variety of agents. The information presented in this chapter identifies several important points in regard to the UGTs and tamoxifen metabolism. When this research began, much of the role of the UGTs in tamoxifen metabolism was unknown, yet this phase II family has been determined to be important in the overview of tamoxifen and its pharmacotherapeutic action. The overall hypothesis for this dissertation is that the UGTs play an important role in the phase II metabolism of the major, active metabolites of tamoxifen and that polymorphisms in the UGTs most active against these metabolites significantly alter enzyme activity. The aims of this dissertation are:

1. To identify which UGTs are important in the metabolism of *trans*-endoxifen and to assess how the polymorphic isoforms alter enzyme activity. The UGTs active against *trans*-4-OH-TAM were previously determined in the laboratory.¹⁴⁹⁻¹⁵⁰
2. To test the hypothesis that *UGT* genotypes are associated with human liver microsomes (HLM) phenotype in the glucuronidation activities of *trans*-endoxifen and *trans*-4-OH-TAM.
3. To test the hypothesis that the potential phosphorylation of UGT2B7 by Src alters enzyme activity against *trans*-endoxifen and *trans*-4-OH-TAM.

Chapter 2

Identification of the UGTs that are active against *trans*-endoxifen

NOTE: The data reported in this chapter are based on the published manuscripts:

Sun D, Sharma AK, Dellinger RW, Blevins-Primeau AS, Balliet RM, Chen G, Boyiri T, Amin S, Lazarus, P. Glucuronidation of active tamoxifen metabolites by the human UDP glucuronosyltransferases. *Drug Metab Dispos.* 2007. 35(11): 2006-2014.

Blevins-Primeau, AS, Sun D, Chen G, Sharma AK, Gallagher CJ, Amin S, Lazarus P. Functional significance of UDP-glucuronosyltransferase variants in the metabolism of active tamoxifen metabolites. *Cancer Res.* 2009. 69(5): 1892-1900.

All figures presented in this chapter are the work of A.S.B-P., except the kinetic analyses of UGT1A8^{173Gly} and UGT1A8^{277Tyr}, which were performed by D.S. Immunoblot analysis of UGT1A8^{277Tyr} was also performed by D.S. (results not shown).

A. Abstract

Tamoxifen (TAM) is a non-steroidal selective estrogen receptor modulator that was approved by the FDA in 1977 for the treatment of breast cancer. Although it is generally well tolerated, significant adverse effects have been reported, including severe hot flashes and an increased risk for venous thromboembolism and endometrial cancer. The phase I metabolism of TAM is primarily catalyzed by CYP2D6 and CYP3A4/5, resulting in the major, active metabolites *N*-desmethyl-4-hydroxy-tamoxifen (endoxifen) and 4-hydroxy-tamoxifen (4-OH-TAM). Interestingly, the presence of *CYP2D6* variant genotypes having an inactive or less active phenotype result in greater levels of circulating endoxifen and are also associated with adverse clinical outcomes. The phase II metabolism of TAM is not yet well characterized. However, glucuronide conjugates of TAM and its metabolites have been observed in the plasma and urine of women treated with TAM. In addition, previous studies have found TAM 4-OH-TAM to be *N*⁺-glucuronidated by UGT1A4. The goal of the present study was to identify which UGTs are involved the *O*-glucuronidation of *trans*-endoxifen. All of the UGT1A family member isoforms catalyzed *O*-glucuronidation of *trans*-endoxifen, except for UGT1A4. In contrast, only UGT2B7 of the UGT2B family was able to perform *O*-glucuronidation of the metabolite. Kinetic analyses indicated that the extra-hepatic UGTs 1A8 and 1A10 and the hepatic UGT2B7 were the most active UGTs against *trans*-endoxifen. In addition, the UGT1A8^{173Lys}, UGT1A8^{277Tyr}, and UGT2B7^{268Tyr} variants exhibited reduced activity against *trans*-endoxifen, as compared to the wild-type isoforms. The UGT1A10^{139Lys} variant demonstrated similar activity as compared to its wild-type

counterpart. These data suggest that UGTs 1A8, 1A10, and 2B7 may play an important role in TAM metabolism. In particular, women with a *UGT1A8* or *UGT2B7* variant genotype may glucuronidate *trans*-endoxifen at a reduced rate, resulting in increased circulating levels of a major, active metabolite, that may ultimately affect therapeutic response.

B. Introduction

i. Tamoxifen. Tamoxifen (TAM; TAM, 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(Z)-ene) is a nonsteroidal triphenylethylene antiestrogen that was approved by the Federal Drug Administration (FDA) in 1977 for the treatment of breast cancer. A 47 percent annual reduction in recurrence rate and a 26 percent annual reduction in death rate are observed in women taking TAM for 5 years.²⁴ The drug is administered to patients in the *trans*-isomer form, due to its higher affinity for the estrogen receptor (ER).²⁸⁻²⁹

TAM is generally considered to be a well-tolerated therapy; however, treatment can produce many side effects, some of which may cause compliance issues and discontinuation of treatment. Side effects include menopausal-like symptoms, such as hot flashes (occurs in at least 50 percent of patients), vaginal dryness and discharge, irregular menses, nausea, insomnia, depression, fatigue, as well as retinopathy, increased risk of endometrial cancer, endometrial hyperplasia, endometrial polyps, increased endometrial thickness, ovarian cysts, and thromboembolic events.²⁹

ii. Tamoxifen metabolism. A large portion of orally administered *trans*-TAM is metabolized by the CYP450s, including demethylation by CYP 3A4/5 to form *N*-desmethyl-TAM³⁸ and hydroxylation by CYP2D6 to form 4-OH-TAM.³⁸⁻⁴⁰ The demethylated and hydroxylated metabolite *N*-desmethyl-4-OH-TAM (endoxifen)²⁷ is formed by either demethylation of 4-OH-TAM by CYP3A4 or the hydroxylation of *N*-desmethyl-TAM catalyzed by CYP2D6.³⁸ Both *trans*-4-OH-TAM and *trans*-endoxifen can be converted to the *cis* isomer either spontaneously⁴⁵ or by CYP1B1.^{39, 46} In

addition, TAM, 4-OH-TAM, and endoxifen are found conjugated to glucuronic acid in the urine, bile, and feces of women taking TAM.⁴⁷⁻⁴⁸ Reports indicate that the *trans* isomers of 4-OH-TAM and endoxifen are more abundant than the *cis* isomers, possibly at a ratio of 70:30, at physiological pH.^{45, 72}

A potentially important route of elimination and detoxification of TAM and its metabolites is via glucuronidation. TAM is excreted largely through the bile which is primarily facilitated by the conjugation of glucuronic acid.⁴⁷ Glucuronide conjugates of TAM and its metabolites have been identified in the urine and serum of women administered TAM therapy.^{27, 47} *N*⁺-glucuronidation by UGT1A4 has been demonstrated to occur for both TAM and 4-OH-TAM. Moreover, the UGT1A4^{48Val} variant was shown to exhibit increased *N*⁺-glucuronidation activity *in vitro* against 4-OH-TAM as compared with the wild-type UGT1A4^{48Leu} isoform.⁴⁴

An important factor associated with endoxifen plasma concentrations *in vivo* is *CYP2D6* genotype.^{50, 69-70} Despite adjusting for *CYP2D6* status, previous studies still detected wide variability of 4-OH-TAM and endoxifen plasma concentrations in the plasma of women treated with TAM.⁶⁹⁻⁷⁰ This suggests that additional mechanisms, such as other metabolic pathways, are important in determining 4-OH-TAM and endoxifen plasma concentrations and, potentially, patient treatment outcomes.

iii. Hypothesis and goals. The identification of the UGTs that are active against *trans*-4-OH-TAM is reported elsewhere.¹⁴⁹⁻¹⁵⁰ The hypothesis of the present study is that the UGTs perform the *O*-glucuronidation of *trans*-endoxifen and that UGT polymorphisms alter enzyme activity against *trans*-endoxifen. The first goal of the present study is to identify which UGTs glucuronidate *trans*-endoxifen. With this

information, the second goal is to determine if prevalent SNPs alter the activity of important *O*-glucuronidating UGTs against the *trans* isomers of 4-OH-TAM and endoxifen, and could therefore play an important role in patient response to TAM.

C. Materials and Methods

i. Chemicals and materials. UDPGA and alamethicin were purchased from Sigma-Aldrich (St. Louis, MO). Endoxifen was synthesized in the Organic Synthesis Core Facility at the Penn State College of Medicine, with the *trans*-endoxifen isomer purified as described previously.¹⁴⁹ HPLC-grade ammonium acetate and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Dulbecco's modified Eagles medium, Dulbecco's phosphate-buffered saline (minus calcium chloride and magnesium chloride), fetal bovine serum, penicillin-streptomycin and geneticin (G418) were purchased from Gibco (Grand Island, NY). The Platinum® *Pfx* DNA polymerase and the pcDNA3.1/V5-His-TOPO mammalian expression vector were obtained from Invitrogen (Carlsbad, CA). The BCA protein assay kit was purchased from Pierce (Rockford, IL) and the QIAEX® II gel extraction kit was purchased from Qiagen (Valencia, CA). The human UGT1A western blotting kit and anti-UGT1A antibody were purchased from Gentest (Woburn, MA). All other chemicals used were purchased from Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

ii. Generation of the UGT stably expressing cell lines. Several new UGT stably expressing cell lines were generated for the present study. The human embryonic kidney (HEK) 293 cell line was utilized for UGT stable expression because it does not endogenously express any UGTs, thereby allowing individual UGT isoforms to be studied. The UGT1A8 stably expressing cell line was a gift previously received from Dr. Thomas Tephly (University of Iowa); however, the *UGT1A8* gene in this cell line was found to have missense polymorphisms located at +362 and +518 (relative to the translational start site). To generate the wild-type *UGT1A8* gene,

cDNA was created by reverse transcription of total RNA from the previously received UGT1A8 stably expressing cell line. Subsequently, *UGT1A8* was PCR amplified from the cDNA using the sense and antisense primers 1A8S and 1A8AS (Table 2-1; Figure 2-1, panel A). PCR amplification was performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) as follows: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min, followed by a final cycle of 7 min at 68°C. Site-directed mutagenesis (SDM) using the QuikChange SDM kit (Stratagene, La Jolla, CA) was performed on *UGT1A8* to change the variant base at nucleotide +362 from the polymorphic C to the wild-type T using the primer set 1A8 Δ S and 1A8 Δ AS. The primer set 1A8P173S and 1A8P173AS were then used to change the codon 173 variant nucleotide +518 from G to the wild-type C. SDM PCR amplification was performed as follows: 1 cycle of 95°C for 4 min, 25 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 16 min, and a final cycle of 68°C for 7 min. The *UGT1A8* wild-type PCR product was purified using the QIAEX II gel extraction kit after electrophoresis in 1.5 percent agarose and subsequently ligated into the pcDNA3.1/V5-His-TOPO mammalian expression vector (Figure 2-2) via T/A cloning sites, according to the manufacturer's protocol. The proper orientation of the PCR product was confirmed by RFLP (Figure 2-3, panel A) and the *UGT1A8* wild-type sequence was confirmed by dideoxy sequencing of the entire PCR-amplified *UGT1A8* cDNA product with the vector primers T7 and BGH (Integrated DNA Technologies, Inc., Coralville, IA) and a *UGT1A8* internal antisense primer (1A8intAS). The *UGT1A8*^{173Gly/277Cys} and *UGT1A8*^{173Ala/277Tyr} variants were generated by SDM of the pcDNA3.1/V5-His-TOPO plasmid containing the original (from Dr.

Table 2-1. Primers utilized for UGT cloning. The sense and anti-sense primers used for cloning UGTs 1A8, 1A8^{173Gly}, 1A8^{277Tyr}, and 2B17 are listed with the primer location relative to the translational ATG start site.

UGT	Primer Name	Primer Set	Location
UGT1A8	1A8S	5'-TTCTCTCATGGCTCGCACAGGG-3'	-7 to +15
	1A8AS	5'-CTCAATGGGTCTTGGATTTGTGGGC-3'	+1570 to +1594
	1A8173S	5'-TTTAACTTATTTT <u>TT</u> TCGCATTGCAGGAG-3'	+349 to +377
	1A8173AS	5'-CTCCTGCAATGCGAA <u>AAAA</u> ATAAGTTAAA-3'	+349 to +377
	1A8ΔS	5'-CCAGGGGAATAG <u>C</u> TTGCCACTATCTTG-3'	+506 to +532
	1A8ΔAS	5'-CAAGATAGTGGCAAG <u>C</u> TATTCCCCTGG-3'	+506 to +532
	1A8intAS	5'-GATAAGTTTCTCCACCACCGAC-3'	+125 to +147
UGT1A8 ^{173Gly}	1A8ΔS	5'-CCAGGGGAATAGCTTGCCACTATCTTG-3'	+506 to +532
	1A8ΔAS	5'-CAAGATAGTGGCAAGCTATTCCCCTGG-3'	+506 to +532
UGT1A8 ^{277Tyr}	1A8277S	5'-GTGGTATCAACT <u>A</u> CCATCAGGGAAAGCC-3'	+815 to +843
	1A8277AS	5'-GGCTTTCCCTGATGGT <u>A</u> GTTGATAACCAC-3'	+815 to +843
UGT2B17	2B17S	5'-CAACAACTGGAAAAGAAGCATTGCA-3'	-35 to -11
	2B17AS	5'-AATAAAGGAGGAGTCCCATCTTTTG-3'	+1622 to +1646

NOTE: Underline represents changed nucleotide in SDM primers

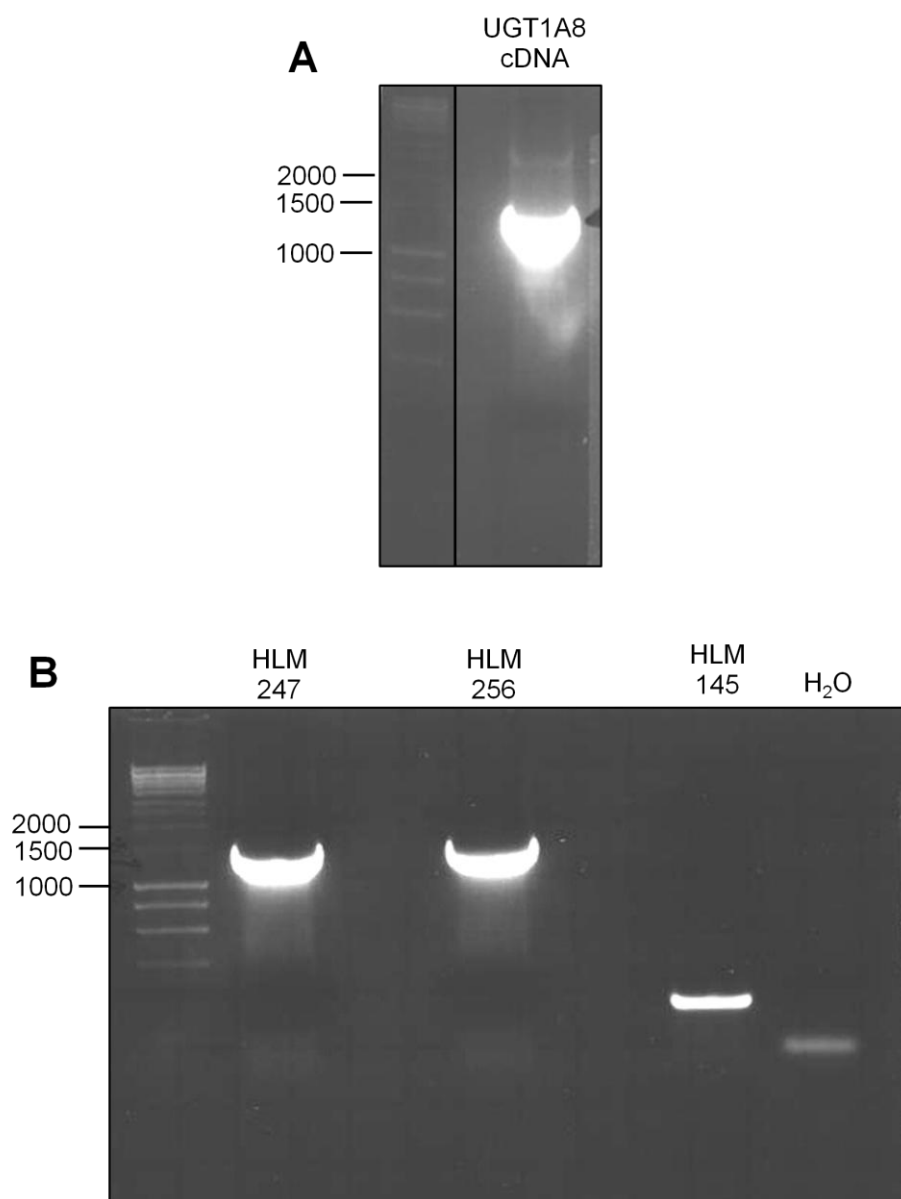


Figure 2-1. PCR products of *UGTs 1A8* and *2B17*. **A)** The *UGT1A8* PCR product was generated from the cDNA of a *UGT1A8* stably expressing cell line with missense polymorphisms. **B)** The *UGT2B17* PCR product was generated from HLM specimens 247 and 256 cDNA. HLM 145 represents the positive control, β -actin, and the negative control contained water instead of cDNA. All PCR product bands were excised and the correct sequence was verified by sequencing. The primer sequences used for each gene can be found in Table 2-1.

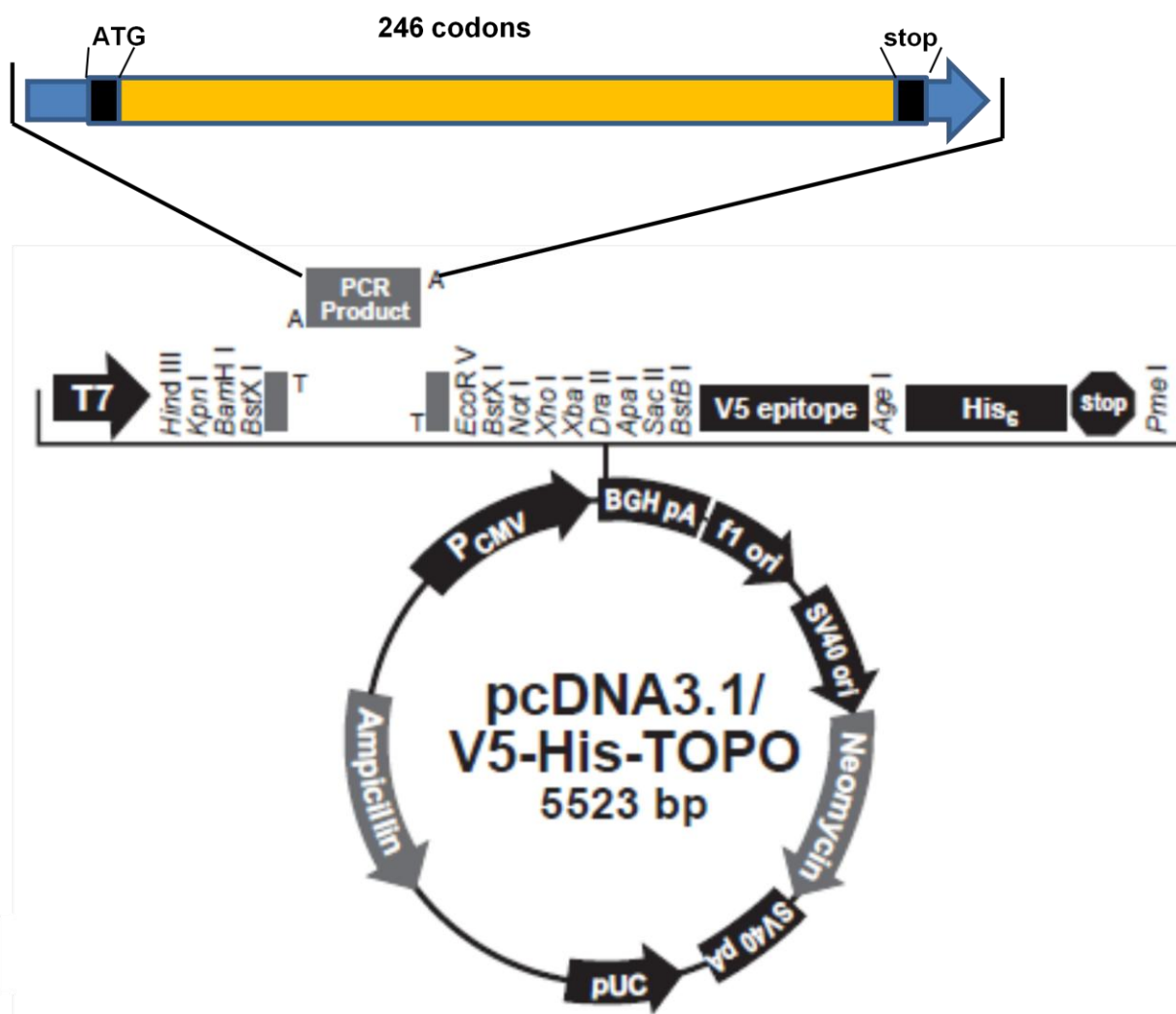


Figure 2-2. Diagram of the pcDNA3.1 vector for stable expression of the UGTs.

The pcDNA3.1/V5-His-TOPO vector manufactured by Invitrogen (Carlsbad, CA) contains an ampicillin resistance gene for bacterial selection and a neomycin resistance gene for mammalian cell selection. A CMV promoter is located upstream of the PCR product insertion site. *This figure was modified from Invitrogen.*

Tephly) or newly created wild-type *UGT1A8 gene*, respectively. To remove the missense polymorphism at +362, the SDM primers 1A8 Δ S and 1A8 Δ AS were used. The missense polymorphism at +518 was that of the codon 173 polymorphism and was not altered. The SDM primers used to create UGT1A8^{277Tyr} were 1A8277S and 1A8277AS (Table 2-1).

The UGT2B17 stably expressing cell line was generated by reverse transcription of adjacent normal human liver total RNA and subsequent PCR amplification (Figure 2-1, panel B) from the cDNA using the sense and antisense primers 2B17S and 2B17AS, respectively (Table 2-1). PCR amplification was performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) as follows: 1 cycle of 94°C for 4 min, 40 cycles of 94°C for 30 s, 59°C for 30 s, and 68°C for 2 min, followed by a final single cycle of 7 min at 68°C. The *UGT2B17* PCR product was purified using the QIAEX II gel extraction kit as above and subsequently ligated via T/A cloning into the pcDNA3.1/V5-His-TOPO mammalian expression vector according to the manufacturer's protocol. The proper orientation of the PCR product was confirmed by RFLP (Figure 2-3, panel B) and the *UGT2B17* sequence was confirmed by dideoxy sequencing of the entire PCR-amplified *UGT2B17* cDNA product using the vector primers T7 and BGH (Integrated DNA Technologies, Inc., Coralville, IA). The cloned wild-type *UGT1A8* and *UGT2B17*, as well as the variant *UGT1A8*, inserts were compared with sequences described in GenBank and were confirmed to be 100 percent identical to their relative sequences.

The UGT-containing plasmids were transfected into HEK293 cells by standard

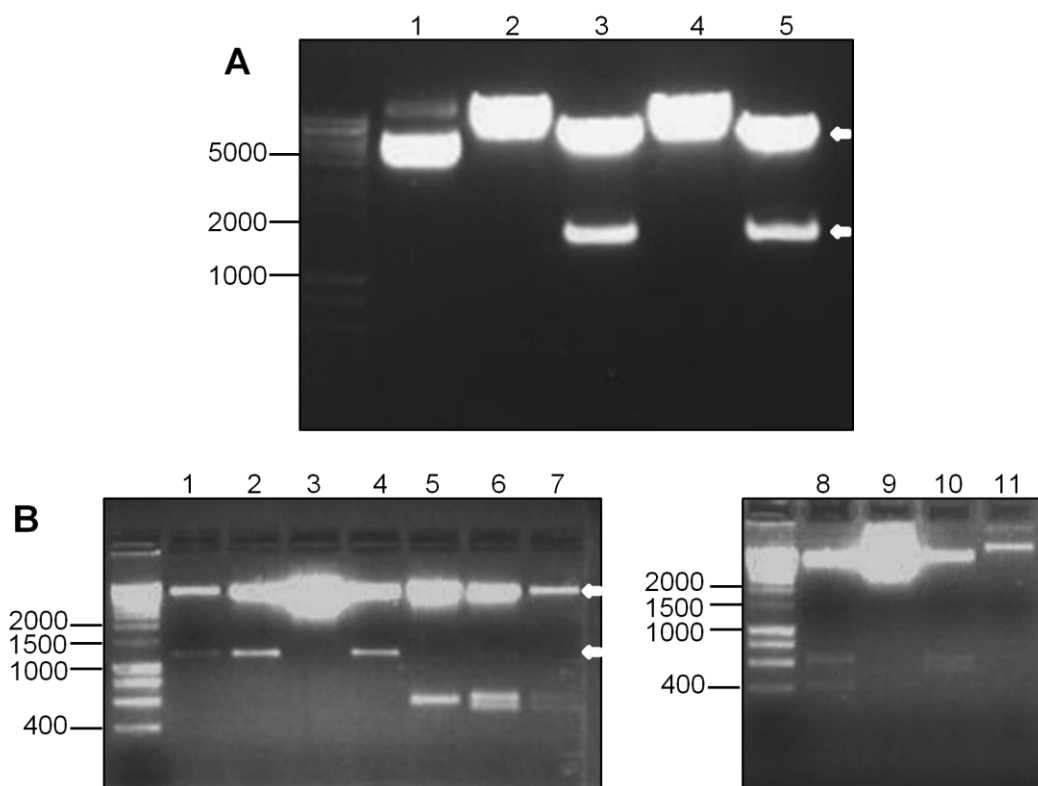


Figure 2-3. Vector digestion with restriction digest enzymes for determination of PCR product orientation. PCR products were ligated into the pcDNA3.1 vector and transformed into bacteria. Multiple independent clones (represented by numbers) were cultured. Following DNA extraction, DNA was incubated with a restriction digest enzyme to determine the orientation of the PCR product by RFLP. **A)** The *UGT1A8* containing vector was incubated with the enzyme KpnI (except lane 11 which was uncut), resulting in 1491 base pair (bp) and 5633 bp fragments if the orientation was correct. **B)** The *UGT2B17* containing vector was incubated with the enzymes BsrGI and XbaI resulting in 1100 bp and 6000 bp fragments, if the orientation was correct.

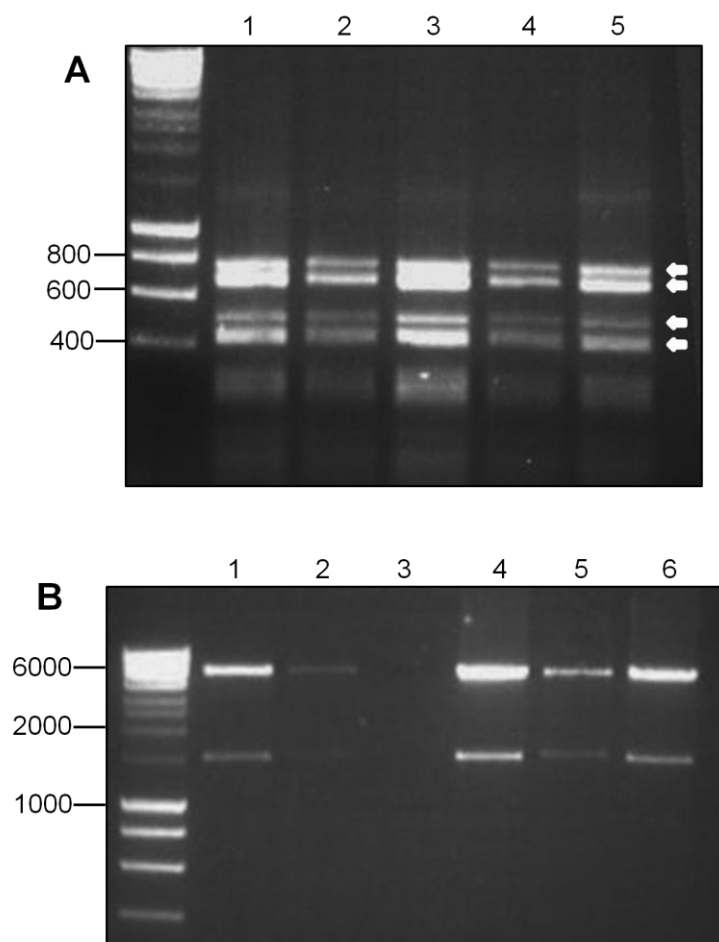


Figure 2-4. Vector digestion with restriction digest enzymes following site-directed mutagenesis. Vectors containing the putative variant UGT gene were incubated with a restriction digest enzyme to verify the success of the previously performed SDM or correct PCR product ligation. **A)** The *UGT1A8* wild-type containing vector was incubated with the enzyme *AluI*, which cuts the wild-type containing vector 4 times resulting in 4 bands. Sequencing confirmed lanes 2, 3, and 4 contained the correct gene. **B)** The *UGT1A8*^{277Tyr} containing vector was incubated with enzyme *KpnI* resulting in 1491 bp and 5633 bp fragments, if the gene contained the polymorphism and was in the correct orientation. Sequencing confirmed lanes 4, 5, and 6 contained the correct sequences.

electroporation techniques in a GenePulser Xcell (Bio-Rad, Hercules, CA) using 10 μ g of pcDNA3.1/V5-His-TOPO/UGT plasmid DNA with 5×10^6 of HEK293 cells in serum-free DMEM media, with electroporation at 250 V and 1000 μ F. Following transfection, cells were grown in 5 percent CO₂ to 80 percent confluence in DMEM supplemented with 4.5 mM glucose, 10 mM HEPES, 10 percent fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 700 μ g/mL of G418 for the batch selection of G418-resistant cells, with selection medium changed every 3 to 4 days. A small number of cells survived selection and were maintained until medium changes and cell detachment from the plate no longer caused cell death. Subsequently, cells were stored with DMEM containing 10 percent sterile DMSO in liquid nitrogen until required. When needed, cells were gently thawed in a 37°C water bath and plated with fresh supplemented DMEM medium, as above, and changed at 24 hours to remove the DMSO. Cells were passaged until an adequate number of plates, typically 20-25, were attained.

Additional cell lines stably expressing the UGT1A and UGT2B isoforms analyzed in this study were previously described.¹⁵¹⁻¹⁵³ All UGT stably expressing cell lines were maintained in DMEM with G418 as described above and were grown to 80 percent confluence before the preparation of cell homogenates by resuspending pelleted cells in Tris-buffered saline (25 mM Tris base, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) and subjecting them to three rounds of freeze-thaws before gentle homogenization with a glass dounce homogenizer. Cell homogenates were stored at -80°C in 200 μ L aliquots. Total homogenate protein concentrations were measured using the BCA protein assay.

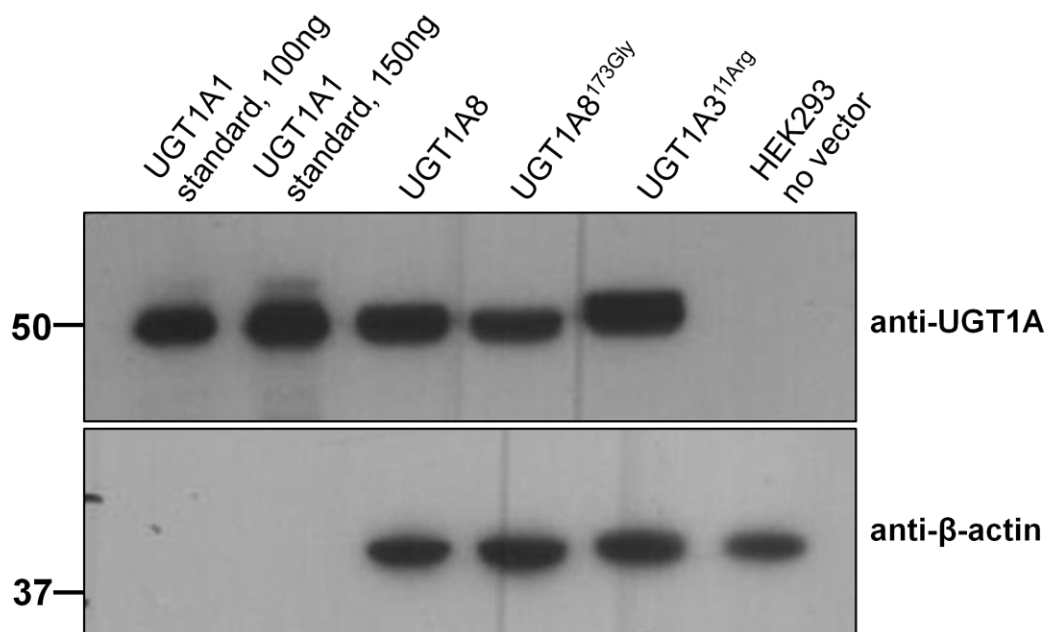


Figure 2-5. Representative immunoblot of UGT1A8 and 1A8^{173Gly} protein expression. 30 µg of protein that was extracted from cell homogenates and varying concentrations of UGT1A1 standard were loaded. Densitometry was performed using β-actin as the loading control. Immunoblot analysis was performed independently, in triplicate.

Protein expression of the UGT over-expressing cell lines was determined by immunoblot analysis, as previously described.⁴⁴ The anti-UGT1A and anti-UGT2B antibodies from Gentest were used to recognize all UGT1A family members and selected UGT2B family members, respectively, (as previously described).¹⁴⁹ Relative UGT protein levels were expressed as the mean of three independent experiments and all glucuronidation assays were normalized relative to UGT expression (as determined by quantitative immunoblots).

iii. Glucuronidation activity assays and kinetic analyses. The glucuronidation activities of UGT stably expressing cell lines against *trans*-endoxifen and 4-methylumbelliferone (4-MU) were performed as previously described.^{44, 149} Briefly, pores were formed in micelles after an initial incubation of 100-1000 μ g UGT cell homogenates with alamethicin (50 μ g/mg protein) for 15 min in an ice bath, then glucuronidation reactions were performed in a final reaction volume of 100 μ L at 37°C in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 4 mM UDPGA, and 8 to 725 μ M of *trans*-endoxifen. The glucuronidation reactions were terminated after 30 to 120 min by the addition of 100 μ L of cold methanol and incubated on ice for 10 min. The reaction mixtures were subsequently centrifuged for 10 min at 4°C at 16,100 g and the supernatant was collected for analysis by HPLC. All experiments were performed in triplicate.

The glucuronidation of *trans*-endoxifen was analyzed by HPLC as previously described^{44, 149} and reactions without *trans*-endoxifen were regularly analyzed as negative controls. HPLC analysis for 4-MU glucuronidation utilized the following gradient program: starting with 98 percent buffer A (100 mM NH₄Ac, pH 5.0)/2 percent

buffer B (100 percent acetonitrile) for 5 min, a linear gradient to 70% buffer B over 17.5 min was performed and then maintained at 70 percent for 10 min. The rate of glucuronide formation was measured based on the ratio of the glucuronide-conjugate and the free parent compound.

iv. Statistical analysis. The Student's t-test (2-sided) was used for comparing kinetic values of glucuronidation formation for UGT wild-type versus variant stably expressing cell lines. Kinetic constants were determined using GraphPad Prism4 software (GraphPad Software, La Jolla, CA).

D. Results

i. Glucuronidation activity screen of the UGTs against *trans*-endoxifen.

In order to determine which UGTs were able to glucuronidate *trans*-endoxifen, a glucuronidation activity screen with the homogenates of all UGT stably expressing cell lines was performed (Figure 2-6, panels A-C). Glucuronide peaks were confirmed by mass spectrometry, as reported elsewhere.¹⁴⁹ Only *O*-glucuronidation was observed, which is consistent with previous studies that demonstrated that UGT1A4 is the only UGT able to perform the *N*⁺-glucuronidation of TAM and 4-OH-TAM⁴⁴ and that endoxifen is not *N*-glucuronidated.¹⁵³ The UGTs 1A4, 2B4, 2B10, 2B15, and 2B17 did not exhibit glucuronidation activity against *trans*-endoxifen (Table 2-2). Glucuronidation of *trans*-endoxifen was observed in the homogenates of UGTs 1A1, 1A3, 1A7, 1A8, 1A9, 1A10, and 2B7 and the homogenates containing these UGTs were subsequently used for kinetic analyses. UGT1A5 was not available for analysis. Interestingly, UGT2B7 was the only UGT2B family member that was active against *trans*-endoxifen.

ii. Kinetic analyses of the UGTs capable of glucuronidating *trans*-endoxifen.

To examine the relative UGT enzyme glucuronidation capabilities of *trans*-endoxifen, kinetic analyses of the active UGTs were performed (Figure 2-7, panels A-C). As discussed in sections C.ii. and C.iii., the $V_{\max}$ was calculated per μg of UGT protein, as determined by immunoblot analyses of UGT stably expressing cell lines. UGT1A10 exhibited the lowest K_M value and UGTs 1A8 and 2B7 exhibited the second lowest K_M values (Table 2-2). In contrast, UGT1A1 demonstrated the greatest K_M value in the glucuronidation of *trans*-endoxifen. UGTs 1A8 and 1A10 demonstrated the

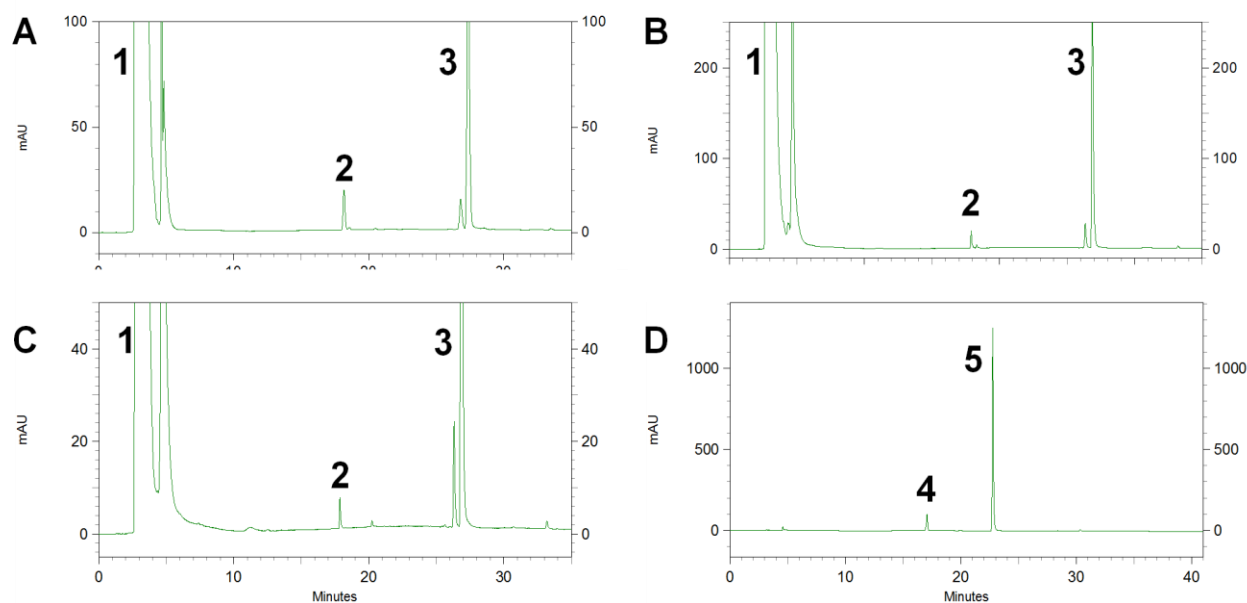


Figure 2-6. Representative HPLC chromatograms of the most active UGTs against *trans*-endoxifen and 4-MU. A) 500 μ g of UGT1A8, B) 250 μ g of UGT1A10, and C) 1 mg of UGT2B7 homogenates were incubated with *trans*-endoxifen for 60 min at 37°C. D) 1 mg of variant UGT1A8^{173Ala/277Tyr} homogenates were incubated with 4-MU for 120 min and analyzed by HPLC. Peak 1 represents UDPGA, the glucuronic acid co-factor; peak 2 represents the *trans*-endoxifen-*O*-glucuronide conjugate; peak 3 represents free *trans*-endoxifen; peak 4 represents 4-MU-*O*-glucuronide; and peak 5 represents free 4-MU.

Table 2-2. Screening results of UGT homogenates against *trans*-endoxifen. 500 µg of cell homogenates were incubated with *trans*-endoxifen from 3 hrs to overnight at 37°C and analyzed by HPLC.

UGT	Activity
1A1	+
1A3	-
1A4	-
1A5	N.D.
1A6	-
1A7	+
1A8	+
1A9	+
1A10	+
2B4	-
2B7	+
2B10	-
2B11	-
2B15	-
2B17	-
N.D., not determined	

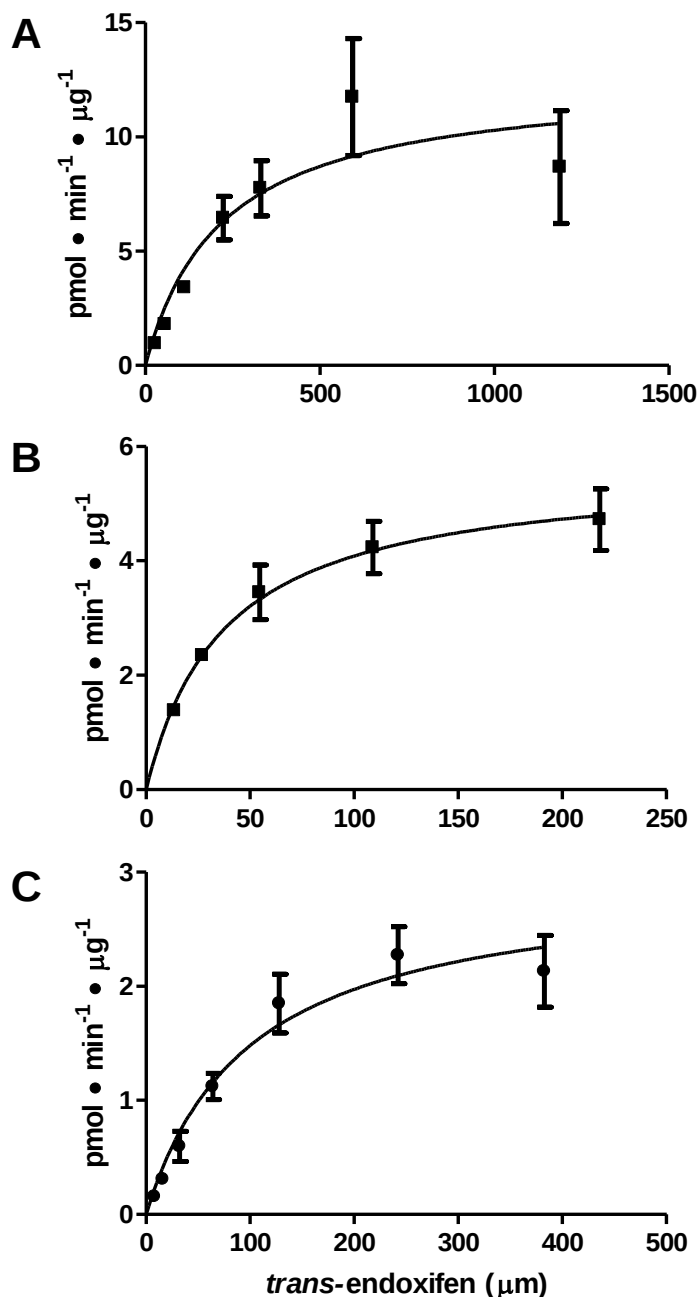


Figure 2-7. Representative kinetic curves of the glucuronidation of the most active UGTs against *trans*-endoxifen. Varying concentrations of *trans*-endoxifen, ranging from 8 to 594 μM , were incubated with **A)** 500 μg of UGT1A8, **B)** 250 μg of UGT1A10, and **C)** 1 mg of UGT2B7 homogenate and analyzed by HPLC. Michaelis-Menten kinetics were determined by GraphPad Prism software and summarized in Table 2-3.

Table 2-3. Kinetic analyses of the glucuronidation of *trans*-endoxifen. Michaelis-Menten kinetics were determined with the GraphPad Prism software based on the reaction rate of cell homogenates incubated with varying amounts of *trans*-endoxifen. The values are per μg of UGT protein, as previously determined by three independent immunoblots and values are representative of three independent experiments.

UGT	<i>trans</i> -endoxifen		
	$V_{\max}$ ($\text{pmol}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$)	K_M ($\mu\text{mol}\cdot\text{L}^{-1}$)	$V_{\max}/K_M$ ($\mu\text{L}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$)
1A1	2.3 ± 0.3	333 ± 60	0.0069 ± 0.0005
1A3	2.9 ± 0.4	158 ± 29	0.018 ± 0.001
1A7		N.D.	
1A8	11.6 ± 1.4	101 ± 13	0.12 ± 0.01
1A9		N.D.	
1A10	5.7 ± 0.7	39.9 ± 3.4	0.14 ± 0.005
2B7	3.0 ± 0.4	101 ± 17	0.030 ± 0.004

N.D., not determined due to low activity

greatest turn-over rate, based on the $V_{\max}$ values, and UGT1A1 had the lowest. According to the $V_{\max}/K_M$ value, a measure of overall enzyme activity, the *trans*-endoxifen glucuronidating activities of the UGTs were: 1A10 > 1A8 > 2B7 > 1A3 > 1A1. UGTs 1A7 and 1A9 demonstrated such low activity that kinetic analyses could not be performed. UGTs 1A8 and 1A10 are not expressed in the liver; therefore, UGT2B7 is the most active UGT expressed in the liver.

iii. Kinetic analyses of UGT variants and *trans*-endoxifen

glucuronidation. To determine if there were functional effects of prevalent UGT polymorphisms on *trans*-endoxifen glucuronidation, kinetic analyses were performed. The variant isoforms of UGTs 1A8, 1A10, and 2B7 were selected for this analysis due to the high activity of their wild-type counterparts against *trans*-endoxifen (Table 2-4). The variant UGT1A8^{173Gly/277Cys} exhibited a significant 1.5-fold decrease ($p < 0.05$) in glucuronidating activity as compared to the wild-type UGT1A8^{173Ala/277Cys} due to an increase in K_M . Interestingly, the UGT1A8^{173Ala/277Tyr} variant demonstrated no detectable glucuronidating activity against *trans*-endoxifen, but did glucuronidate 4-MU (Figure 2-6, panel D), a well known substrate of UGT glucuronidation and a positive control for activity. The UGT1A10^{139Lys} variant and the wild-type UGT1A10^{139Gly} isoforms demonstrated similar overall enzyme activity, despite a significant 3-fold reduction in $V_{\max}$ and a 13-fold reduction in K_M ($p < 0.01$). A significant 5-fold decrease ($p < 0.01$) in overall enzyme activity was observed in the homogenates of UGT2B7^{268Tyr}, as compared to the wild-type UGT2B7^{268His}. This difference was driven by a significant 5.5-fold decrease in $V_{\max}$ ($p < 0.01$).

Table 2-4. Kinetic analyses of the variant isoforms of the most active UGTs against *trans*-endoxifen. Activity assays of the wild-type (UGTs 2B7^{268His}, 1A10^{139Glu}, and 1A8^{173Ala/277Cys}) and variant cell homogenates were performed simultaneously under similar conditions. The values are per μg of UGT protein, as previously determined by three independent immunoblots and values are representative of three independent experiments. No activity was detected in homogenates of UGT1A8^{173Ala/277Tyr} against this substrate however, 4-MU glucuronidation was detected.

UGT isoform	<i>trans</i> -endoxifen		
	$V_{\max}$ ($\text{pmol}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$)	K_M ($\mu\text{mol}\cdot\text{L}^{-1}$)	$V_{\max}/K_M$ ($\mu\text{L}\cdot\text{min}^{-1}\cdot\mu\text{g}^{-1}$)
UGT2B7 ^{268His}	3.0 ± 0.44	101 ± 17	0.030 ± 0.004
UGT2B7 ^{268Tyr}	$0.55 \pm 0.01^*$	101 ± 15	$0.006 \pm 0.001^*$
UGT1A10 ^{139Glu}	5.7 ± 0.7	40 ± 3	0.14 ± 0.005
UGT1A10 ^{139Lys}	$1.9 \pm 0.2^*$	$13 \pm 2^*$	0.14 ± 0.004
UGT1A8 ^{173Ala/277Cys}	5.4 ± 0.2	98 ± 9	0.060 ± 0.004
UGT1A8 ^{173Gly/277Cys}	5.9 ± 0.2	135 ± 26	$0.040 \pm 0.005^{**}$
UGT1A8 ^{173Ala/277Tyr}	no detectable activity		

* $p < 0.01$, ** $p < 0.05$

E. Discussion

Glucuronide conjugates of TAM and TAM metabolites have been observed in the plasma and urine of women treated with TAM.⁴⁷⁻⁴⁸ In addition, previous studies demonstrated that UGT1A4 performed *N*⁺-glucuronidation of TAM and a major, active metabolite, 4-OH-TAM, *in vitro*.⁴³⁻⁴⁴ Demethylation of the dimethylaminoethoxy side chain appears to inhibit *N*-glucuronidation, as only *O*-glucuronide conjugates have been observed for endoxifen, the other major, active metabolite of TAM.¹⁵³

The goal of the present study was to identify which UGTs perform *O*-glucuronidation of *trans*-endoxifen, *in vitro*. Initially, the homogenates of all UGT stably expressing cell lines were screened for activity against these substrates. Only *O*-glucuronidation was observed, which was consistent with previous reports.¹⁵³ Interestingly, all isoforms of the UGT1A family, except for UGT1A4, performed *O*-glucuronidation against *trans*-endoxifen. In contrast, only UGT2B7 of the UGT2B family was able to glucuronidate *trans*-endoxifen.

UGTs that demonstrated activity were further examined by kinetic analysis. Based on the $V_{\max}/K_M$ values, an indication of overall enzyme activity *for a given substrate*, the exclusively extra-hepatic enzymes UGT1A8 and UGT1A10 were the most active UGTs against *trans*-endoxifen. UGT2B7 was the third most active UGT, but the most active of the hepatically-expressed UGTs. Interestingly, UGTs 1A8 and 2B7 expression have been observed in breast tissue,^{113, 154-155} the target of tissue of TAM. UGT2B7 is highly expressed in the liver,^{113, 155} the major detoxification organ. Therefore, the activity of these three UGT isoforms may play an important role in the

inactivation and elimination of the major, active metabolites of TAM, both systemically and at the target site of TAM.

The second goal of the present study was to examine how the previously identified polymorphisms (SNPs) in *UGTs* 1A8, 1A10, and 2B7 (Table 2-5)¹⁵⁶⁻¹⁵⁸ alter the glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM. Kinetic analyses were performed on the variant UGT stably expressing cell lines. Interestingly, homogenates of the polymorphic UGT1A10^{139Lys} exhibited similar glucuronidation activity as the homogenate of the wild-type UGT1A10 stably expressing cell line. In addition, the UGT1A10^{139Lys} variant is observed at very low prevalence in Caucasians (< 1 percent).¹⁵⁷ The prevalences of the polymorphic UGT1A8^{173Gly} and 1A8^{277Tyr} are up to 40 percent^{93, 158} and 2.4 percent,^{91, 93, 158} respectively (Table 2-5). In contrast to the polymorphic UGT1A10^{139Lys}, the variants UGT1A8^{173Gly}, UGT1A8^{277Tyr}, and UGT2B7^{268Tyr} resulted in a decrease in glucuronidation activity. These data suggest that SNPs in *UGT1A8* and *UGT2B7* result in a reduced activity enzyme and therefore, decreased *O*-glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM.

The pharmacogenetics of the phase I metabolism of TAM by CYP2D6 was previously examined. Lower plasma levels of endoxifen in women treated with TAM are associated with intermediate and poor metabolizers of TAM due to the presence of variant *CYP2D6* genotypes.^{50, 69, 71, 159} In addition, when the dose of TAM is increased in patients based on their *CYP2D6* genotypes, endoxifen levels also increase.¹⁶⁰ However, large variability in endoxifen plasma levels remain following stratification with *CYP2D6* genotype, suggesting that other mechanisms may be important in determining endoxifen plasma levels.^{69, 159}

Table 2-5. Frequencies of relevant SNPs in UGTs 1A8, 1A10, and 2B7.

<i>UGT</i> gene	mRNA		amino acid		frequency (%)**	source
	location*	change	codon	change		
<i>UGT1A8</i>	+518	C > G	173	Ala > Gly	14.5 - 40.6	Huang <i>et al.</i> , 2002; Bernard <i>et. al.</i> 2006; HapMap
<i>UGT1A8</i>	+830	G > A	277	Cys > Tyr	1.2 - 2.4	Thibaudeau <i>et al.</i> , 2006; Huang <i>et. al.</i> , 2002; Bernard <i>et. al.</i> , 2006
<i>UGT1A10</i>	+415	G > A	139	Glu > Lys	< 1†	Elahi <i>et al.</i> , 2003; HapMap
<i>UGT2B7</i>	+802	T > C	268	His>Tyr	49 - 54	Lampe <i>et al.</i> , 2000; Wiener <i>et al.</i> , 2004; Mehlotra <i>et al.</i> , 2007

*from ATG translational start site, **in Caucasian populations

†2.5 - 4% in blacks (Elahi *et al.*, 2003 and HapMap)

The pharmacogenetics of the phase II metabolism of the *trans*-endoxifen and *trans*-4-OH-TAM was examined in the present chapter of this dissertation.

Polymorphic UGTs 1A8 and 2B7 have significantly reduced enzyme activity against *trans*-endoxifen and *trans*-4-OH-TAM, *in vitro*. Decreased UGT enzyme activity may cause an increase in plasma levels of endoxifen or 4-OH-TAM in women treated with TAM who are heter- or homozygous for the variant *UGT1A8* and/or *UGT2B7* genotype. Higher levels of circulating endoxifen or 4-OH-TAM may improve the clinical response to TAM, but may also result in greater or more severe adverse events.

In summary, the present data identified the extra-hepatic UGTs 1A8 and 1A10, and the hepatic UGT2B7, as having important roles in the metabolism of the major, active metabolites of TAM. Individuals who have variant genotypes for *UGT1A8* and *UGT2B7* may glucuronidate *trans*-endoxifen and *trans*-4-OH-TAM at a reduced rate, thereby resulting in higher circulating levels of the TAM metabolites. Long-term clinical studies are needed to fully assess the outcomes of women that bear variant *UGT* genotypes and undergoing TAM therapy, to better personalize treatment regimens.

Chapter 3

***UGT2B7* genotype and glucuronidation phenotype of *trans*-endoxifen and *trans*-4-OH-TAM**

NOTE: The results reported in this chapter represent unpublished data and portions of the published manuscript:

Blevins-Primeau, AS, Sun D, Chen G, Sharma AK, Gallagher CJ, Amin S, Lazarus P. Functional significance of UDP-glucuronosyltransferase variants in the metabolism of active tamoxifen metabolites. *Cancer Res.* 2009. 69(5): 1892-1900.

All figures presented in this chapter are the work of A.S.B.-P., except the mass spectra of *trans*-endoxifen-*O*-glucuronide and *trans*-4-OH-TAM-*N*⁺-glucuronide in HLM and breast tissue, which were performed by G.C., and the *UGT2B7* mRNA expression analysis by real-time PCR, which was performed by C.J.G.

A. Abstract

Tamoxifen (TAM) is a selective estrogen receptor modulator widely used in the prevention and treatment of breast cancer. A major mode of metabolism of the major active metabolites of TAM, 4-OH-TAM and endoxifen, is by glucuronidation via the UDP-glucuronosyltransferase (UGT) family of enzymes. A previous report and Chapter 2 of this dissertation indicated that hepatic UGTs 1A4 and 2B7 are highly active against the TAM metabolites. In addition, their respective variants have significantly altered glucuronidation activity. To determine if *UGT* genotype influences human liver microsome (HLM) phenotype, glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM by 111 HLM specimens was performed. The rate of O-glucuronidation against *trans*-4-OH-TAM and *trans*-endoxifen was 28% ($p<0.001$) and 27% ($p=0.002$) lower, respectively, in HLM homozygous for the polymorphic *UGT2B7*^{268Tyr} genotype, as compared to specimens that were homozygous for the wild-type *UGT2B7*^{268His} genotype. There was a significant trend for decreasing O-glucuronidation activity against *trans*-4-OH-TAM ($p<0.001$) and *trans*-endoxifen ($p=0.009$) with increasing numbers of the polymorphic *UGT2B7*^{268Tyr} allele. In contrast, HLM homozygous for the variant *UGT1A4*^{24Thr} and *UGT1A4*^{48Val} genotypes exhibited similar *N*⁺-glucuronidation rates of *trans*-4-OH-TAM as HLM homozygous for wild-type *UGT1A4*^{24Pro/48Leu}. These results suggest that functional polymorphisms in TAM-metabolizing UGTs, particularly in *UGT2B7*, may be important in inter-individual variability in TAM metabolism and ultimately, response to TAM therapy.

B. Introduction

i. Tamoxifen pharmacogenetics. TAM is extensively metabolized by several enzymes, which allows the potential for polymorphic enzymes to affect TAM and TAM metabolite levels, and therefore influence patient outcomes. As described in section 1.E.iii, TAM undergoes phase I metabolism, mostly by CYP2D6 and/or CYP3A4/5 to form endoxifen and 4-OH-TAM. Early studies found 4-OH-TAM to have a greater affinity for the estrogen receptor (ER) than the parent drug TAM⁴⁹ and both 4-OH-TAM and endoxifen exhibit up to 100-fold greater potency than TAM at inhibiting the estrogen-dependent proliferation of cells.⁵⁰ In addition, 4-OH-TAM and endoxifen have been shown to be essentially equal in affinity for ER binding, inhibition of estrogen-dependent cell line proliferation,⁵ antagonism of estradiol (E₂)-induced expression of the progesterone receptor,⁵¹ and induction of estrogen-responsive global gene expression in MCF-7 cell lines.⁵² Importantly, both metabolites are abundant in the plasma of TAM-treated women, although endoxifen is often present at levels 5- to 10-fold higher than 4-OH-TAM.^{27, 47, 53} This evidence supports the theory that TAM is a pro-drug and endoxifen and 4-OH-TAM are major contributors to TAM's therapeutic benefits. Therefore, the activity of the enzymes that convert TAM to its active metabolites and the activity of the enzymes responsible for the inactivation and elimination of endoxifen and 4-OH-TAM are important in determining plasma levels of these compounds and ultimately, therapeutic response.

The pharmacogenetics of TAM due to *CYP2D6* genotype has been recognized by the FDA, although an advisory committee was undecided as to recommending genotyping or to offer genotyping as an option for women who are

candidates for TAM therapy.¹⁶¹ *CYP2D6* is a highly polymorphic gene, with 19 inactive and 7 reduced activity alleles currently identified in the population.⁶⁹ An inactive or less active enzyme would result in less TAM converted to 4-OH-TAM and endoxifen. Indeed, *in vivo* studies have found that *CYP2D6* status in women treated with TAM was associated with changes in endoxifen plasma concentrations. Namely, *CYP2D6* genotypes that resulted in reduced activity or an inactive enzyme had lower plasma levels of endoxifen.^{50, 69-70} In addition, TAM treated breast cancer patients that were *CYP2D6* poor metabolizers, due to reduced activity or inactive *CYP2D6*, experienced increased recurrence, mortality rates and fewer side effects as compared to patients that were extensive metabolizers.^{50, 69} However, despite the apparent importance of *CYP2D6* genotype, large variability in the plasma levels of endoxifen and 4-OH-TAM are still observed in women treated with TAM, despite stratification by *CYP2D6* genotype,⁶⁹⁻⁷⁰ suggesting that additional factors influence plasma levels.

ii. UDP-glucuronosyltransferases in the metabolism of tamoxifen.

TAM is administered to patients in the *trans*-isomer form, due to its higher affinity for the estrogen receptor (ER).²⁸⁻²⁹ Previous studies demonstrated the glucuronidation of both *trans*-endoxifen and *trans*-4-OH-TAM by the UGTs.^{44, 149} Wild-type UGT1A4^{24Pro/48Leu} and the variant UGT1A4^{24Thr/48Leu} performed similar levels of *N*⁺-glucuronidation of *trans*-4-OH-TAM, whereas the polymorphic UGT1A4^{24Pro/48Val} exhibited increased enzyme activity against *trans*-4-OH-TAM, as compared to the wild-type UGT1A4^{24Pro/48Leu} enzyme activity.⁴⁴ In addition, all of the UGT1A family members, except UGT1A4, and UGT2B7 performed *O*-glucuronidation of *trans*-

endoxifen and *trans*-4-OH-TAM, as demonstrated in Chapter 2 of this dissertation and elsewhere.¹⁴⁹ Kinetic analyses indicated that, of the hepatically-expressed UGTs, UGT2B7 was the most active against *trans*-endoxifen and *trans*-4-OH-TAM.¹⁴⁹ Interestingly, the polymorphic UGT2B7^{268Tyr} results in a significant decrease in enzyme activity against both substrates.¹⁵⁰

UGTs 1A8 and 1A10 demonstrated the highest overall *O*-glucuronidation activity against *trans*-endoxifen and *trans*-4-OH-TAM.¹⁴⁹ Both enzymes are exclusively extra-hepatic.^{113, 155} The UGT1A10 polymorphism resulting in a Glu-to-Lys amino acid change at codon 139 has a very low frequency in Caucasians and occurs up to 4 percent in Blacks (Table 2-5).¹⁵⁷ However, the variant UGT1A8^{173Gly} has an allelic frequency up to 40 percent in Caucasians^{93, 158} and results in a significant decrease in *trans*-endoxifen and *trans*-4-OH-TAM glucuronidation.¹⁵⁰ Interestingly, UGTs 1A8 and 2B7 expression have been detected in human breast tissue,^{91, 162} the target organ of TAM.

iii. Hypothesis. Our previous results indicated that UGT2B7 glucuronidation activity is significantly impaired by the His-to-Tyr change at codon 268 in the homogenates of UGT2B7 stably expressing cell lines.¹⁵⁰ Therefore, the hypothesis of the present study was that HLM prepared from specimens that harbored the polymorphic UGT2B7^{268Tyr} genotype would exhibit decreased *O*-glucuronidation activity against *trans*-endoxifen and *trans*-4-OH-TAM, as compared to wild-type UGT2B7^{268His} glucuronidation activity. In addition, HLM that were polymorphic for UGT1A4^{24Pro/48Val} were expected to exhibit an increase in *N*+-glucuronidation of *trans*-4-OH-TAM.

UGT1A8 and UGT2B7 protein have been previously detected in human breast tissue.^{91, 162} Due to the high activity of UGTs 1A8 and 2B7 against *trans*-endoxifen and *trans*-4-OH-TAM,¹⁴⁹⁻¹⁵⁰ a second hypothesis for the current study is that the polymorphic UGT1A8^{173Gly}, UGT1A8^{277Tyr}, and UGT2B7^{268Tyr} decrease the rate of glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM in human breast homogenates. The overall goal of the present study was to determine if there was an association between *UGT* genotype and *trans*-endoxifen and *trans*-4-OH-TAM glucuronidation phenotype in HLM and human breast homogenates.

C. Materials and Methods

i. Chemicals and materials. *trans*-4-OH-TAM (98% pure), UDPGA, alamethicin, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Endoxifen was synthesized in the Organic Synthesis Core Facility at the Penn State College of Medicine, with the *trans*-endoxifen isomer purified as previously described.¹⁴⁹ HPLC-grade ammonium acetate and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Dulbecco's modified Eagles medium, Dulbecco's phosphate-buffered saline (minus CaCl_2 and MgCl_2), fetal bovine serum, penicillin-streptomycin and geneticin (G418) were purchased from Gibco (Grand Island, NY). The BCA protein assay kit was purchased from Pierce (Rockford, IL). The human UGT1A and UGT2B Western blotting kits were purchased from Gentest (Woburn, MA). All other chemicals used were purchased from Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

ii. Human liver microsome (HLM) preparation. Normal human liver tissue specimens (n=111) were obtained from the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center (Tampa, FL) and include 78 liver specimens that were examined in previous studies.¹⁶³⁻¹⁶⁴ HLM were prepared as previously described (Figure 3-1)¹⁶⁴ and stored at -80°C . Matching genomic DNA was prepared from nuclei isolated during the microsomal differential centrifugation preparation procedure for each specimen using standard phenol chloroform techniques. Microsomal protein concentrations were measured using the BCA protein assay. Matching total RNA was obtained for each specimen directly from Moffitt's Tissue Procurement Facility and was stored at -80°C .

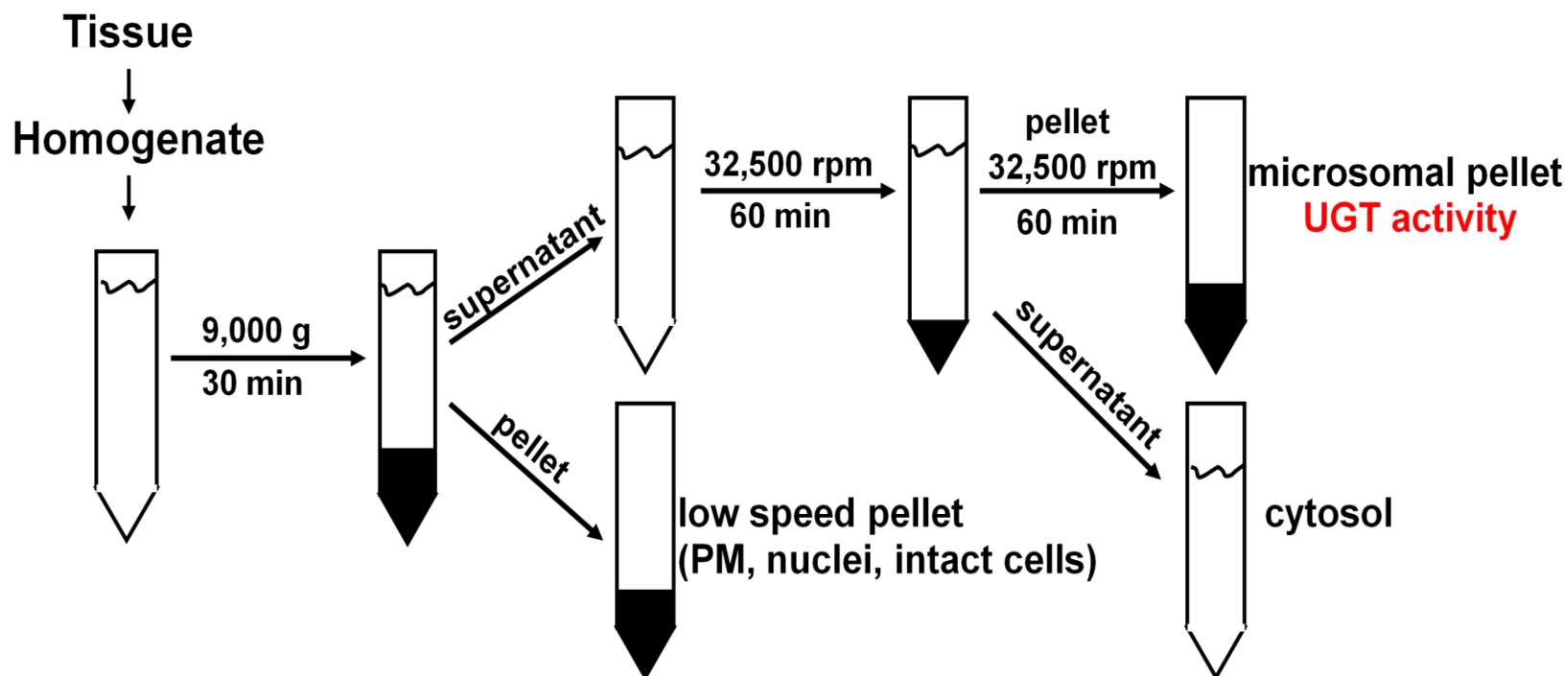


Figure 3-1. Schematic of the procedure for microsome preparation. Adjacent normal human liver and breast tissue were homogenized with an electric homogenizer on ice. Aliquots of breast homogenate were saved and stored at -80°C . Homogenates were centrifuged at 4°C at 9,000 g for 30 min. The pellet was saved for DNA extraction and stored at -80°C . The supernatant was subjected to two rounds of ultracentrifugation at 4°C at 105,000 g for 60 min each. The supernatant, considered to be the cytosolic fraction, was saved and stored at -80°C . The pellet was considered to be the microsomal fraction and was resuspended with 0.25 M sucrose, flash-frozen in an ethanol and dry-ice bath and stored at -80°C .

iii. Human breast homogenate and microsome preparation. Visibly adjacent normal human breast tissues were obtained from the Pennsylvania State University College of Medicine during breast tumor excision surgery and flash-frozen in liquid nitrogen within 15 min. Tissues were stored at -80°C until homogenate and microsome preparation. Visible adipose tissue was manually removed while the tissue was on ice prior to the preparation of microsomes, similar to the HLM procedure (Figure 3-1), except that aliquots of the homogenized tissue were saved as breast tissue homogenate. Homogenate and microsomal protein concentrations were determined by the BCA protein assay.

iv. Glucuronidation assays. The glucuronidation activities of HLM and breast tissue homogenate and microsomes against *trans*-4-OH-TAM and *trans*-endoxifen were performed as previously described.^{44, 149} HLM (2.5 µg total protein), breast homogenate (250 µg total protein), or breast microsome (250 µg total protein) were incubated with alamethicin (50 µg/mg protein) for 15 min in an ice bath to create pores in the micelles. Glucuronidation reactions were performed in a final reaction volume of 25 µL at 37°C in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 4 mM UDPGA, and the subsaturating concentrations of 4 µM or 30 µM of *trans*-4-OH-TAM or *trans*-endoxifen, respectively, for HLM and breast homogenate or microsome glucuronidation assays. 4-methylumbelliferone (4-MU) was used as a positive control at 100 µM with breast homogenate and microsomes. Kinetic analysis of HLM from subjects with varying *UGT2B7* genotypes was performed using 0.5-15 µM *trans*-4-OH-TAM and 2-60 µM *trans*-endoxifen. This analysis was performed with three randomly chosen HLM. Reactions were terminated by the addition of 25 µl cold

methanol on ice. Mixtures were centrifuged for 10 min at 4°C at 16,100 g prior to the collection of the supernatant. All HLM glucuronidation assays were performed in duplicate independent experiments.

Assays of TAM metabolite formation were performed by UPLC/MS/MS using a Waters (Milford, MA) ACQUITY UPLC consisting of a binary gradient pump, an autosampler maintained at 4°C, and a UV detector operated at 254 nm, attached to a Waters TQD triple quadrupole mass spectrometer. Similar to that described previously,⁷² each sample was injected onto an ACQUITY UPLC BEH C18 1.7 µm, 2.1X100 mm column (Waters) with the following gradient elution conditions for *trans*-4-OH-TAM and 4-MU: starting with 69% buffer A (0.01mol/L NH₄Ac, pH 5.0)/31% acetonitrile for 2 min with a subsequent linear gradient to 75% acetonitrile over 2 min. The gradient elution conditions for *trans*-endoxifen, using the same buffers, were as follows: starting with 30% acetonitrile for 4 min and a subsequent linear gradient to 75% acetonitrile for 2 min. The elution flow rate was 0.5 mL/min and 5 µL of the reaction was injected for all assays. Electrospray ionization mass spectrometry (ESI-MS) daughter scans of 564, 550, and 353 (*m*+1/*z*) verified the glucuronides of *trans*-4-OH-TAM, *trans*-endoxifen, and 4-MU, respectively. The formation of the glucuronides of *trans*-endoxifen, *trans*-4-OH-TAM, and 4-MU were quantified by UPLC based on the ratio of the glucuronide versus free *trans*-4-OH-TAM or *trans*-endoxifen. Glucuronidation assays without TAM metabolite were regularly analyzed as negative controls for glucuronidation activity as previously described.¹⁶⁴

v. UGT genotyping. Genomic DNA from the 111 liver specimens examined for glucuronidation activity in this study were used to genotype the *UGT2B7* codon

268 (His>Tyr) polymorphism (Genbank accession #NM_001074), the *UGT1A1**28 TATAA box polymorphism (GenBank wild-type accession number NM_000463 and SNP rs8175347), and the *UGT1A4* codon 24 (Pro>Thr) and codon 48 (Leu>Val) polymorphisms (Genbank accession #NM_007120). *UGT2B7* and *UGT1A4* genotypes were determined by real-time PCR and direct sequencing of PCR-amplified PCR products spanning the codon 268 polymorphism for *UGT2B7*, and codon 24 and 48 for *UGT1A4* using the primers 2B7S, 2B7AS, 1A4S, and 1A4AS (Table 3-1). Sequencing was performed using an ABI 3130 Capillary Sequencer at the Functional Genomics Core Facility at the Penn State College of Medicine.

The *UGT1A1**28 polymorphism was genotyped utilizing DNA fragment analysis by capillary electrophoresis on the ABI 3130 Capillary Sequencer at the Molecular Genetics Core Facility at the Penn State College of Medicine using primers and PCR conditions identical to those described previously¹⁶⁵ with the exception that the PCR product was not diluted prior to electrophoresis and that 0.5 µL of the size standard ladder (GeneScan 500 LIZ Size Standard, Applied Biosystems, Foster City, CA) was used as a DNA size marker. Informative results were obtained for 105 of the 111 liver specimens examined in this study.

UGT2B7 codon 268 genotypes were determined primarily by real-time PCR assays using the TaqMan Drug Metabolism Genotyping Assay C__32449742_20 (Applied Biosystems, Foster City, CA) in the ABI 7900HT sequence detection system equipped with an autoloader in the Functional Genomics Core Facility at the Penn State College of Medicine. Forty percent of all samples within each of the three potential *UGT2B7* genotype groups (His268His, His268Tyr and Tyr268Tyr; as

Table 3-1. Primer sequences utilized for UGT genotyping.

UGT	Primer Name	Primer Set	Location*
1A4	1A4S	5'-GGCTTCTGCTGAGATGGCCAG-3'	-13 to +8
	1A4AS	5'-CCTTGAGTGTAGCCCAGCGT-3'	+277 to +306
2B7	2B7S	5'-CTATAGTGCTTTACTTTGACTTTTGGTTCG-3'	+642 to +670
	2B7AS	5'-GCTAGAAAAGCAAAGAAGGGAAAAAATGATTAGTTATATCTGA-3'	+1555 to +1597
	2B7ScDNA	5'-TGCAGATGCTATTTTTCCCTGTA-3'	+456 to +478
	2B7AScDNA	5'-GAACCTTTTGTGGGATCTGGGCC-3'	+984 to +1006

*relative to the ATG translational start site

identified by real-time PCR) were further confirmed by standard PCR and cycle sequencing performed with the ABI 3130XL Capillary Sequencer at the Molecular Genetics Core Facility at the Penn State College of Medicine. For those samples for which real-time assays of *UGT2B7* genotypes were inconclusive (n=13), PCR and direct sequencing analysis was performed as described above. For eight of these samples, both real-time and direct sequencing failed to provide informative results. Direct sequencing of *UGT2B7* cDNA was then performed successfully for these samples using matching total RNA as template. Briefly, after reverse transcription (RT)-PCR was performed using standard conditions with 5 µg total liver RNA as template and oligo(dT) as primer, PCR was performed using 2 µL of cDNA as template the primers 2B7ScDNA and 2B7AScDNA (Table 3-1). Direct sequencing of these RT-PCR-amplified fragments was performed using the ABI 3130XL Capillary Sequencer at the Molecular Genetics Core Facility at the Penn State College of Medicine using the same primers used for PCR.

vi. *UGT2B7* mRNA expression analysis. Matching total RNA was available for expression analysis for 99 of the 111 liver specimens analyzed in this study. Five µg of RNA was used for cDNA synthesis using standard reverse transcription methods as described above. 20 ng of cDNA was used for expression analysis using the ABI gene expression kit assay for *UGT2B7* (Hs02556232_s1; Applied Biosystems, Foster City, CA) with the ABI 7900HT detection system equipped with an autoloader in the Functional Genomics Core Facility at the Penn State College of Medicine. Expression assays were performed in triplicate with expression

normalized relative to the expression levels of the housekeeping gene *PPIA* within the same samples.

vii. Immunoblot analyses. Breast tissue homogenate and microsome protein expression were analyzed by immunoblot analyses. Protein was extracted from fresh breast homogenate or microsomes and 100 μ g were loaded onto denaturing 8 percent SDS-PAGE gels. Proteins were transferred to a PVDF membrane by a traditional wet transfer for 2 hours at 30 volts. Blots were blocked for 1 hr at room temperature with 5 percent milk in TBST and subsequently incubated in primary antibody, also in 5 percent milk in TBST, overnight at 37°C. HRP-conjugated anti-rabbit or anti-mouse secondary antibodies were used at 1:5,000 for 1 hour at room temperature. Blots were incubated with SuperSignal West Dura extended duration chemiluminescence substrate (Pierce/Thermo Scientific, Pittsburgh, PA) for 5 min. Film was developed with a standard film processor. The blots were stripped and probed for the loading control β -actin, as stated above. The concentrations of the primary antibodies were as follows: UGT1A 1:5000, UGT2B 1:2500, and β -actin 1:2000.

viii. Statistical analysis. The Student's t-test (2-sided) was used for comparing kinetic values of glucuronidation formation for HLM glucuronidation rates stratified by *UGT* genotypes. The one-way ANOVA trend test was used to compare HLM glucuronidation rates across multiple *UGT* genotypes. Kinetic constants were determined using Graphpad Prism4 (GraphPad Prism, La Jolla, CA) software.

D. Results

i. Glucuronidation activities of HLM. To determine the rate of *O*- and *N*⁺-glucuronidation of *trans*-4-OH-TAM and *trans*-endoxifen, glucuronidation activity assays were performed for 111 independent HLM samples and analyzed by UPLC and UPLC/MS/MS. The concentrations of *trans*-4-OH-TAM or *trans*-endoxifen substrate used in the HLM glucuronidation activity assays were determined by kinetic analyses of three randomly-chosen HLM specimens. The resulting *K*_M's were 4 μM and 30 μM for *trans*-4-OH-TAM and *trans*-endoxifen, respectively (data not shown).

HLM glucuronidation activity assays, containing 4 μM *trans*-4-OH-TAM for 60 min, resulted in two major putative glucuronide peaks, the TAM-4-*O*-glucuronide and the 4-OH-TAM-*N*⁺-glucuronide, which exhibited retention times of 1.76 and 3.35 min, respectively, distinct from free *trans*-4-OH-TAM which eluted at 3.95 min (Figure 3-2, panel A). HLM incubated with 30 μM *trans*-endoxifen for 30 min exhibited a single major putative glucuronide peak observed at a retention time of 1.95 min, which was distinct from the endoxifen peak eluting at 3.90 min (Figure 3-2, panel B). All putative glucuronide peaks eluted at retention times identical to previously characterized TAM-glucuronide standards,⁷² and were confirmed by tandem MS (Figure 3-2, panels C-D; the MS/MS patterns observed for *trans*-TAM-4-*O*-glucuronide and *trans*-4-OH-TAM-*N*⁺-glucuronide are identical). For 4-OH-TAM glucuronidation assays, a third, smaller peak eluting at a retention time of 2.02 min was confirmed to be *cis*-TAM-

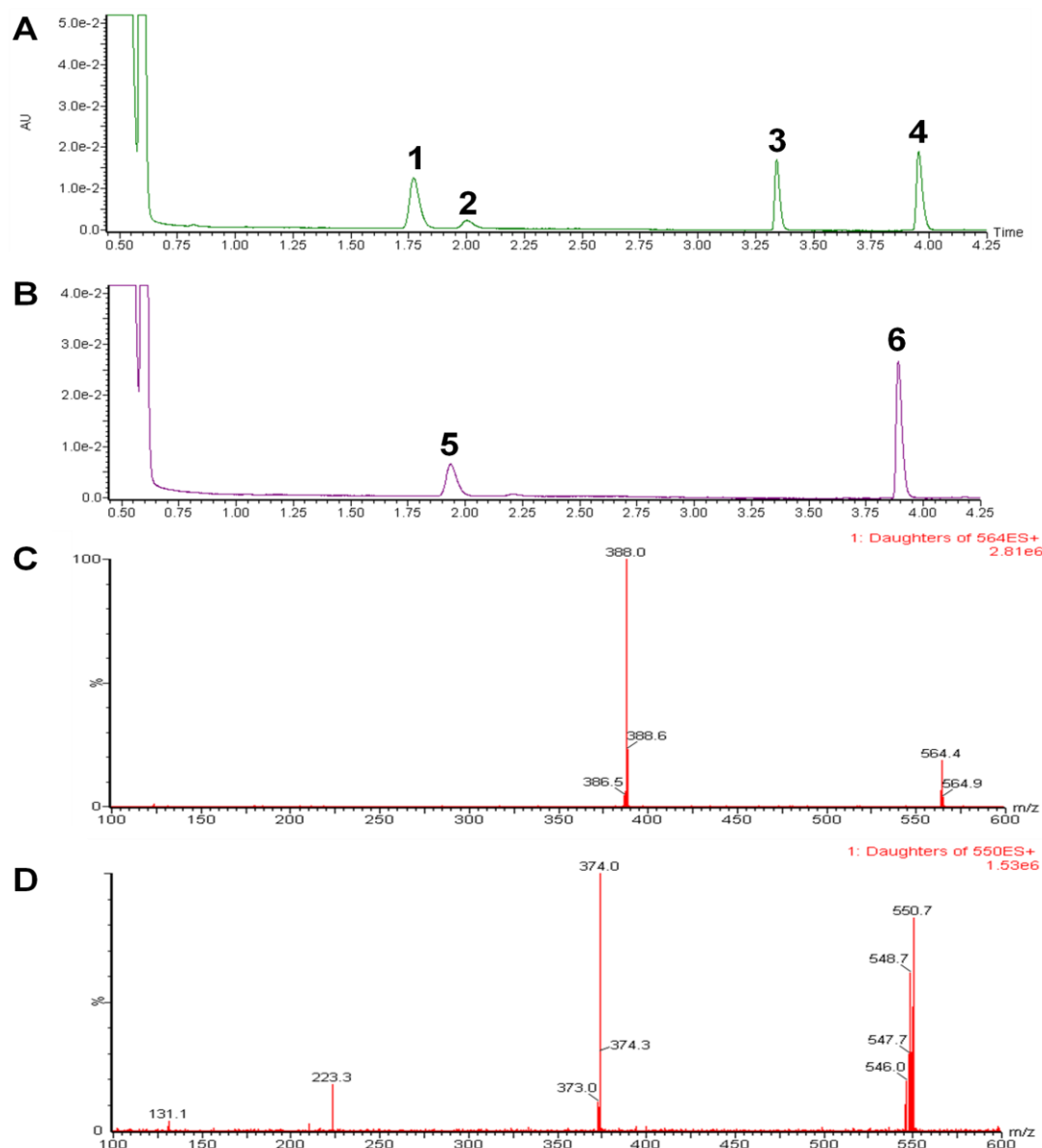


Figure 3-2. Representative UPLC chromatograms and mass spectra of HLM activity against *trans*-endoxifen and *trans*-4-OH-TAM. Glucuronidation activity assays were performed with 40 μ g of HLM and **A)** 30 μ M of *trans*-endoxifen or **B)** 4 μ M of *trans*-4-OH-TAM. Tandem MS/MS confirmed the glucuronide peaks of HLM glucuronidation reactions against **C)** *trans*-endoxifen and **D)** *trans*-4-OH-TAM. Peak 1 represents *trans*-TAM-4-O-glucuronide; peak 2 represents *cis*-TAM-4-O-glucuronide; peak 3 represents *trans*-4-OH-TAM- N^+ -glucuronide; peak 4 represents free *trans*-4-OH-TAM; peak 5 represents *trans*-endoxifen-O-glucuronide; and peak 6 represents free *trans*-endoxifen,

4-*O*-glucuronide, likely formed due to spontaneous interconversion between the *trans* and *cis* 4-OH-TAM isomers. Similar to that observed in previous studies for three HLM specimens,¹⁴⁹ *N*-glucuronidation was not observed in any of the glucuronidation activity assays of *trans*-endoxifen by the 111 HLM examined in the present analysis. The mean rate of formation $\pm$ the standard deviation of TAM-4-*O*-glucuronide, 4-OH-TAM-*N*⁺-glucuronide and endoxifen-*O*-glucuronide in HLM were 141 ± 45 , 175 ± 52 and 168 ± 66 pmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹, respectively. The minimum and maximum glucuronide formation in all HLM ranged from 4.5-, 10-, and 17-fold for TAM-4-*O*-glucuronide, 4-OH-TAM-*N*⁺-glucuronide, and endoxifen-*O*-glucuronide, respectively. The range of the ratio of TAM-4-*O*-glucuronide : 4-OH-TAM-*N*⁺-glucuronide in the HLM samples was 8.0-fold.

ii. *UGT* genotype and glucuronidation phenotype analysis. Previous studies indicated that the polymorphic UGT2B7^{268Tyr} results in a significant reduction in the glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM in UGT2B7^{268Tyr} over-expressing cell lines, as compared to the wild-type UGT2B7^{268His} cell line.¹⁵⁰ To examine the effect of *UGT* genotype on HLM formation of *trans*-endoxifen- and *trans*-TAM-4-*O*-glucuronides, HLM glucuronidation rates (n = 111) were stratified by *UGT* genotype.

Stratification of HLM *O*-glucuronidation activities by UGT2B7 codon 268 genotype resulted in a significant 27% decrease in the *O*-glucuronidation of *trans*-endoxifen in HLM that were homozygous for the variant UGT2B7^{268Tyr} genotype (p = 0.002; n = 28), as compared to HLM homozygous for the wild-type

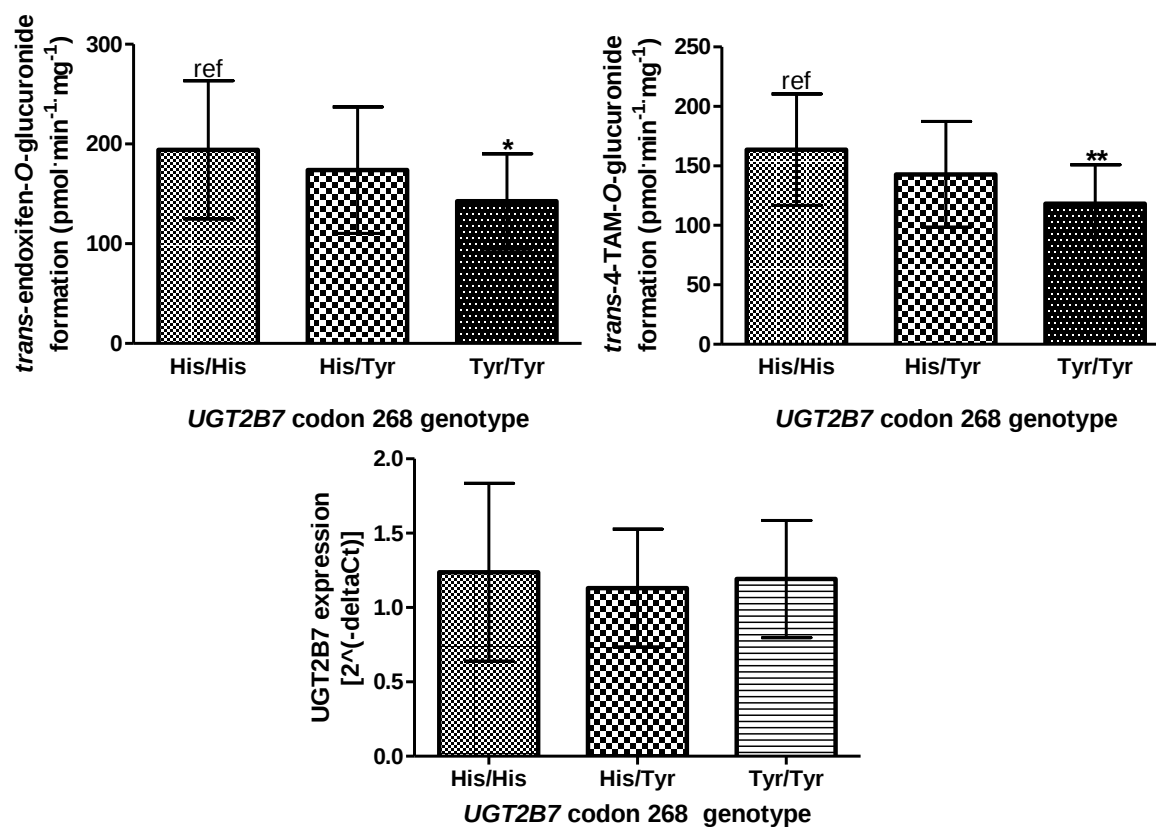


Figure 3-3. *UGT2B7* genotype association with HLM glucuronidation phenotype of *trans*-endoxifen and *trans*-4-OH-TAM . The rate of O-glucuronidation of **A) *trans*-endoxifen** and **B) *trans*-4-OH-TAM** was stratified by *UGT2B7* genotype. **C) *UGT2B7* mRNA expression levels**, as measure by real-time PCR, were stratified by *UGT2B7* genotype. *p < 0.002, **p < 0.001, error bars represent standard deviation

UGT2B7^{268His} genotype (n = 25; Figure 3-3, panel A). In addition, a significant trend of decreasing *O*-glucuronidation of *trans*-endoxifen was observed in HLM with increasing numbers of the *UGT2B7*^{268Tyr} allele ($p=0.009$). Similarly, a 13% decrease in TAM-4-*O*-glucuronide formation in HLM heterozygous for the polymorphic *UGT2B7*^{268Tyr} genotype ($p = 0.059$; n = 28) and a significant 28% decrease in TAM-4-*O*-glucuronide formation in HLM homozygous for the *UGT2B7*^{268Tyr} genotype ($p < 0.001$), as compared to HLM homozygous for the wild-type *UGT2B7*^{268His} genotype (Figure 3-3, panel B). A significant 17% decrease in TAM-4-*O*-glucuronide formation was observed in HLM heterozygous for the *UGT2B7*^{268Tyr} genotype ($p = 0.01$), as compared to HLM homozygous for the variant *UGT2B7*^{268Tyr} genotype. A significant trend of decreasing *O*-glucuronidation of *trans*-4-OH-TAM was observed in HLM with increasing numbers of the *UGT2B7*^{268Tyr} allele ($p<0.001$).

A previous study reported the single nucleotide polymorphism (SNP) of a thymidine to a cytosine located within the *UGT2B7* promoter at -161, relative to the ATG translational start site, that is in complete linkage disequilibrium with the SNP that results in the *UGT2B7*^{268Tyr} allele.¹⁶⁶ Similarly, the SNP at -161 was in 100 percent linkage disequilibrium with the *UGT2B7*^{268Tyr} allele in the present population. *UGT2B7* mRNA expression was analyzed to examine the possibility that *UGT2B7* expression levels due to the -161 SNP were influencing the *UGT2B7* genotype-phenotype relationship. The mean level of *UGT2B7* expression was similar in liver specimens within each of the *UGT2B7* genotype groups, with specimens homozygous for the wild-type *UGT2B7*^{268His} genotype exhibiting a 4 percent higher

level of expression than liver specimens homozygous for the variant *UGT2B7*^{268Tyr} genotype (Figure 3-3, panel C).

A previous report demonstrated that UGT1A4 performs *N*⁺-glucuronidation of 4-OH-TAM, and that the polymorphic *UGT1A4*^{24Pro/48Val} exhibited increased glucuronidation activity.⁴⁴ HLM glucuronidation of *trans*-TAM-4-*O*-glucuronide was stratified by *UGT1A4* genotype. Surprisingly, no significant associations were observed for the variant *UGT1A4*^{24Pro/48Val}, as well as the variant *UGT1A4*^{24Thr/48Leu}, and HLM *N*⁺-glucuronidation activity against 4-OH-TAM (Figure 3-4, panels A and B).

Although UGT1A1 was not as active as UGTs 1A4 and 2B7 against *trans*-endoxifen and *trans*-4-OH-TAM, due to its high level of expression in liver, glucuronidation rates of HLM were also stratified by *UGT1A1**28 genotype. Non-significant decreases in *O*-glucuronidation activity of 14 and 11% were observed against the *trans* isomers of 4-OH-TAM and endoxifen, respectively, in HLM homozygous for the polymorphic *UGT1A1**28 genotype, as compared to HLM homozygous for the wild-type *UGT1A1**1 genotype (Figure 3-5, panels C and D). This decrease remained non-significant when combining HLM that were either homozygous polymorphic for *UGT1A1**28 or heterozygous polymorphic for *UGT1A1**28 genotypes (data not shown).

iii. Glucuronidation activity of breast tissue. In addition to UGT2B7, UGT1A8 was also found to have high activity against *trans*-endoxifen and *trans*-4-OH-TAM.¹⁴⁹ Both enzymes are also expressed in breast tissue.^{91, 162} Therefore, to determine the feasibility of a genotype-phenotype analysis in breast tissue, the

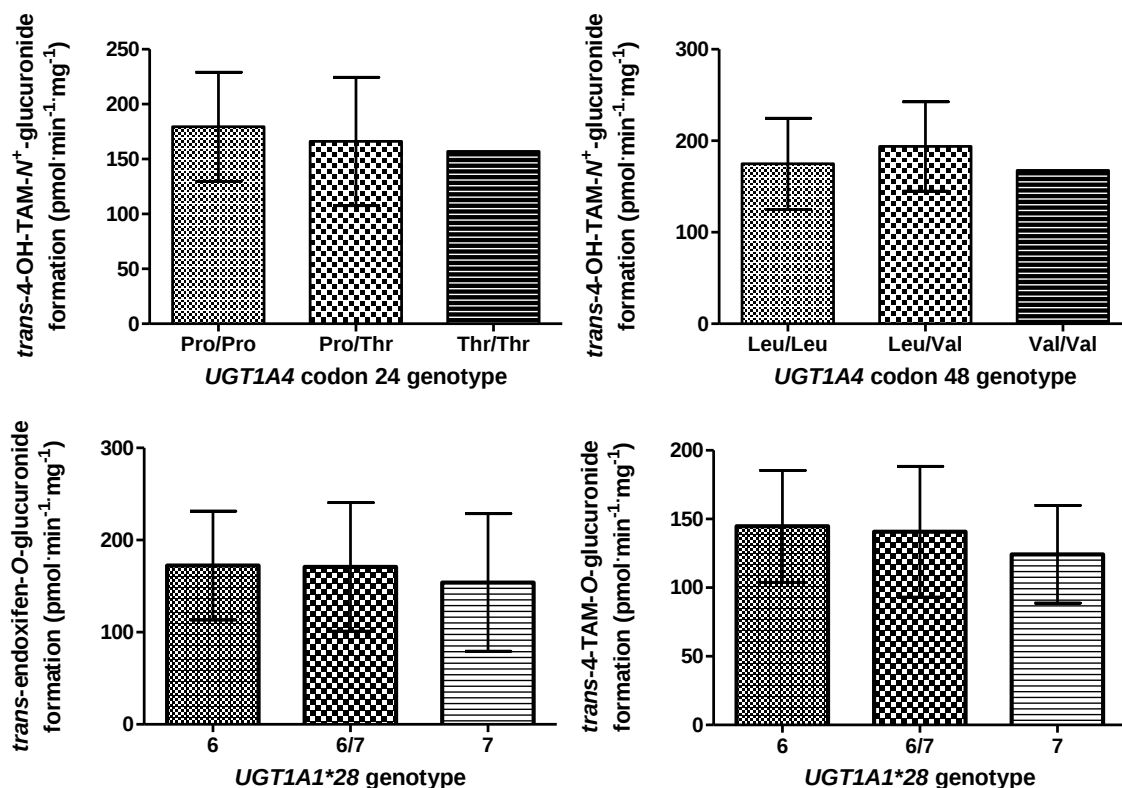


Figure 3-4. HLM glucuronidation activity stratified by *UGT1A4* and *UGT1A1* genotype. HLM N^+ -glucuronidation activity against *trans*-4-OH-TAM stratified by **A)** *UGT1A4*^{24Thr/48Leu} genotype and **B)** *UGT1A4*^{24Pro/48Val} genotype. HLM O-glucuronidation activity against **C)** *trans*-endoxifen and **D)** *trans*-4-OH-TAM stratified by *UGT1A1**28 genotype. Error bars represent standard deviation.

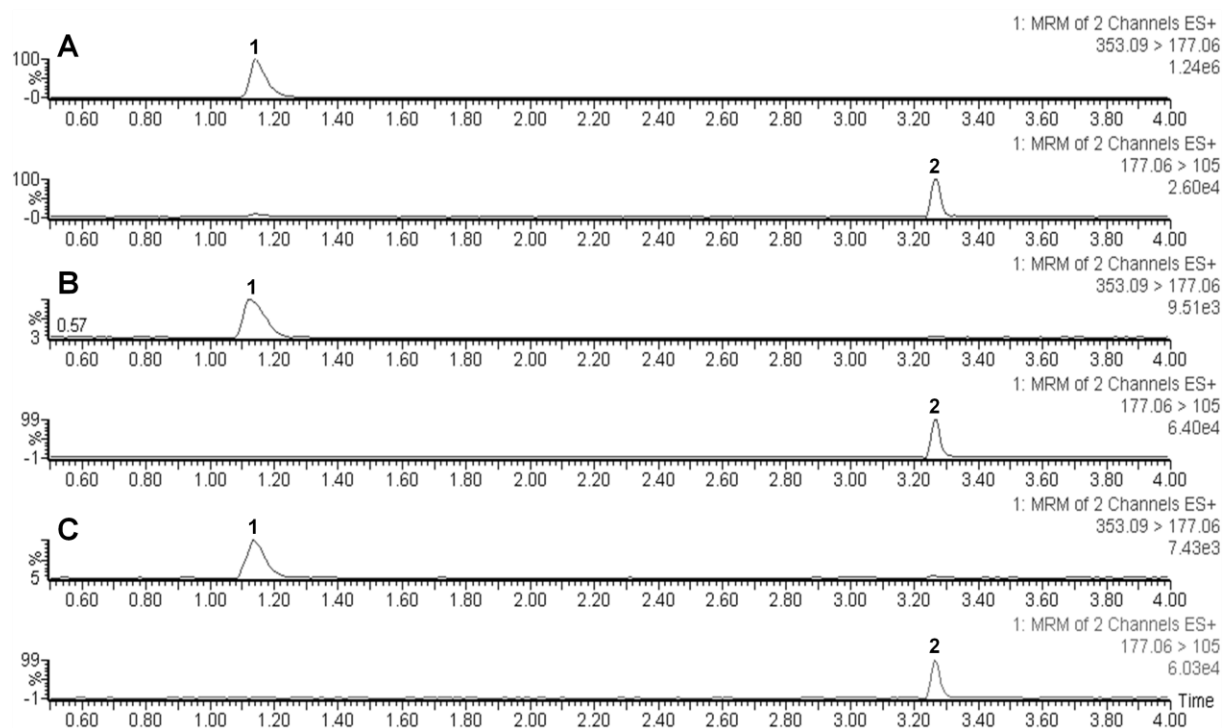


Figure 3-5. Representative mass spectra of 4-MU glucuronidation in human breast tissue. A) HLM glucuronidation of 4-MU, as a positive control for UGT activity. 4-MU glucuronidation by human breast tissue B) microsomes and C) homogenates. Mass spectral channels recognized the mass of 4-MU (177.06) and 4-MU conjugated to glucuronic acid (353.09), +1 m/z. Peak 1 represents 4-MU-O-glucuronide and peak 2 represents free 4-MU.

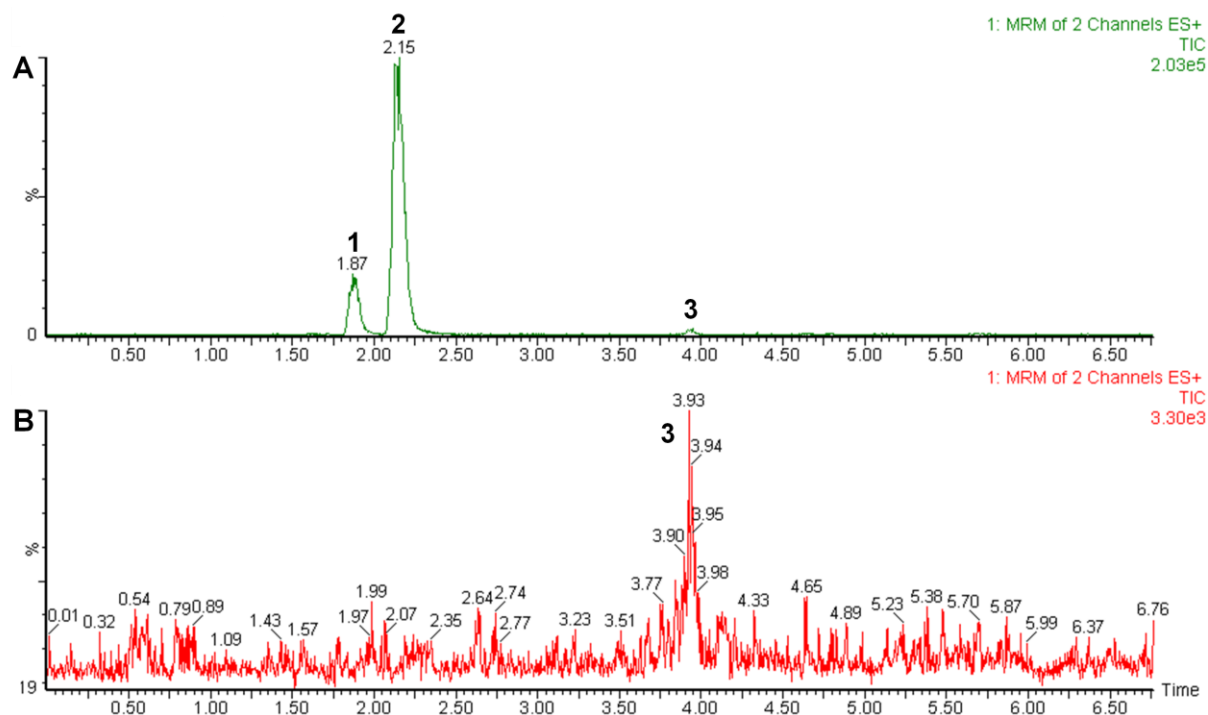


Figure 3-6. Representative mass spectra of the breast tissue glucuronidation activity assays against *trans*-endoxifen. **A)** 20 μ g of HLM was incubated with *trans*-endoxifen as a positive control and compared to **B)** 670 μ g of breast tissue homogenate that was incubated with *trans*-endoxifen in a 100 μ L reaction, and then vacuum-dried and resuspended in 6 μ L prior to MS analysis. Breast tissue homogenate glucuronidation was not observed. Peak 1 represents *cis*-endoxifen-*O*-glucuronide; peak 2 represents *trans*-endoxifen-*O*-glucuronide; and peak 3 represents residual unconjugated *trans*-endoxifen.

glucuronidation activity of breast tissue homogenates and microsomes were examined. Both breast homogenates and microsomes exhibited glucuronidation activity against 4-MU, a marker of UGT activity (Figure 3-5, panels B and C). However, glucuronidation activity by multiple specimens against both *trans*-endoxifen (Figure 3-6, panel B) and *trans*-4-OH-TAM (Figure 3-6, panel C) was not observed, despite pre-concentrating the samples by 10-fold prior to analysis.

iv. Immunoblot analysis of breast tissue. To ensure that UGT protein was present in breast homogenates and microsomes, immunoblot analysis was performed. UGT1A and UGT2B family members were observed in immunoblots of breast tissue homogenate and microsomes and the immunoreactive species were similar to the UGT1A1 and UGT2B7 positive control standards (Figure 3-7). A small difference in size is not unusual due to post-translational modifications, such as glycosylation, that occurs to UGT enzymes stably expressed in cell lines. The UGT standards are purified recombinant enzymes and are not expected to have post-translational modifications. Due to the absence of glucuronidation activity in multiple breast homogenate and microsomal specimens, further analyses were not pursued.

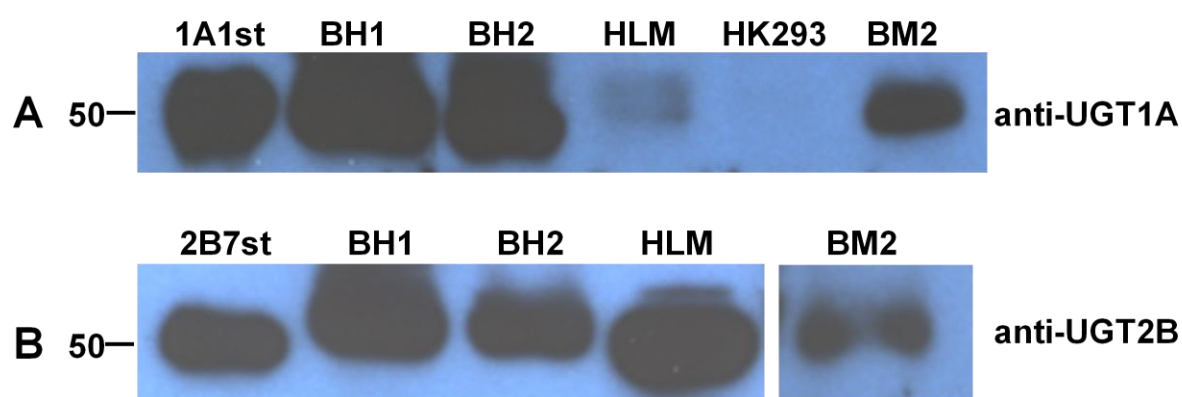


Figure 3-7. Immunoblot analysis of UGT protein expression in human breast tissue. Protein was extracted from human breast tissue homogenate and microsomes and 75 μ g of total protein was analyzed for **A)** UGT1A family member protein expression in purified UGT1A1 standard (250 ng of UGT protein), breast homogenate (BH) specimen 1, BH specimen 2, HLM as a positive control, HK293 as a negative control, and breast microsome (BM) specimen 2. **B)** UGT2B family member expression was examined in purified UGT2B7 standard (250 ng of UGT protein), BH specimen 1, BH specimen 2, HLM, and BM specimen 2.

E. Discussion

The present study examines the potential role of UGT polymorphisms in the metabolism of the *trans* isomers of 4-OH-TAM and endoxifen, the major active metabolites of tamoxifen. Several UGTs were previously shown to be active against these metabolites, with UGT2B7 being the most active hepatic UGT. As UGT2B7 expression has been detected in a variety of tissues including the liver, the gastrointestinal tract, and the breast,^{152, 162, 166-169} variations in UGT2B7 function or expression could potentially significantly impact individual response to drugs or chemotherapeutic agents. The data presented in this study demonstrate that *O*-glucuronidation of both *trans*-endoxifen and *trans*-4-OH-TAM in HLM was significantly associated with *UGT2B7* genotype, with lower activities correlated with specimens that were hetero- or homozygous for the *UGT2B7*^{268Tyr} allele. These data were consistent with the observation that HEK293 cells that stably expressed the *UGT2B7*^{268Tyr} variant exhibited lower glucuronidation activity against both TAM metabolites, as compared to cells stably expressing wild-type *UGT2B7*^{268His}. These results are also consistent with a study showing a functional role for this polymorphism against other substrates, including tobacco carcinogen metabolites like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).¹⁶⁴

Previous studies have observed a SNP located at -161(T>C) relative to the ATG translational start site in the promoter of *UGT2B7* that is in complete linkage disequilibrium with the SNP at *UGT2B7* codon 268.¹⁷⁰ The SNP at -161 was in complete linkage disequilibrium in the present study as well. Unlike a previous report that found this SNP to alter *UGT2B7* expression and alter morphine

metabolism,¹⁷⁰ in the present study, *UGT2B7* genotype was not associated with a change in *UGT2B7* mRNA expression, as there was less than a 4 percent difference in expression levels between HLM homozygous for the wild-type *UGT2B7*^{268His} versus the polymorphic *UGT2B7*^{268Tyr} genotypes. These data suggest that the decrease in *O*-glucuronidation activity against TAM metabolites in HLM associated with the *UGT2B7* codon 268 polymorphism is indeed due to functional changes within the *UGT2B7* enzyme.

In addition to *UGT2B7*, polymorphisms in UGTs 1A1 and 1A4, which were previously shown to be active against 4-OH-TAM and endoxifen,^{44, 149} were examined for their effect on HLM glucuronidation activity. A microsatellite (TA)-repeat polymorphism present in the TATAA box of the *UGT1A1* promoter has been linked to lower *UGT1A1* expression¹⁷¹⁻¹⁷² and lower activity against a variety of endogenous and exogenous substrates, including bilirubin,^{142, 171, 173} carcinogens such as metabolites of benzo(a)pyrene,¹⁷¹ and chemotherapeutic agents such as SN-38, the major, active metabolite of irinotecan.¹⁷⁴⁻¹⁷⁵ The non-statistically significant trend of decreased glucuronidation activity against TAM metabolites that was observed in HLMS from subjects with one or more *UGT1A1**28 alleles is consistent with previous studies indicating that *UGT1A1* exhibits between 4.4 and 5.4-fold lower overall activity against these substrates *in vitro* than *UGT2B7*,¹⁴⁹ and may therefore play a less significant role in TAM metabolism.

The *N*-glucuronidation of TAM and 4-OH-TAM was previously shown to be performed exclusively by *UGT1A4*.^{44, 176} Although a previous study demonstrated that the *UGT1A4* codon 48 polymorphism was linked to small, but significant,

alterations in N^+ -glucuronidation activity against TAM and 4-OH-TAM *in vitro*,⁴⁴ a significant difference was not observed in HLM against *trans*-4-OH-TAM in the present study. This may be due to the relatively low number of HLM specimens that were heterozygous (n=13) or homozygous (n=1) for the UGT1A4^{48Val} variant in this study. An absent association between the UGT1A4 codon 24 polymorphism and HLM N^+ -glucuronidation phenotypes of *trans*-4-OH-TAM glucuronidation is consistent with previous data that demonstrated that wild-type UGT1A4^{24Pro} and variant UGT1A4^{24Val} exhibited similar enzyme activities against this substrate.⁴⁴

The extra-hepatic UGTs 1A10 and 1A8 exhibited the highest levels of activity *in vitro* against the *trans* isomers of 4-OH-TAM and endoxifen in previous studies.¹⁴⁹ However, the polymorphic UGT1A10^{139Lys} is observed at very low prevalence in the population; less than 1 percent in Caucasian and Asians, and only up to 4 percent in blacks (Table 2-5).¹⁵⁷ Therefore, the functional effect of the UGT1A10 polymorphism would be difficult to examine due to the low prevalence. In contrast, UGT1A8 has a polymorphism at codons 173 and 277 that have allelic prevalences of up to 40 percent and 2.5 percent, respectively, in Caucasians (Table 2-5).^{91, 93, 158} In addition, the previous observations that UGT1A8 is highly active against TAM metabolites and is well-expressed in the breast^{91, 177} suggests that, like the UGT2B7 codon 268 polymorphism, the UGT1A8 polymorphism may be important in individual response to TAM.

Human breast tissue was procured for the preparation of homogenate and microsomes for screening of glucuronidation activity. Glucuronidation activity by these specimens was not detected for *trans*-endoxifen or *trans*-4-OH-TAM. If

glucuronidation activity against *trans*-endoxifen and *trans*-4-OH-TAM were observed, a genotype-phenotype study of breast tissue UGT genotype and glucuronidation phenotype would have been performed. UGT1A and UGT2B family immunoreactive proteins were detected in both breast tissue homogenate and microsomes, suggesting that the UGTs are present in the present breast tissue specimens. This is consistent with previous studies that demonstrated the presence of UGT2B7 protein in breast tissue by immunohistochemistry and immunoblot analyses.^{91, 162} In addition, 4-MU glucuronidation by breast homogenate and microsomes was observed in the present study. A majority of the UGTs are highly active against 4-MU, making it a favorable marker of UGT activity. Similarly, a published report demonstrated UGT2B7 activity in normal breast tissue against 4-hydroxy-estrone (4-OH-estrone) that was reduced in invasive breast tumor tissue.¹⁶² However, breast tissue homogenate and microsomes did not exhibit glucuronidation activity against *trans*-endoxifen or *trans*-4-OH-TAM. Multiple protein concentrations, as well as concentrating the glucuronidation reaction mixtures prior to analysis, did not yield positive results.

The previous report by Gestl *et al*¹⁶² that demonstrated UGT2B7 activity in breast tissue detailed a careful dissection of adipose tissue from breast tissue specimens. Visible adipose tissue was also dissected from the breast tissue specimens utilized in the present study; however, during the centrifugation steps for preparing microsomal fractions, a fatty layer was observed. Although this layer was avoided during preparation, it is possible that enough lipids were remaining to interfere with the glucuronidation activity assays. Both *trans*-endoxifen and *trans*-4-

OH-TAM are lipophilic and to a greater extent than 4-MU or 4-OH-estrone. It is possible that the TAM metabolites were “trapped” by the excess lipids, preventing their glucuronidation by UGTs present in the breast homogenate and microsomes. Alternatively, the breast tissue specimens used in this study may have simply had very low levels of UGT1A8 and UGT2B7 enzyme activity. In this case, TAM metabolism catalyzed in the liver is even more important because it would be the major site of endoxifen and 4-OH-TAM inactivation by glucuronidation.

The results of the HLM *UGT2B7* genotype and *trans*-endoxifen and *trans*-4-OH-TAM glucuronidation phenotype analysis examined in the present study are consistent with that observed previously for functional polymorphisms in the *CYP2D6* gene. Decreased levels of endoxifen were observed in the serum of TAM-treated women following stratification by the *CYP2D6* deletion genotype.⁵⁰ Similarly, a reduction in endoxifen plasma levels was also observed when CYP2D6 inhibitors were co-administered with TAM.⁶⁹ The *CYP2D6*4* deletion allele has been associated with time until breast cancer recurrence, relapse-free survival, disease-free survival, and overall survival in patients treated with TAM.^{50, 69, 159} In addition, patients with the *CYP2D6*4* genotype report few, if any, occurrences of hot flashes.⁵⁰ These data suggest an important role for endoxifen in TAM therapeutic efficacy. Despite a strong correlation between *CYP2D6* genotype and serum levels of endoxifen in patients treated with TAM, large variability in circulating endoxifen levels are still observed after controlling for CYP2D6 genotype.^{50, 69} The evidence presented in the present study suggests that inter-individual differences in TAM glucuronidation pathways may help explain this variability.

In summary, this study indicates that genetic variants in UGTs that are highly active against TAM metabolites significantly alter TAM metabolism in HLM and, potentially, TAM elimination in TAM-treated individuals. Similar to that described above for CYP2D6, this could potentially affect overall patient response to TAM. Additional studies examining the effect of *UGT2B7* genotypes in women undergoing TAM therapy, such as plasma TAM metabolite levels and overall patient response to TAM, will be required to further examine the role of UGT polymorphisms on the therapeutic efficacy of TAM.

Chapter 4

Src over-expression and UGT2B7 enzyme activity against the major, active metabolites of tamoxifen

A. Abstract

Src, a non-receptor tyrosine kinase, has been shown to be upregulated in several cancer cell types, including breast cancer. UGT2B7 is the predominant hepatic UGT that glucuronidates the major, active metabolites of the anti-breast cancer drug TAM, *trans*-endoxifen and *trans*-4-OH-TAM. Previous studies in HLM specimens have shown that heter- or homozygotes with the *UGT2B7*2* (*UGT2B7*^{268Tyr}) allele have significantly decreased rates of glucuronidating *trans*-endoxifen and *trans*-4-OH-TAM. In addition, recent reports indicate that UGT2B7 enzyme activity is altered by phosphorylation at Tyr236 and/or Tyr438 by Src. The goal of the present study was to determine if tyrosine kinase status within the cell influences the wild-type *UGT2B7*^{268His} or the variant *UGT2B7*^{268Tyr} enzyme activity against *trans*-endoxifen and *trans*-4-OH-TAM, *in vitro*. HEK293 cell lines stably expressing *UGT2B7*^{268His} or *UGT2B7*^{268Tyr} were transfected with c-Src or v-Src and kinetic analyses were performed. Wild-type *UGT2B7*^{268His} cells over-expressing c-Src or v-Src exhibited a significant 3- and 5-fold reduction, respectively, in the glucuronidation of *trans*-endoxifen, as compared to the parent *UGT2B7*^{268His} cell line ($p \leq 0.009$). Similarly, a 5- and 8-fold reduction in the glucuronidation of *trans*-4-OH-TAM was observed. However, no significant difference in glucuronidation capacity was observed in the c-Src or v-Src over-expressing polymorphism-bearing *UGT2B7*^{268Tyr} cell lines, as compared to the parent variant *UGT2B7*^{268Tyr} cell line ($p \leq 0.0002$).

Decreased glucuronidation activity of HLM against TAM metabolites has been observed previously for the *UGT2B7*^{268Tyr} polymorphic enzyme (Chapter 3). Src

over-expression caused a significant decrease in wild-type UGT2B7^{268His} enzyme activity against several substrates, including the major, active metabolites of TAM, *trans*-endoxifen and *trans*-4-OH-TAM, and the activity is reduced to the level observed for polymorphic UGT2B7^{268Tyr} enzyme. These results suggest that Src protein expression status combined with *UGT2B7*2* genotype may play an important role in the metabolism of TAM and ultimately, patient response to TAM therapy.

B. Introduction

i. Phosphorylation by Src. Phosphorylation is a post-translational modification whereby a γ -phosphoryl moiety is transferred from ATP to the amino acids Ser, Thr, or Tyr of a protein,¹⁷⁸ that is catalyzed by serine/threonine kinases or tyrosine kinases, respectively. Src is a non-receptor tyrosine kinase that phosphorylates a variety of protein substrates, including those of well studied pathways, such as focal adhesion kinase¹⁷⁹ (FAK) and Crk-and Src-associated substrate¹⁸⁰ (CAS), both involved in important focal adhesion pathways. In addition to cytoplasmic and plasma membrane-bound substrates, Src has been observed to phosphorylate protein targets located within the nucleus, such as Sam68,¹⁸¹⁻¹⁸² an RNA-binding protein that is implicated in the G₁/S transition.¹⁸³ This interaction may be important in regulating mitosis-related events during cell division.¹⁸⁴

Cellular-Src (c-Src) is a proto-oncogene and can become an oncogene. c-Src is important in a variety of normal cellular processes, such as differentiation, proliferation, cell division,¹²⁴⁻¹²⁵ survival,¹²⁶ cell adhesion, cell motility,¹²⁶⁻¹²⁸ morphology, and bone remodeling and reabsorption.^{124, 129-130} Aberrant Src gene expression can cause dysregulation of the cell cycle and is associated with cancer.

Increased protein and/or activity levels of c-Src have been observed in human neoplasms such as breast,¹⁸⁵ colon,¹⁸⁶ and pancreatic cancers.¹⁸⁷ Interestingly, cells that acquire TAM resistance exhibit increased levels of Src and there is an association in ER-positive breast cancer patients of activated Src in the cytoplasm of their breast tumor and reduced survival time with endocrine therapy. MCF7 cells, a breast cancer cell line that over-expresses active Src, develop a greater migratory

and invasive behavior and their growth is not inhibited when treated with TAM. When these cells are treated with a Src specific inhibitor, their morphology changes so that the cells appear to regain their cell-to-cell contacts and both migratory and invasive behavior is decreased.¹³⁹

ii. Phosphorylation of UGT2B7 by Src. A recent study suggested that UGT2B7 is phosphorylated by Src.¹¹¹ A database search of UGT2B7 identified three putative protein kinase C (PKC) sites, located at Thr123, Ser132, and Ser437 and two tyrosine kinase (TK) sites, located at Tyr236 and Tyr438. Curcumin, which is both a PKC and a Src inhibitor, decreased UGT2B7 activity without affecting PKC-site phosphorylation. In addition, site-directed mutations of the PKC sites did not alter enzyme activity, suggesting that PKC site phosphorylation was not important for UGT2B7 activity. However, site mutations at either or both TK sites resulted in decreased enzyme activity. Transient co-transfection of UGT2B7 with c-Src or v-Src caused a 1.5-fold increase in UGT2B7 activity against 4-OH-estrone, an endogenous substrate for UGT2B7.¹¹¹ These data suggest that UGT2B7 is phosphorylated by Src and that phosphorylation alters enzyme activity.

iii. Hypothesis. The evidence presented above for 4-OH-estrone led to the hypothesis that UGT2B7 enzyme activity altered by Src-mediated phosphorylation affects the glucuronidation activity of UGT2B7 against the substrates *trans*-endoxifen and *trans*-4-OH-TAM. In addition, the highly prevalent UGT2B7^{268Tyr} polymorphism (Table 2-5), which adds a novel Tyr residue to UGT2B7, if coupled with phosphorylation by Src, may cause additional complexities in the glucuronidation activity of these substrates.

C. Materials and methods

i. Chemicals and materials. *trans*-4-OH-TAM (98% pure), UDPGA, alamethicin, and bovine serum albumin (for immunoblot) were purchased from Sigma-Aldrich (St. Louis, MO). *trans*-endoxifen was purchased from Toronto Research Chemicals (North York, Ontario, Canada). HPLC-grade ammonium acetate, acetonitrile and peptide synthesis grade triethylamine were purchased from Fisher Scientific (Pittsburgh, PA). Dulbecco's modified Eagles medium, Dulbecco's phosphate-buffered saline (minus CaCl₂ and MgCl₂), fetal bovine serum (for tissue culture), penicillin-streptomycin and geneticin (G418) were obtained from Gibco (Grand Island, NY). The Platinum® *Pfx* DNA polymerase and the pcDNA6.2/V5/GW/D-TOPO mammalian expression vector were obtained from Invitrogen (Carlsbad, CA). The BCA protein assay kit was purchased from Pierce (Rockford, IL), while the QIAEX® II gel extraction kit was purchased from Qiagen (Valencia, CA). The anti-UGT2B7 antibody was purchased from Millipore (Woburn, MA), the anti-activate Src antibody (specific for Src without phosphorylation at amino acid 529) was purchased from Invitrogen, the anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO), and the anti-calnexin, anti-Src, and anti-phospho-tyrosine antibodies were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals used were procured from Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

ii. Src over-expressing cell lines. The HEK293 cell lines stably expressing the wild-type UGT2B7^{268His} and variant UGT2B7^{268Tyr} used in this study have been described previously.^{44, 164, 188} pOTB7 non-mammalian expression vectors containing

c-Src and v-Src were purchased from Open Biosystems (Huntsville, AL) and the Src transcripts were copied by PCR using the primers cSrcS, cSrcAS, vSrcS, and vSrcAS (Table 4-1). In order to ligate the Src PCR products into the vector, the lead sequence CACC was added to the products by PCR utilizing the same anti-sense primers and the sense primers cSrcCACC and vSrcCACC (Table 4-1). The Src PCR products, led by CACC at the 5' end, were ligated into the pcDNA6.2/V5/GW/D-TOPO vector containing blasticidin resistance, for selection of the Src-containing vector (Figure 4-1). All Src cDNA sequences were confirmed by dideoxy sequencing prior to transfection by Lipofectamine into the HEK293 cell lines stably expressing UGT2B7^{268His} or UGT2B7^{268Tyr}. Cells were grown in Dulbecco's Modified Eagle's medium supplemented with 10 percent fetal bovine serum, 100 U/mL penicillin and maintained in 350 µg/mL G418, for selection of the UGT-containing vector, and 9 µg/mL blasticidin, for selection of the c-Src- or v-Src-containing vector, in a humidified incubator in an atmosphere of 5 percent CO₂. Cells were grown to 80 percent confluence prior to the preparation of cell homogenates as described in Chapter 2 of this dissertation. For a subset of glucuronidation activity assays, 200 µM protein tyrosine phosphatase inhibitor, sodium orthovanadate, was added to the cell pellets of UGT2B7^{268His} cells and UGT2B7^{268His} cells over-expressing c-Src or v-Src prior to the homogenate preparation. Total homogenate protein concentrations were measured using the BCA protein assay. UGT2B7 protein levels were determined by immunoblot analysis, as previously described.⁴⁴ Relative UGT2B7 protein levels were expressed as the mean of three independent experiments, and all activity assays were normalized relative to UGT2B7 protein expression.

Table 4-1. Primer sequences utilized for the cloning of c-Src and v-Src.

Gene	Primer Name	Primer Set	Location
c-Src	cSrcS	5'-ACGCCAGAGCTCCTGAGAAGATGTCAG-3'	-20 to +7
	cSrcAS	5'-AGTCCCCACAGGCCCAT-3'	+1375 to +1391
	cSrcCACC	5'-CACCACGCCAGAGCTCCTGAGAAGATGTCAG-3'	5' PCR product
v-Src	vSrcS	5'-CAGGACCATGGGTAGCAACAAGA-3'	-7 to +16
	vSrcAS	5'-CCCGCCTGTGCCTAGAGGTTC-3'	+1602 to +1622
	vSrcCACC	5'-CACCCAGGACCATGGGTAGCAACAAGA-3'	5' PCR product

*relative to the ATG translational start site

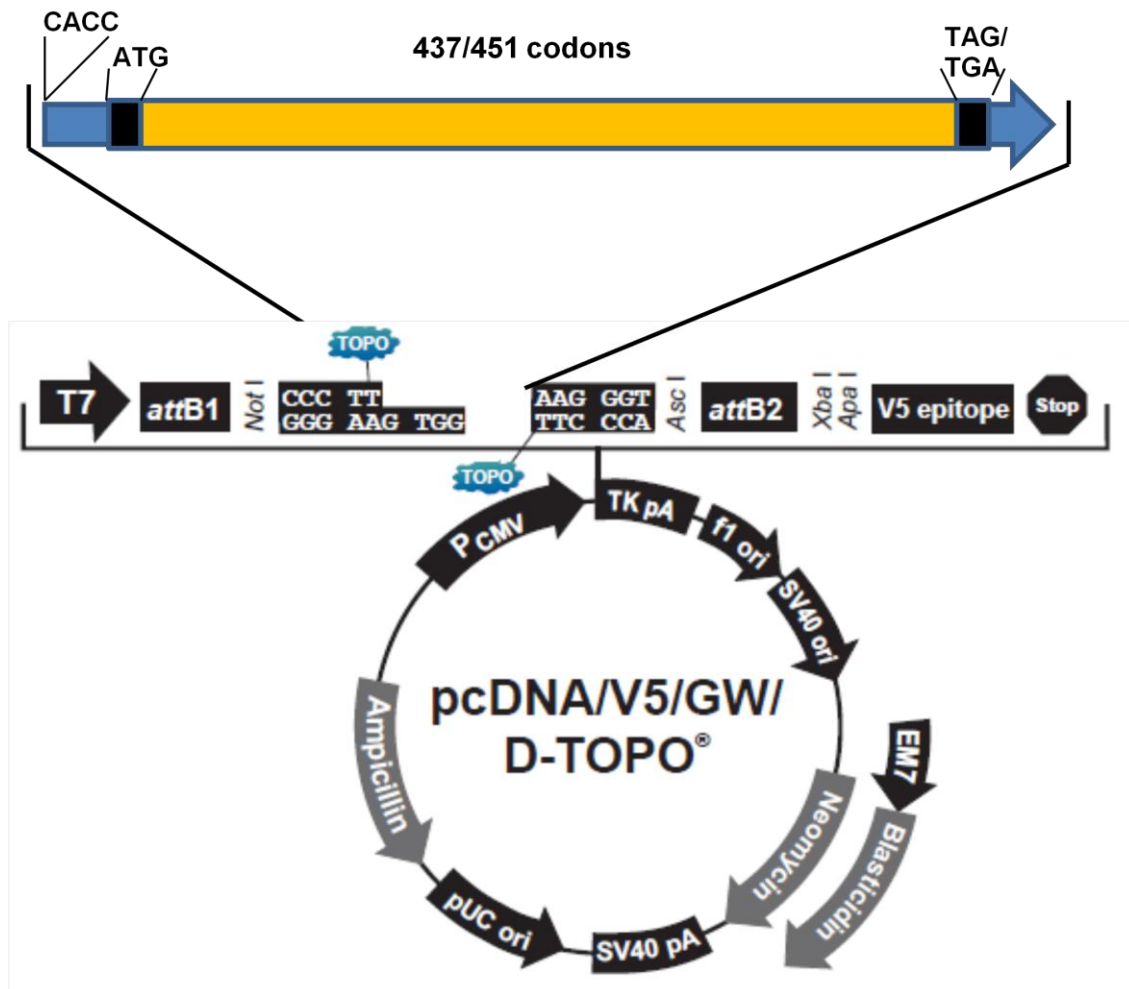


Figure 4-1. Diagram of the pcDNA vector for stable expression of c-Src and v-Src in the UGT2B7-HEK293 cell line. The pcDNA6.2/V5/GW/D-TOPO vector manufactured by Invitrogen (Carlsbad, CA) contains an ampicillin resistance gene for bacterial selection and a blasticidin gene for mammalian cell selection. A CMV promoter is located upstream of the PCR product insertion site. c-Src and v-Src with the lead CACC sequence were ligated to the vector, resulting in a 451 or 437 of translatable codons. *This figure was modified from Invitrogen.*

iii. Glucuronidation assays. The glucuronidation activities of homogenates from HEK293 cell lines stably expressing UGT2B7^{268His} or UGT2B7^{268Tyr} and either c-Src or v-Src against *trans*-4-OH-TAM, *trans*-endoxifen, and 4-OH-estrone were performed as previously described.^{44, 149} Following an initial incubation of cell homogenate protein (15-500 µg) with alamethicin (50 µg/mg protein) for 15 min in an ice bath, glucuronidation reactions were performed in a final reaction volume of 25 µL at 37°C in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 4 mM UDPGA, and 8 to 536 µM *trans*-endoxifen, 1 to 172 µM *trans*-4-OH-TAM, or 2-140 µM 4-OH-estrone for 15 min to 2 hours. Negative controls were incubated simultaneously with either ethanol (instead of TAM metabolites) or DMSO (instead of 4-OH-estrone). The reactions were terminated by the addition of 25 µL of cold acetonitrile on ice and, following a 10 min incubation on ice, the reactions were centrifuged for 10 min at 4°C at 16,100 g and the supernatant was collected. All glucuronidation assays were performed in triplicate.

Glucuronidation activity assays were analyzed by a Waters (Milford, MA) AQUITY UPLC consisting of a binary gradient pump, an autosampler maintained at 4°C, and a UV detector operated at 254 nm for the TAM metabolites and 280 nm for 4-OH-estrone. Samples were injected onto an AQUITY UPLC BEH C18 1.7 µm, 2.1X100 mm column (Waters) with the following gradient elution conditions: starting with 74% buffer A (0.01mol/L NH₄Ac, pH 5.0)/26% acetonitrile for the TAM metabolites or 86% buffer A/14% buffer B for 4-OH-estrone for 2 min, with a subsequent linear gradient to 75% acetonitrile over 2 min. The elution flow rate was 0.5 mL/min and 5-7 µL of the reactions were injected for all assays. The formation of

the O- glucuronides of 4-OH-TAM, endoxifen and 4-OH-estrone were quantified by UPLC based on the ratio of the glucuronide versus the free parent compound. Glucuronidation assays without TAM metabolites or 4-OH-estrone were regularly analyzed as negative controls for glucuronidation activity.

iv. Src inhibitor treatment. UGT2B7^{268His} cells and UGT2B7^{268His} cells over-expressing c-Src or v-Src were treated with 0, 0.5, 1, 10, 50, 100, or 150 μ M of Src inhibitor-1 (Sigma-Aldrich, St. Louis, MO) for 1 hour and incubated at 37°C prior to collection and homogenization. Src inhibitor-1 was dissolved in DMSO and cells were treated with equal volumes of Src inhibitor-1 and/or DMSO. Cell homogenates were utilized in glucuronidation assays as described above.

v. Immunoblot analyses. Protein expression of Src, active Src, phospho-tyrosine, β -actin, and calnexin were analyzed by immunoblot analyses. Protein was extracted from fresh homogenate of UGT2B7 cell lines and UGT2B7 cell lines over-expressing c-Src and v-Src and 75 or 100 μ g were loaded onto denaturing 8 percent SDS-PAGE gels. For the immunoblots of Src and active Src, the proteins were transferred to a PVDF membrane by the iBlot system (Invitrogen) using program 3 for 4 min and 30 sec. For the anti-phospho-tyrosine blots, the proteins were transferred to a PVDF membrane by a traditional wet transfer for 2 hours at 30 volts. Blots were blocked for 1 hr at room temperature with 5 percent milk or bovine serum albumin (phospho-tyrosine only) in TBST and subsequently incubated in primary antibody in either 5 percent milk or fetal bovine serum (phospho-tyrosine only) in TBST overnight at 37°C. HRP-conjugated secondary antibodies were used at 1:5,000 for 1 hour at room temperature. Blots were incubated with SuperSignal West Dura extended

duration chemiluminescence substrate (Pierce/Thermo Scientific, Pittsburgh, PA) for 5 min. Film was developed with a standard film processor. The blots were stripped and probed for the loading controls β -actin and/or calnexin, as stated above. The concentrations of the primary antibodies were as follows: Src 1:1000, activated Src 2.5 $\mu\text{g}/\mu\text{L}$, phospho-tyrosine 1:2000, β -actin 1:5000, and calnexin 1:2000.

vi. Statistical analysis. The Student's t-test (2-sided) was performed to compare kinetic values and glucuronidation rates. The one-way ANOVA trend test was performed to compare glucuronidation rates across multiple variables. Kinetic constants were determined using Graphpad Prism4 (GraphPad Software, La Jolla, CA) software.

D. Results

i. Over-expression of Src in UGT2B7 stably expressing cell lines. To determine the effect of Src over-expression on UGT2B7 enzyme activity, cell lines that stably expressed UGT2B7^{268His} or UGT2B7^{268Tyr} were transfected with either c-Src or v-Src. Immunoblot analysis indicated that c-Src and v-Src were successfully over-expressed in wild-type UGT2B7^{268His} or variant UGT2B7^{268Tyr} expressing HEK293 cell lines (Figure 4-2, panel A). Analysis by ImageJ software indicated that in both UGT2B7^{268His} and UGT2B7^{268Tyr} cell lines, c-Src and v-Src were expressed about 2- and 200-fold, respectively, over the levels in the original UGT2B7 cell lines (Figure 4-2, panel B). Active Src, which is phosphorylated at 419Tyr but not at Tyr529, is clearly present in the UGT2B7 cell lines over-expressing v-Src; however, no active c-Src was detected in the c-Src over-expressing and the original UGT2B7^{268His} or UGT2B7^{268Tyr} cell lines (Figure 4-2, panel C).

ii. Kinetic analyses of UGT2B7 and Src over-expressing cell lines against *trans*-4-OH-TAM, *trans*-endoxifen, and 4-OH-estrone. Several other reports demonstrated that UGT2B7 is the most important hepatic-expressed UGT in the glucuronidation of active TAM metabolites.¹⁴⁹⁻¹⁵⁰ A previous report indicated that transient co-expression of Src and UGT2B7 in COS-1 cells resulted in a 1.5-fold increase in UGT2B7 activity against 4-OH-estrone.¹¹¹ To determine if over-expression of Src alters UGT2B7 glucuronidation activity against *trans*-endoxifen and *trans*-4-OH-TAM, *in vitro* kinetic analyses of the original UGT2B7^{268His} and UGT2B7^{268Tyr} cell lines, and those that also over-express c-Src or v-Src, were performed (Table 4-1).

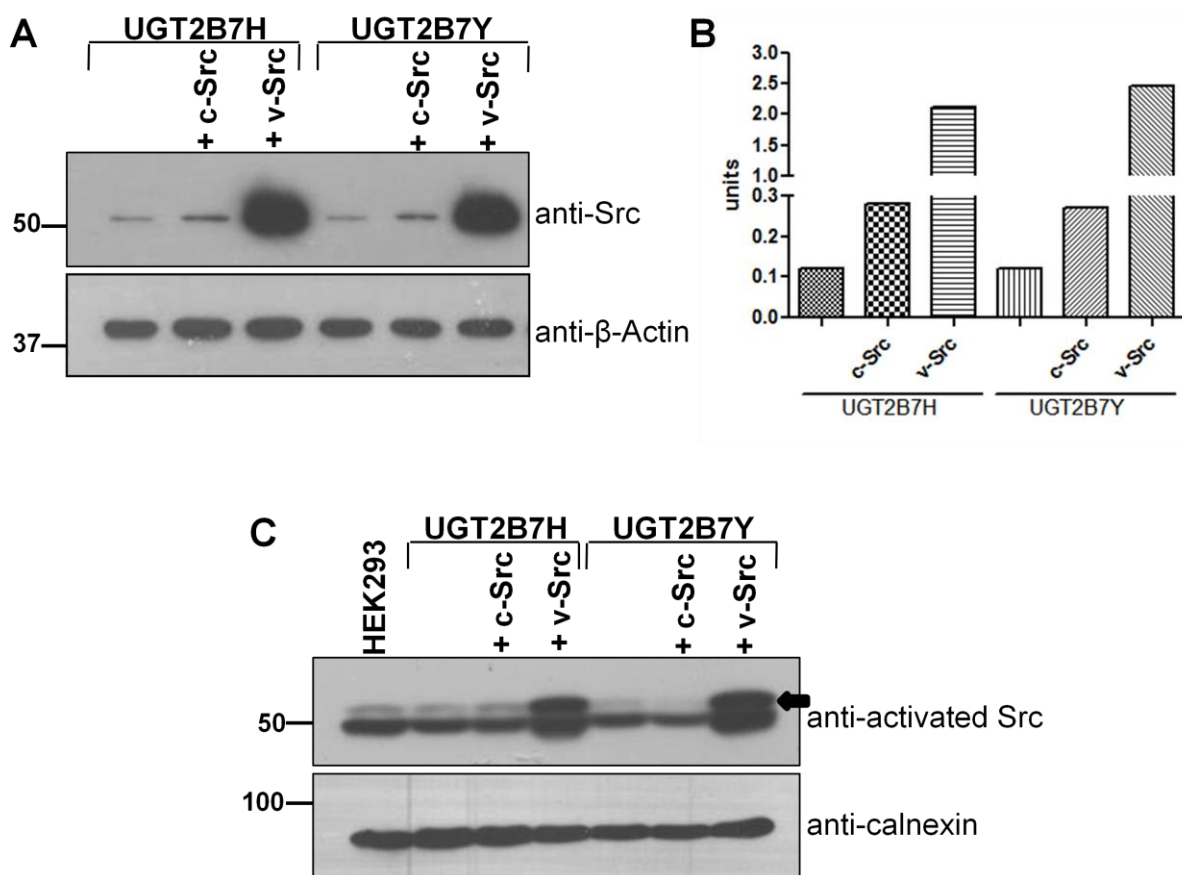


Figure 4-2. Immunoblot analysis of c-Src and v-Src over-expression in UGT2B7-HEK293 cell lines. Protein was extracted from the parent UGT2B7^{268His} (2B7H) or UGT2B7^{268Tyr} (2B7Y) cell lines and over-expressing c-Src and v-Src and 100 µg was analyzed for **A)** Src protein expression with β-actin as the loading control and **C)** activated Src protein expression and calnexin as the loading control. **B)** The relative expression levels of Src in UGT2B7H or UGT2B7Y cell lines from panel A, as measured by ImageJ software and expressed in units relative to β-actin.

Table 4-2. Kinetic analyses of the glucuronidation of *trans*-endoxifen, *trans*-4-OH-TAM, and 4-OH-estrone by Src over-expressing UGT2B7^{268His} cell lines. 15 or 500 µg of cell homogenate proteins were incubated with varying concentrations of *trans*-endoxifen (8-536 µM), *trans*-4-OH-TAM (1-172 µM), and 4-OH-estrone (2-140 µM) for 15 to 60 min and analyzed by UPLC. Kinetic analyses were performed by GraphPad Prism software and experiments were performed in triplicate. * $p \leq 0.005$, ** $p \leq 0.02$, [†] $p \leq 0.05$.

cell line	<i>trans</i> -endoxifen			<i>trans</i> -4-OH-TAM		
	V _{max} (pmol/min/µg)	K _m (µmol/L)	V _{max} /K _m (µL/min/µg)	V _{max} (pmol/min/µg)	K _m (µmol/L)	V _{max} /K _m (µL/min/µg)
2B7H	0.240 ± 0.065	31.3 ± 11.4	0.008 ± 0.002	1.38 ± 0.682	57.3 ± 28.4	0.024 ± 0.004
2B7H+ c-Src	0.147 ± 0.044	97.9 ± 73.7	0.002 ± 0.0008*	0.362 ± 0.409	93.2 ± 95.4	0.004 ± 0.002*
2B7H+ v-Src	0.115 ± 0.037 [†]	80.2 ± 9.08*	0.001 ± 0.0006*	0.239 ± 0.101 [†]	34.5 ± 2.70	0.007 ± 0.002**

cell line	4-OH-estrone		
	V _{max} (pmol/min/µg)	K _m (µmol/L)	V _{max} /K _m (µL/min/µg)
2B7H	589 ± 161	15.9 ± 5.49	37.8 ± 3.89
2B7H + c-Src	195 ± 35.4**	16.2 ± 4.22	12.5 ± 3.76*
2B7H + v-Src	222 ± 27.2**	17.3 ± 3.16	13.4 ± 3.83*

Contrary to the findings of Mitra *et al*,¹¹¹ the present study found that 4-OH-estrone glucuronidation was significantly decreased by 3-fold in the homogenates of UGT2B7^{268His} cell lines over-expressing c-Src or v-Src, as compared to the original UGT2B7^{268His} homogenate ($p \leq 0.005$), due to a significant 3-fold decrease in $V_{\max}$ ($p \leq 0.02$; Figure 4-3, row A, column III). Similarly, UGT2B7^{268His} homogenates over-expressing c-Src or v-Src exhibited significant 4- and 8-fold decreases, respectively, in overall enzyme activity against *trans*-endoxifen ($p \leq 0.005$; Figure 4-3, row A, column I). This was due to an approximate 2-fold decrease in $V_{\max}$ in the homogenates of UGT2B7^{268His} cell lines over-expressing c-Src and a significant 2-fold decrease in $V_{\max}$ ($p \leq 0.05$) and a 2.5- to 3-fold increase in K_M ($p \leq 0.005$), respectively, in the homogenates of cell lines over-expressing v-Src, as compared to the original UGT2B7^{268His} cell line. A significant 6- and 3.4-fold reduction in UGT2B7^{268His} glucuronidating activity was observed in the c-Src and v-Src over-expressing cell line homogenates against *trans*-4-OH-TAM ($p \leq 0.005$ and 0.02, respectively), due to a 4- and 6-fold decrease in $V_{\max}$, as compared to the original cell line (Figure 4-3, row A, column II).

As discussed in Chapter 3 of this dissertation, hetero- and homozygosity at the UGT2B7*2 allele caused a significant reduction in individual HLM glucuronidation activities against *trans*-4-OH-TAM and *trans*-endoxifen.¹⁵⁰ To determine if variant UGT2B7^{268Tyr} phosphorylation by Src alters enzyme activity against the active TAM metabolites, kinetic analyses were performed in the homogenates of cell lines stably expressing both the variant UGT2B7^{268Tyr} and c-Src or v-Src (Table 4-2; Figure 4-4, row B, columns I-III). Interestingly, no significant difference was observed in the

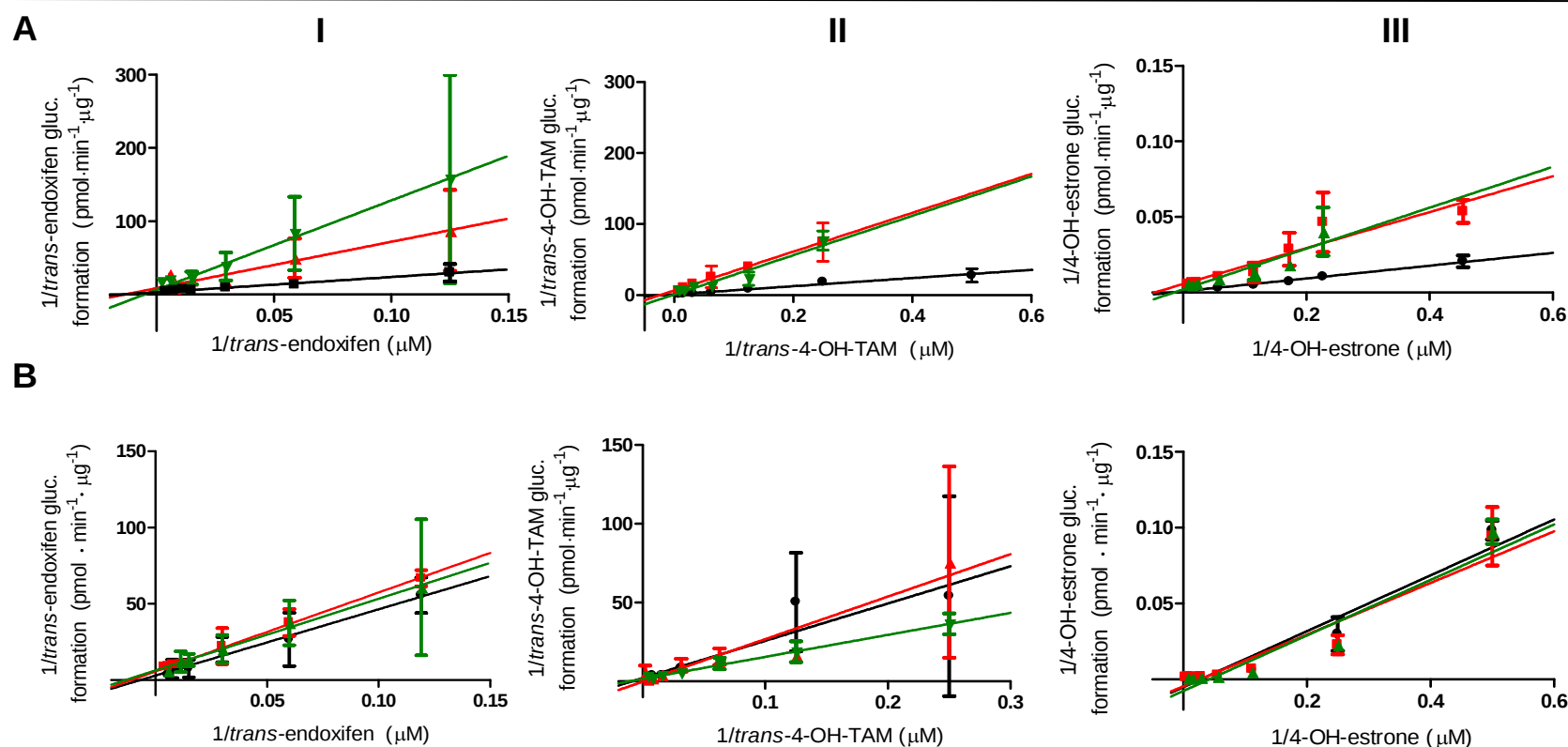


Figure 4-3. Lineweaver-Burk graphs of the glucuronidation of *trans*-endoxifen, *trans*-4-OH-TAM, and 4-OH-estrone by UGT2B7 cells over-expressing Src. 15 or 500 μg of **A)** wild-type UGT2B7^{268Hi} or **B)** the variant UGT2B7^{268Tyr} stably expressing cell lines were incubated with varying concentrations of *trans*-endoxifen (**column I**), *trans*-4-OH-TAM (**column II**), or 4-OH-estrone (**column III**) for 15 min to 1 hr at 37°C. The black lines with boxes represents the parent cell line, the red lines with triangles represents the cell lines over-expressing c-Src, and the green lines with up-side down triangles represents the cell lines over-expressing v-Src. Kinetic analyses were performed by GraphPad Prism software and are summarized in Tables 4-2 and 4-3. The error bars represent the standard deviation of three independent experiments.

Table 4-3. Kinetic analyses of the glucuronidation of *trans*-endoxifen, *trans*-4-OH-TAM, and 4-OH-estrone by variant UGT2B7^{268Tyr} cell lines over-expressing Src. 15 or 500 µg of cell homogenate protein was incubated with varying concentrations of *trans*-endoxifen (8-268 µM), *trans*-4-OH-TAM (4-516 µM), and 4-OH-estrone (2-279 µM) for 15 to 60 min and analyzed by UPLC. Kinetic analyses were performed by GraphPad Prism software and experiments were performed in triplicate.

cell line	<i>trans</i> -endoxifen			<i>trans</i> -4-OH-TAM		
	V _{max} (pmol/min/µg)	K _m (µmol/L)	V _{max} /K _m (µL/min/µg)	V _{max} (pmol/min/µg)	K _m (µmol/L)	V _{max} /K _m (µL/min/µg)
2B7Y	0.372 ± 0.352	47.7 ± 23.0	0.014 ± 0.019	0.693 ± 0.523	78.1 ± 44.9	0.008 ± 0.002
2B7Y + c-Src	0.201 ± 0.088	102 ± 71.9	0.002 ± 0.001	0.738 ± 0.402	90.9 ± 30.2	0.008 ± 0.002
2B7Y + v-Src	0.256 ± 0.134	134 ± 149	0.003 ± 0.002	0.973 ± 0.685	134 ± 132	0.009 ± 0.003

cell line	4-OH-estrone		
	V _{max} (pmol/min/µg)	K _m (µmol/L)	V _{max} /K _m (µL/min/µg)
2B7Y	1204 ± 452	30.3 ± 12.9	40.2 ± 1.98
2B7Y + c-Src	795 ± 126	23.6 ± 6.10	35.3 ± 10.3
2B7Y + v-Src	1347 ± 126	29.6 ± 8.15	47.3 ± 9.21

glucuronidation activities of the homogenates of all three cell lines bearing polymorphic UGT2B7 against *trans*-endoxifen, *trans*-4-OH-TAM, and 4-OH-estrone. Although up to a 7-fold decrease in UGT2B7 glucuronidation activity is observed against *trans*-endoxifen, it is not consistent with the trend observed against *trans*-4-OH-TAM and 4-OH-estrone, and is most likely due to the large standard deviation of the $V_{\max}/K_m$ of the parent variant UGT2B7 cell line activity (Table 4-3). This finding suggests that the amino acid change at codon 268 alters the effect of Src on UGT2B7 enzyme activity, as compared to the observations in the wild-type cell lines over-expressing c-Src or v-Src.

iii. Treatment of UGT2B7 and Src over-expressing cell lines with Src

inhibitor-1. To further demonstrate that over-expression of c-Src or v-Src alters UGT2B7 enzyme activity, UGT2B7^{268His} cells and UGT2B7^{268His} cells over-expressing c-Src or v-Src were treated with 0, 0.5, 1, or 10 μ M of Src inhibitor-1. A non-significant decrease in glucuronidation activity against all substrates was observed with increasing concentrations of Src inhibitor-1 treatment in the original UGT2B7^{268His} cell line, which is contrary to the expected result (data not shown). However, Src inhibitor-1 treatment of UGT2B7^{268His} cells over-expressing c-Src or v-Src did not alter the protein expression of phospho-tyrosines, nor did it significantly alter UGT2B7 enzyme activity against *trans*-endoxifen, *trans*-4-OH-TAM, or 4-OH-estrone (data not shown). Due to the over-expression of c-Src and v-Src, the concentrations of Src inhibitor-1 may not have been great enough to inhibit Src activity. Therefore, cells were subsequently treated with 0, 50, 100, and 150 μ M of Src inhibitor-1. Immunoblot analyses indicated that the increased concentrations of

Src inhibitor-1 were toxic to the cells, which resulted in a decrease in the protein expression of phospho-tyrosines, β -actin, and calnexin (data not shown). In addition, at 100 and 150 μ M of Src inhibitor-1, the anti-calnexin antibody recognized two distinct bands, indicating that protein degradation had occurred. Due to cellular toxicity, UGT2B7 enzyme glucuronidation activity could not be analyzed.

E. Discussion

Src is an important proto-oncogene that plays a critical role in a variety of normal cellular processes such as differentiation, proliferation, cell division,¹²⁴⁻¹²⁵ survival,¹²⁶ cell adhesion, cell motility,^{24, 126-128} morphology, and bone remodeling and reabsorption.^{124, 129-130} Interestingly, increased c-Src expression has been observed in human neoplasms such as breast, colon, gastric, lung, pancreatic, neural, and ovarian cancers.¹²³ Cell lines that express high levels of activated Src become more invasive *in vivo* and are associated with metastases in animal models. In addition, breast cancer exhibits increased Src activity as compared to normal tissue. Interestingly, cells that acquire TAM resistance exhibit increased levels of Src and there is an association in ER-positive patients of activated Src in the cytoplasm of their breast tumor and reduced survival time with endocrine therapy. MCF-7 cells that over-express active Src develop a greater migratory and invasive behavior and their growth is not inhibited when treated with TAM. When these cells are treated with a Src specific inhibitor, their morphology changes so that the cells appear to regain their cell-to-cell contacts and both migratory and invasive behavior is decreased.¹³⁹

A previous study demonstrated that UGT2B7 has the highest overall enzyme activity of all the hepatic UGTs against both *trans*-endoxifen and *trans*-4-OH-TAM, *in vitro*.¹⁴⁹ In addition, individual HLM specimens that were hetero- or homozygous for the *UGT2B7*2* allele exhibited a significant reduction in the glucuronidation of both *trans*-endoxifen and *trans*-4-OH-TAM.¹⁵⁰ Therefore, women with increasing numbers of the *UGT2B7*2* allele may have increased levels of circulating *trans*-endoxifen and

trans-4-OH-TAM, which may alter their overall response to TAM therapy. These findings suggest that UGT2B7 and the UGT2B7^{268Tyr} variant play an important role in TAM metabolism and are an important factor in patients undergoing TAM therapy.

Recent studies have indicated that UGT2B7 is phosphorylated by Src, which results in altered UGT2B7 enzyme activity.¹¹⁰⁻¹¹¹ Due to the importance of UGT2B7 in the metabolism of TAM, the finding that UGT2B7 is phosphorylated could have important implications in women administered TAM. Src is often expressed at high levels in breast tumors¹⁸⁵ and UGT2B7 is also expressed in breast tissue.⁹¹ Interestingly, Mitra *et al.* found that Src can co-localize with UGT2B7¹¹¹ and that affinity purified UGT2B7 via a His tag incorporated greater amounts of radio-labeled ATP when incubated with Src, as compared to incubations without Src,¹¹⁰ suggesting that the phosphorylation of UGT2B7 by Src is feasible. Therefore, the goal of this study was to determine if the stable transfection of Src into the wild-type UGT2B7 cell line altered enzyme activity against either *trans*-endoxifen or *trans*-4-OH-TAM, *in vitro*. Additionally, the His-to-Tyr amino acid change in the variant UGT2B7 isoform represents a potential additional phosphorylation site, which could create additional complexities in the TAM metabolism pathway. A second goal of the present study was to determine if the stable transfection of Src in the polymorphic UGT2B7^{268Tyr} cell line resulted in additional alteration in enzyme activity, beyond what the amino acid change causes.

UGT2B7^{268His} and UGT2B7^{268Tyr} stably expressing HEK293 cells were transfected with c-Src or v-Src cDNA, resulting in over-expression of these kinase isoforms. UGT2B7 cells over-expressing v-Src clearly showed an increase in

activated Src protein expression, indicating that additional Src in the active conformation was available for phosphorylating targets and potentially simulating an oncogenic transformation. UGT2B7 expression remained the same following transfection with c-Src or v-Src (data not shown).

Kinetic analyses found that increased expression of either c-Src or v-Src in UGT2B7^{268His} cells caused a significant decrease in glucuronidation activity against *trans*-endoxifen and *trans*-4-OH-TAM. Interestingly, the reduction in UGT2B7 activity was incremental for *trans*-endoxifen and *trans*-4-OH-TAM, where the v-Src over-expressing cell line had lower activity than the cell line over-expressing c-Src. This is consistent with reports in the literature that v-Src activity is greater than that of c-Src.¹²² This finding could impact TAM metabolism, as a decrease in UGT2B7 activity would result in less glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM, thereby producing higher circulating levels of active TAM metabolites. *CYP2D6* variant genotypes that result in a decrease in endoxifen plasma levels⁶⁹ are associated with an impaired clinical response to TAM and fewer side effects.^{50, 159} Additional studies are needed to determine if phosphorylation of UGT2B7 by Src affects the circulating levels of *trans*-endoxifen and *trans*-4-OH-TAM, *in vivo*.

A major limitation of the kinetic analyses was the small peaks observed by UPLC corresponding to the endoxifen- or 4-OH-TAM-glucuronide conjugates, particularly in the cell lines over-expressing Src. This difficulty led to the greater than ideal standard deviations reported in the kinetic analyses summaries (Tables 4-2 and 4-3). Despite the broad standard deviations, the difference between the wild-type UGT2B7 parent cell line and the cell lines over-expressing c-Src and v-Src were

highly significant and ranged from 3- to 8-fold. This large difference, combined with the statistical significance, supports the trend of the data and indicates that if the standard deviations were narrower, the same trend would most likely be observed.

Interestingly, the glucuronidation activity against 4-OH-estrone was also significantly decreased, in contrast to the finding of Mitra *et al.* where 4-OH-estrone glucuronidation was increased when c-Src or v-Src were over-expressed.¹¹¹ There are several methodological differences that might account for these discrepant findings. The previous study reported percent of glucuronidation activity, which was performed at one substrate concentration, whereas the present study reported the kinetic constants that involved a concentration range of each substrate that would encompass the K_M . In addition, the previous study incubated the 4-OH-estrone glucuronidation reactions for 2 hours. Based on the high reactivity of UGT2B7^{268His} to its endogenous substrate 4-OH-estrone, the present study utilized 15 min incubations as the optimal glucuronidation reaction time for accurate results. In addition, the cell line system used in the present study was the human cell line HEK293 for stable expression of the proteins of interest. In contrast, the previous study used African green monkey COS-1 cells and transient co-transfections were performed for both the expression of UGT2B7 and the over-expression of c-Src and v-Src. Interestingly, murine SYF^{-/-} cells, which do not endogenously express Src or the Src family members Yes or Fyn, exhibited a decrease in glucuronidation activity against 4-OH-estrone when transfected with both UGT2B7 and c-Src, as compared to SYF^{-/-} cells only transfected with UGT2B7.¹¹⁰ This indicates that cell type may play a role in the effect phosphorylation has on UGT2B7 enzyme activity. A human cell line is

probably a more accurate depiction of what may occur *in vivo*, as it is the most physiologically similar to humans, unlike cell lines derived from other species. However, we must acknowledge that all of these experiments (including our own) represent artificial cellular expression systems.

The variant UGT2B7^{268Tyr} exhibited decreased glucuronidation activity against *trans*-endoxifen and *trans*-4-OH-TAM and an increase in glucuronidation activity against 4-OH-estrone, in a similar pattern to what was previously described in Chapter 3 of this dissertation and elsewhere.^{91, 150} Interestingly, the increased expression of c-Src or v-Src in the variant UGT2B7^{268Tyr} cell line did not significantly alter overall enzyme activity against *trans*-endoxifen, *trans*-4-OH-TAM, and 4-OH-estrone. Although there was a trend of increasing K_m with the variant UGT2B7 cell lines over-expressing c-Src and v-Src against *trans*-endoxifen and *trans*-4-OH-TAM, it was not significant and was not sufficient to significantly alter the V_{max}/K_m . In addition, the UGT2B7^{268His} cell lines over-expressing c-Src or v-Src had similar levels of overall UGT2B7 enzyme activity as the UGT2B7^{268Tyr} variant cell line, or lower, as in the case of 4-OH-estrone. Potentially, the His-to-Tyr amino acid change, which results in an additional phosphorylation site, prevented an additive decrease in glucuronidation activity due to both the variant amino acid and phosphorylation by Src. Alternatively, the amino acid change may alter protein folding in such a way that it may cause no change in enzyme activity when phosphorylated at Tyr236 and/or Tyr438 or altered folding may prevent the phosphorylation of Tyr 268. Additional studies are needed to determine if Tyr268 is phosphorylated and to further

investigate why c-Src or v-Src over-expression does not alter UGT2B7^{268Tyr} enzyme activity against these substrates.

To further test the theory that the decrease in glucuronidation activity seen in the c-Src and v-Src over-expressing cell lines is due to the phosphorylation of UGT2B7 by Src, the cell lines were treated with a Src-specific inhibitor, Src inhibitor-1. If Src phosphorylation altered UGT2B7 activity, increasing levels of glucuronidation activity should be observed with increasing concentrations of Src inhibitor-1 in the c-Src and v-Src over-expressing UGT2B7 cells, to about the level of the original UGT2B7 cell line. However, treatment with 0, 0.5, 1, and 10 μ M of Src inhibitor-1 did not change the glucuronidation activity of UGT2B7 against *trans*-endoxifen, *trans*-4-OH-TAM, or 4-OH-estrone in the UGT2B7 cell lines over-expressing c-Src or v-Src. Treatment of the parent UGT2B7^{268His} cell line with 10 μ M of Src inhibitor-1 did cause a decrease in the intensity and the number of bands in an immunoblot analysis for phospho-tyrosine (data not shown). These data suggest that Src inhibitor-1 is able to enter the cell and inhibit Src mediated tyrosine phosphorylation. Failure to see the decrease in glucuronidation activity observed in Src inhibitor treated UGT2B7^{268His} cells over-expressing v-Src may be a result of insufficient concentrations of the inhibitor due to the high levels of activated Src in the v-Src over-expressing cells. Therefore, cells were treated with greater concentrations with 0, 50, 100, and 150 μ M of Src inhibitor-1. However, immunoblot analyses indicate that these concentrations caused toxicity in the cells, as indicated by the decrease in band intensity in the anti-phospho-tyrosine, anti- β -actin, and anti-calnexin antibodies. Additional studies should be performed evaluating intermediate

concentrations of Src inhibitor-1 or using another Src-specific inhibitor, such as PP1 or PP2 (discussed further in Chapter 5).

The data in the present study suggests that the hypothesis that Src directly interacts with UGT2B7 resulting in a phosphorylation event is false. Therefore, other mechanisms that can cause a decrease in UGT2B7 activity following over-expression of Src must be considered. Src is involved in a multitude of signaling pathways and it is conceivable that one of these pathways may interact with the UGTs, in particular, UGT2B7.

Src signaling results in the activation of other phosphorylating kinases, such as phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC). Interestingly, PKC has been implicated in the phosphorylation of UT1A family members, although additional studies are needed to confirm this finding. UGT2B7 has putative PKC phosphorylation sites, although evidence provided by Mitra *et al* suggests that PKC is not involved in UGT2B7 phosphorylation. It is possible that over-expression of Src activates a greater number of PKC or PI3K enzymes than is typical, resulting in increased phosphorylation events of UGT2B7.

The limitation of the theory that UGT2B7 is phosphorylated by a downstream substrate of Src is the localization of UGT2B7. The UGTs, including UGT2B7, are localized to the endoplasmic reticulum (ER) membrane, with a majority of the enzyme located within the lumen. In the artificial environment of cell homogenates, the majority of the UGT2B7 enzyme is probably located within the lumen of a micelle. Although the antibiotic alamethicin is used to create pores in the micelles, it is unlikely that PKC or PI3K are able to reach the putative phosphorylation sites located

on UGT2B7. In contrast, Src has been observed to localize to the perinuclear membrane, which is continuous with the EPR, and membrane encased vesicles,^{131, 133-135} lending feasibility to the co-localization of UGT2B7 and Src.

An alternative mechanism as to the cause of the decrease in UGT2B7 enzyme activity when Src is over-expressed is the phosphorylation of multidrug resistance proteins by downstream Src substrates, leading to an increase in the efflux of endoxifen and 4-OH-TAM from the cell prior to glucuronidation. Multidrug resistance protein (MDRP) is phosphorylated by PKC, causing an increase in transport activity.¹⁸⁹ In addition, the multidrug resistant-associated protein, P-glycoprotein, appears to also be phosphorylated by PKC. Interestingly, multidrug resistant cell lines that are treated with a PKC activator cause an increase in P-glycoprotein phosphorylation¹⁹⁰⁻¹⁹⁴ as well as reduced drug accumulation^{190, 195-196} and drug resistance.¹⁹⁵⁻¹⁹⁶ The opposite occurs when multidrug resistant cells are treated with a PKC inhibitor.^{190, 192, 197} A recent study has observed that endoxifen is a substrate for P-glycoprotein transport¹⁹⁸ and based on structure and activity similarities, it can be hypothesized that 4-OH-TAM is also transported by P-glycoprotein.

In the cell, many multidrug resistance proteins are localized to the plasma membrane. In the artificial environment of cell homogenate, multidrug resistance proteins are most likely located in the micelle membrane. Endoxifen and 4-OH-TAM diffuse into the micelle, where they can be glucuronidated. However, in the case of this theory, the drug transport activity of multidrug resistant-associated proteins, such as P-glycoprotein, are increased due to phosphorylation by PKC and endoxifen and

4-OH-TAM are instead transported out of the micelle before glucuronidation by UGT2B7 can occur. The rapid efflux of endoxifen and 4-OH-TAM could explain the reduced $V_{\max}/K_m$ observed in UGT2B7 cell lines over-expressing Src, as compared to the parent cell line. In addition, this theory is also consistent with the observation of the small endoxifen and 4-OH-TAM glucuronide conjugate peaks observed by UPLC.

The conclusion of Src downstream signaling, including following the activation of PI3K and PKC, typically involves transcriptional regulation. An obvious mechanism of decreased UGT2B7 activity is inhibition of *UGT2B7* transcription and, ultimately, a decrease in the number of UGT2B7 enzymes available for glucuronidation of endoxifen and 4-OH-TAM. However, preliminary data in the present study found UGT2B7 protein levels to be similar in the cell lines over-expressing Src, as compared to the parent UGT2B7 cell line. This suggests that protein levels are not affected by the over-expression of Src and other mechanisms, such as those suggested above, are involved. However, confirmation of the protein levels should be pursued in future studies, as well as determining *UGT2B7* mRNA level, to further rule out the mechanism of decreased protein levels.

In conclusion, over-expression of Src reduces stably-expressed wild-type UGT2B7 enzyme activity to the level of the variant UGT2B7 activity against *trans*-endoxifen, *trans*-4-OH-TAM, and 4-OH-estrone. However, the mechanism for this remains uncharacterized and it is likely that the initial hypothesis of the present study is false. Other mechanisms involving indirect activity of Src are possible, including phosphorylation of downstream Src substrates and the upregulation of MDR pathways. Additional studies are clearly required to determine the mechanism

behind the effect of Src over-expression on UGT2B7 activity and to assess potential clinical implications.

Chapter 5

Final considerations and clinical implications

A. Final conclusions

The evidence presented in this dissertation provides additional knowledge regarding the metabolism of tamoxifen (TAM) and specifically, how the pharmacogenetics of the UDP-glucuronosyltransferase (UGT) family of phase II metabolizing enzymes cause inter-individual differences in TAM metabolism (Figure 5-1). UGTs 1A8, 1A10, and 2B7 were identified as the most active UGTs against a major, active metabolite of TAM, *trans*-endoxifen, in HEK293 cells individually stably expressing the UGTs (Chapter 2). Interestingly, UGTs 1A8 and 1A10 are exclusively extra-hepatic, while UGT2B7 is expressed in the liver, as well as other tissues (Table 1-1).^{113, 155} In addition, UGTs 1A8 and 2B7 protein have been detected in breast tissue,^{91, 162} the target tissue of TAM treatment. The present dissertation also demonstrated that *UGT2B7* genotype is associated with the glucuronidation phenotype of human liver microsomes (HLM) against both *trans*-endoxifen and *trans*-4-hydroxy(OH)-TAM (Chapter 3). HLM specimens that were hetero- or homozygous for the polymorphic *UGT2B7*^{268Tyr} allele exhibited a significant decrease in the glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM. Human breast tissue homogenates and microsomes did not glucuronidate the TAM metabolites. However, these samples did glucuronidate 4-methylumbelliferone (4-MU), a positive control, demonstrating that the samples maintained their integrity during processing and preparation. Finally, the effect of over-expression of Src in UGT2B7 cells on the glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM was examined (Chapter 4). Stable over-expression of Src in the wild-type UGT2B7 cells resulted in a significant decrease in the glucuronidation of both TAM metabolites, similar to the level

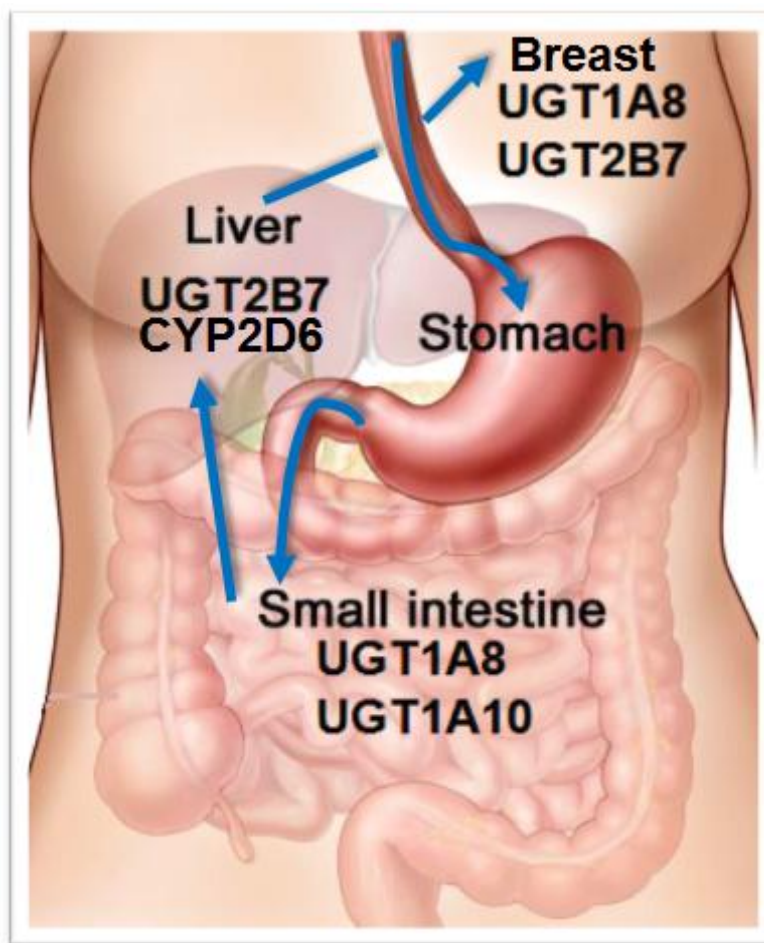


Figure 5-1. Illustration highlighting the location of the UGTs most active against TAM metabolites. TAM (blue arrows) is ingested orally and absorbed in the small intestine, where UGTs 1A8 and 1A10 are expressed. However, probably only a minimal amount of endoxifen or 4-OH-TAM is glucuronidated at this point because TAM must first be metabolized by the CYP2D6 and/or CYP3A4/5. Following absorption, TAM and its metabolites travel to the liver, where UGT2B7 and CYP2D6 are expressed. Following metabolism in the liver, TAM and its metabolites (blue arrows) travel systemically, including to the target tissue of TAM, the breast, where UGTs 1A8 and 2B7 are expressed. Research presented in this dissertation found that polymorphic UGT1A8 and 2B7 glucuronidated *trans*-endoxifen and *trans*-4-OH-TAM at a reduced rate, as compared to their wild-type counterparts. A decreased rate of glucuronidation would increase the concentration of circulating endoxifen and 4-OH-TAM, potentially affecting clinical response and acquired TAM resistance.

observed in cell lines stably expressing the polymorphic UGT2B7^{268Tyr}. Interestingly, over-expression of Src in the variant UGT2B7^{268Tyr} cell line did not alter glucuronidation activity, possibly due to the presence of the polymorphic Tyr residue. The additional Tyr residue may be phosphorylated by Src or may alter protein folding in such a way that it prevents the phosphorylation of the other Tyr residues.

B. Future directions

i. Chapter 4. Chapter 4 of this dissertation presented evidence that over-expression of c-Src or v-Src in wild-type UGT2B7 stably expressing cell lines resulted in a significant decrease in the glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM. However, there are additional experiments that can be performed to enhance the findings of this project.

In the present dissertation, UGT2B7 cells over-expressing Src were treated with Src inhibitor-1 to inhibit Src activity, with the expectation that UGT2B7 glucuronidation of *trans*-endoxifen and *trans*-4-OH-TAM in these cells would regain glucuronidation activity similar to the levels observed for the original UGT2B7 cell line. However, initial treatment concentrations were too low to cause Src inhibition in the cell lines over-expressing Src and higher concentrations resulted in cell toxicity. This indicates that the therapeutic window is narrow and a more careful evaluation of Src inhibitor-1 treatment is necessary. Therefore, a future experiment involving treatment with intermediate levels of Src inhibitor-1, or other Src inhibitors such as PP1 or PP2, should be performed.

Regardless of the Src inhibitor, the first step undertaken should be a dose-response experiment in which the concentration of the inhibitor is varied. Immunoblot

analyses of phospho-tyrosine and β -actin protein expression should be performed to determine if Src inhibition was successful without causing cell toxicity. A time-course analysis should be performed to determine the optimal amount of time cells should be treated with the Src inhibitor. The final experiment would combine four optimal concentrations with the optimal time point which should provide a dose-dependent increase in Src inhibition as determined by immunoblot analyses of phospho-tyrosine and β -actin protein expression. If the immunoblots are positive, glucuronidation activity assays should then be performed to determine the effect of Src inhibition on UGT2B7 enzyme activity. If the difference in UGT2B7 enzyme activity is related to Src function, the UGT2B7 cells over-expressing c-Src or v-Src treated with the highest concentration of Src inhibitor-1 should exhibit similar UGT2B7 enzyme activity as the original UGT2B7 cell line. If the data demonstrate no alteration in UGT2B7 enzyme activity or a decrease in enzyme activity, then the possibility that Src over-expression is not directly causing the observed decrease in UGT2B7 glucuronidation activity against *trans*-endoxifen and *trans*-4-OH-TAM would have to be addressed. Other mechanisms, such as protein interactions, downstream effectors, and other signaling pathways tied to Src, must be examined.

Another interesting future direction is the characterization of the putative Src phosphorylation sites located on both the wild-type and polymorphic UGT2B7 enzyme. This study would determine the exact location(s) of tyrosine phosphorylation on the wild-type UGT2B7 enzyme and if the location(s) are altered in the polymorphic UGT2B7 enzyme. Additionally, this study would provide further information regarding whether UGT2B7 is phosphorylated by a tyrosine kinase.

Following cell collection and protein extraction that is protected by the phosphatase inhibitor sodium orthovanadate, proteins can be visualized by denaturing SDS-PAGE gels stained with coomassie-blue. The putative UGT2B7 band, as illustrated by band size and similarity to the location of the UGT2B7 pure protein standard band, would be excised, purified, and digested by trypsin into short peptides. The short peptides would be sequenced by mass spectrometry.¹⁹⁹

ii. *in vivo* studies. The data in the present dissertation should be confirmed by studies performed *in vivo*. A detailed pharmacokinetics study based on *CYP2D6* and *UGT2B7* genotypes should be performed. Specifically, women undergoing TAM therapy should be recruited for a study that would measure plasma levels of endoxifen and 4-OH-TAM, as well as document clinical outcomes, reported adverse events, and acquired TAM resistance. In addition, their respective *CYP2D6* and *UGT2B7* genotypes in breast tissue should be examined in order to form a correlation between genotypes and protein levels of enzymes versus clinical outcome and resistance.

C. Clinical implications

i. Acquired tamoxifen resistance. Acquired TAM resistance will eventually occur for many women treated with TAM,⁵⁸ often due to unknown mechanisms. However, some studies have suggested that multidrug resistance (MDR) pathways may play an important role in TAM resistance.⁵⁸⁻⁵⁹ MDR is a phenomenon whereby a tumor acquires resistance to multiple anticancer drugs due to increased activity or expression of drug transporters, enabling the tumor cells to

remove drugs at an increased rate. This mechanism protects the tumor from the toxic effects of the drug. MDR can be accomplished by induction of the efflux transporters by the particular anticancer drug. Alternatively, drug treatment has been observed to result in over-expression⁶² and/or mutations that alter substrate specificity in transporter genes associated with MDR.⁶³⁻⁶⁴

MDR causes challenges in the treatment of many diseases, including cancer. A classic example of MDR occurs with breast cancer treatment with the chemotherapy, doxorubicin (DOX). A major mechanism for DOX resistance is the over-expression of P-glycoprotein, causing increased drug efflux, resulting in a reduction in the amount of DOX accumulated in the cell.²⁰⁰ The low level of DOX within the cancer cell is not able to cause toxicity, resulting in decreased tumor response to DOX treatment.

ii. Multidrug resistance pathways in tamoxifen resistance. Alterations the MDR pathways have been associated with acquired TAM resistance due to the increased expression of proteins that provide transport and efflux functions to cancer cells. For example, the multidrug resistance-associated protein (MRP) is expressed at higher levels in TAM resistant MCF-7 cells, as compared to TAM sensitive MCF-7 cells.⁶⁶ Expression of multidrug resistant protein 8 (MRP8, or more commonly ABCC11) is also increased in TAM resistant MCF-7 cells. Interestingly, treatment of MCF-7 cells with E₂ reduced *ABCC11* mRNA expression, which was reversed when the cells were treated with TAM.⁶⁷ Implications of MDR in TAM resistance have been observed *in vivo*; the variant *ABCC11* genotype has been associated with longer recurrence-free survival in breast cancer patients treated with TAM.⁶⁸

A recent study identified endoxifen as a substrate for P-glycoprotein. The hepatic disposition of endoxifen in mice appeared to not be affected in P-glycoprotein-deficient mice, but endoxifen accumulation was observed to be significantly reduced in brain tissue when P-glycoprotein was present. The authors suggest that high P-glycoprotein expression, such as at the blood-brain barrier, or over-expression, such as in a breast tumor, could lead to an important reduction in endoxifen accumulation (Figure 5-2).¹⁹⁸ Although additional studies are needed to determine the effect of P-glycoprotein and MDR in regards to endoxifen and 4-OH-TAM, these data provide the feasibility of an important connection between the two.

Acquired TAM resistance due to MDR is an example of a major mechanism of TAM resistance—low TAM concentrations within tumor cells. Interestingly, breast cancer patients treated with TAM that have a *CYP2D6* genotype resulting in poor conversion of TAM to endoxifen and 4-OH-TAM are more likely to acquire TAM resistance.^{50, 201} The potential mechanism is that low levels of active TAM metabolites are not able to elicit a strong clinical response, but instead cause TAM resistance, possibly by induction of MDR pathways.

iii. *UGT2B7* genotype and MDR of tamoxifen. In breast cancer patients that are wild-type for *UGT2B7*, the endoxifen and 4-OH-TAM that diffuses into tumor cells are efficiently glucuronidated. Endoxifen and 4-OH-TAM are inactivated by glucuronidation. This low level of active drug within the tumor cells may cause the tumor to acquire TAM resistance by inducing MDR pathways, similar to the mechanism of DOX resistance. In contrast, if a breast cancer patient has a variant genotype for *UGT2B7*, then their glucuronidation activity against endoxifen and 4-

OH-TAM is decreased, allowing greater concentrations of active drug to accumulate in the tumor cells. In this scenario, the TAM metabolites are able to elicit their anti-estrogenic effect before MDR induction occurs, preventing cell growth. Therefore, genotyping breast cancer patients prior to determining their course of treatment is potentially important to prevent acquired TAM resistance. Women who are wild-type for UGT2B7 may benefit more from an alternative treatment, such as with aromatase inhibitors (AI).

iv. A personalized medicine approach to tamoxifen. The work presented in this dissertation has important implications in the pharmacogenetics of TAM. Although the findings presented must be validated *in vivo*, the data have the potential to play an important role in a clinician's breast cancer treatment design, such as determining which breast cancer therapeutic to prescribe, the dose to be administered, and patient counseling on potential adverse events. The findings presented in this dissertation support a personalized medicine approach to breast cancer treatment.

v. Aromatase inhibitors compared to tamoxifen. In addition to TAM, AI are also frequently used in the treatment of estrogen receptor (ER)-positive breast cancer. AIs inhibit CYP19A1, also referred to as aromatase, which converts androstenedione to estrone and testosterone to 17 β -estradiol (E₂) in tissues other than the ovaries. The third generation of AIs includes anastrozole, exemestane, and letrozole. Recent clinical trials indicated that AIs may be more efficacious than TAM in outcomes such as disease-free survival, time to recurrence, and time to distant recurrence.²⁰² However, a modeling analysis compared data from the BIG 1-98 trial,

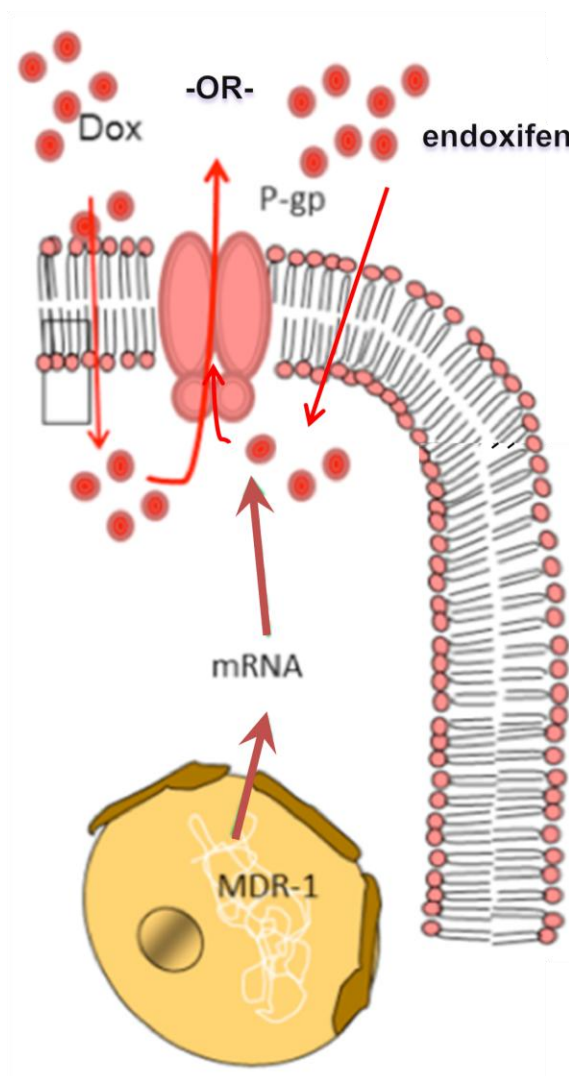


Figure 5-2. P-glycoprotein transport of doxorubicin and endoxifen. In MDR tumor cells, low concentrations of doxorubicin (DOX) or endoxifen potentially induce the over-expression of P-glycoprotein. DOX or endoxifen enter the cell by diffusion through the plasma membrane and are rapidly removed from the cell by P-glycoprotein, preventing the therapeutic effect of the drug. *This figure was modified from Sawant, R.*²⁰³

which included patients that were not genotyped for *CYP2D6*, and patients from the North Central Cancer Treatment Group (NCCTG) trial that were genotyped for *CYP2D6* by Goetz *et. al.*^{50, 204} The modeling data suggested that TAM monotherapy in a population that was homozygous for wild-type *CYP2D6* genotype demonstrated equal efficacy to AI monotherapy. In contrast, clinical trials comparing TAM to AIs were performed in populations that were not genotyped for *CYP2D6* and were unselected,²⁰⁴ which may account for the discrepancies in outcomes. Clearly, a study involving actual patients whose dosing is selected based on genotype is needed to address the discrepancy. In addition, a recent study reported that adjusting TAM dose based on *CYP2D6* genotype improved the plasma levels of endoxifen in patients. Women who were genotyped to be intermediate (reduced *CYP2D6* activity) or poor metabolizers (no *CYP2D6* activity) of TAM were given 40 mg of TAM, instead of the standard 20 mg, which was given to the women who were genotyped as extensive TAM metabolizers (wild-type *CYP2D6* activity).¹⁶⁰

Interestingly, AIs are metabolized by different phase I and phase II enzymes than TAM. Oxidation of anastrozole is performed predominantly by CYP3A4/5 and *N*-glucuronidation is performed by UGT1A4.²⁰⁵⁻²⁰⁶ *O*-glucuronidation of anastrozole is not possible due to the absence of oxygen in its chemical structure (Figure 5-3). Exemestane is also oxidized by CYP3A4, but 17-keto reduction seems to be the major mode of metabolism and forms the major metabolite, 17-dihydroexemestane. This metabolite is thought to be more active than the parent drug exemestane.²⁰⁷ Glucuronidation of 17-dihydroexemestane is predominantly performed by the hepatic UGT2B17, but the exclusively extra-hepatic UGTs 1A10 and 1A8 are also

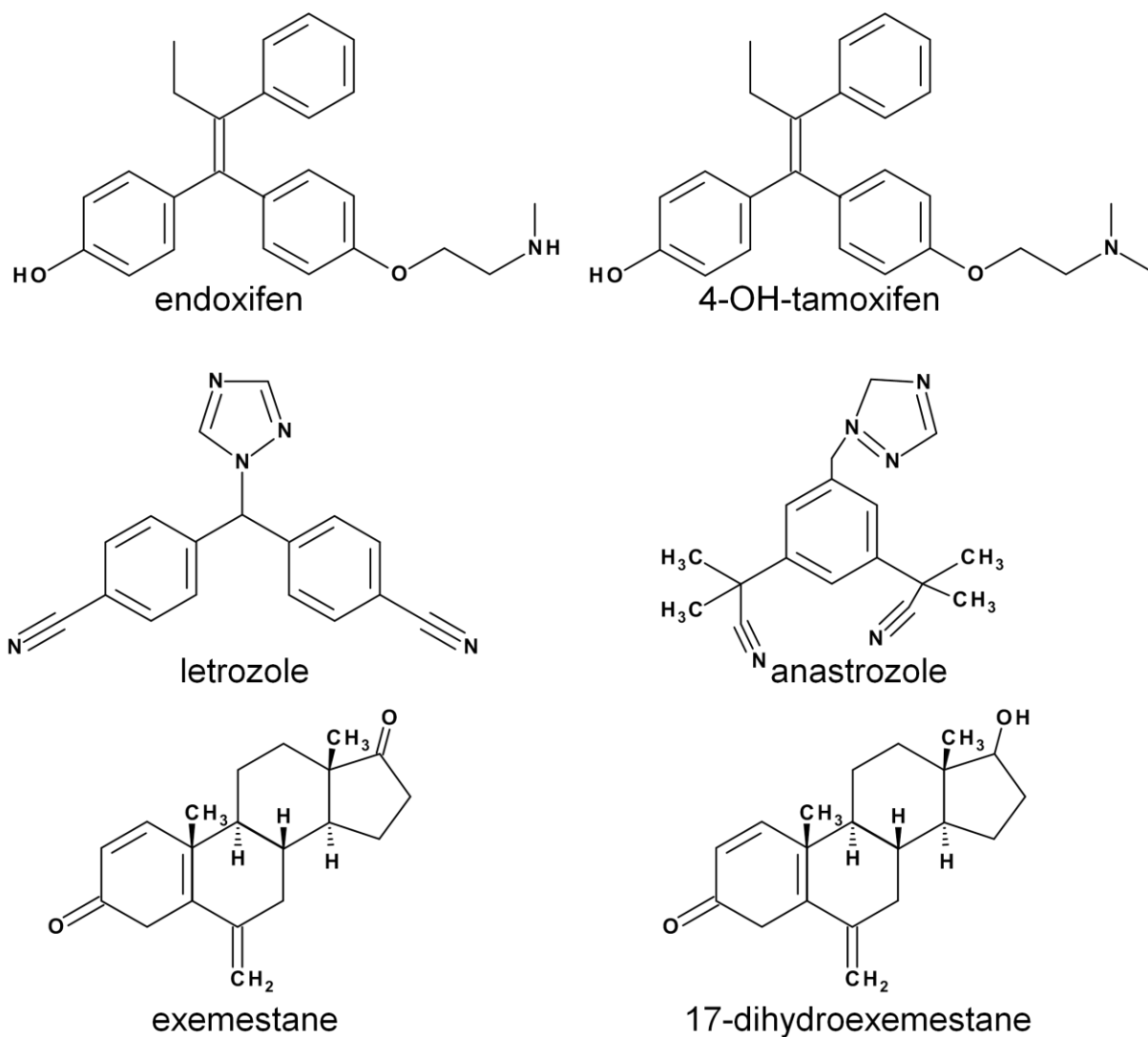


Figure 5-3. Chemical structures of TAM metabolites and AIs. The major, active metabolites of TAM, endoxifen and 4-OH-TAM, as well as the aromatase inhibitors (AI) letrozole, anastrozole, exemestane, and exemestane's active metabolite, 17-dihydroexemestane, are illustrated.

involved.²⁰⁸ CYPs 2A6 and 3A4 metabolize letrozole to inactive metabolites. Direct glucuronidation of letrozole does not occur²⁰⁹ and investigations of the specific UGTs involved in the glucuronidation of its metabolites have not been reported. Although CYP3A4 demethylates TAM and 4-OH-TAM to form *N*-desmethyl-TAM and endoxifen,³⁸ respectively, CYP3A4 has not been associated with plasma levels of TAM metabolites.⁵⁰ In contrast, CYP2D6, which hydroxylates TAM and *N*-desmethyl-TAM to form 4-OH-TAM and endoxifen,³⁸ respectively, has been correlated with endoxifen plasma levels.^{69-70, 159}

vi. *CYP2D6* extensive metabolizers. Individuals who have a *CYP2D6* genotype that codes for the wild-type enzyme, or an isoform with similar enzyme activity, are referred to as extensive metabolizers of TAM. The wild-type isoform of *CYP2D6* catalyzes the hydroxylation of TAM and *N*-desmethyl-TAM to the major, more active metabolites, 4-OH-TAM and endoxifen, respectively (Figure 1-1).³⁸⁻⁴⁰ Studies suggest that endoxifen and 4-OH-TAM are the predominant species that elicit a therapeutic response. Therefore, higher circulating levels of endoxifen and 4-OH-TAM may result in a more positive clinical response to TAM therapy, albeit with the potential for greater or more severe adverse events.⁴⁹⁻⁵⁰ Extensive metabolizers of TAM are expected to have greater circulating levels of endoxifen and 4-OH-TAM.^{69-70, 159} A patient that is an extensive metabolizer of TAM and homozygous or heterozygous for the polymorphic *UGT2B7*^{268Tyr} allele is expected to have the highest circulating levels of endoxifen and 4-OH-TAM because of the extensive conversion from TAM. In addition, these patients would also exhibit the highest levels of endoxifen and 4-OH-TAM accumulation in tumor cells because of their reduced

inactivation due to a reduced rate of glucuronidation. Therefore, these patients should be prescribed TAM for their anti-breast cancer therapy (Table 5-1) because the highest amount of the active drug is available to the tumor and there is a lower risk of acquired TAM resistance due to a low rate of inactivation within the tumor cell.

vii. *CYP2D6* intermediate metabolizers. Individuals who have a *CYP2D6* genotype that codes for a CYP2D6 enzyme with reduced activity are referred to as intermediate metabolizers of TAM. These individuals experience less conversion of TAM to endoxifen and 4-OH-TAM by CYP2D6 than individuals who have a wild-type CYP2D6 enzyme and therefore lower levels of circulating endoxifen and 4-OH-TAM.^{50, 69-70, 159} If the patient is an intermediate metabolizer, but is homozygous for the variant *UGT2B7*^{268Tyr} allele, then TAM should still be considered for therapy because relatively high levels of active drug would accumulate in the tumor cells. The reduced rate of glucuronidation may result in high enough levels of endoxifen and 4- OH-TAM for therapeutic efficacy and low risk of acquired TAM resistance. In contrast, if an intermediate metabolizer of TAM is hetero- or homozygous for the wild-type *UGT2B7*^{268His} allele, then AIs may be a better choice for anti-breast cancer therapy (Table 5-1). The higher level of glucuronidation activity may result in low levels of endoxifen and 4-OH-TAM that would limit a clinical response and potentially lead to induction of MDR pathways and ultimately, acquired TAM resistance.

vi. *CYP2D6* poor metabolizers. Individuals with a *CYP2D6* genotype that results in an inactive CYP2D6 enzyme are only able to convert small amounts of TAM to endoxifen and 4-OH-TAM, due to other CYP450s. Very low levels of

Table 5-1. Proposed clinical matrix for estrogen receptor-positive breast cancer treatment.

Genotypes		Treatment
<i>CYP2D6 extensive</i>	<i>UGT2B7His</i>	TAM TAM
	<i>UGT2B7Tyr</i>	TAM TAM
<i>CYP2D6 intermed.</i>	<i>UGT2B7His</i>	AI AI
	<i>UGT2B7Tyr</i>	TAM TAM
<i>CYP2D6 poor</i>	<i>UGT2B7His</i>	AI AI
	<i>UGT2B7Tyr</i>	AI AI

circulating endoxifen and 4-OH-TAM levels are observed and have been associated with poor clinical outcomes.^{50, 69-70, 159} In this case, the *UGT2B7* genotype is irrelevant, because very little endoxifen or 4-OH-TAM is present. Therefore, individuals that are poor metabolizers of TAM should not receive TAM and AIs should be prescribed instead (Table 5-1).

viii. Endoxifen as the parent drug. The importance of endoxifen as a major, active metabolite of TAM has led to the active development of endoxifen as a breast cancer therapeutic. Researchers at Jina Pharmaceuticals (Libertyville, IL) have described the pharmacokinetics of endoxifen in 32 human subjects and demonstrated that up to 4 mg of oral endoxifen is rapidly absorbed and systemically available.²¹⁰ This small study established the safety and feasibility of endoxifen as an oral therapeutic in humans, but larger clinical trials are required to better determine safety and efficacy. If larger clinical trials demonstrate positive results, administration of endoxifen would eliminate the need for *CYP2D6* genotyping. However, the UGTs involved in endoxifen glucuronidation will remain important to the inactivation and elimination of endoxifen.

D. Conclusion

The research presented in this dissertation provides improved understanding of the pharmacogenetics of TAM. Genotyping patients for the enzymes involved in both TAM and AI metabolism is important for personalized medicine and has the potential to improve outcomes and prevent acquired resistance by administering the

optimal therapy for each individual. Although additional studies are required, this work supports a personalized medicine approach to TAM therapy.

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PUBLICATIONS (N=7)

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