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Inductive Effect of Tamoxifen

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Pharmacogenetic Impact on Metabolism and Cytochrome P450 (CYP) 3A Inductive Effect of Tamoxifen

A dissertation submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in the Department of Pharmaceutical Sciences of the College of Pharmacy 2009

by

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ABSTRACT

Tamoxifen (TAM) is an effective selective estrogen receptor modulator (SERM) widely used for the treatment and chemoprevention of estrogen receptor (ER) positive tumors. Notwithstanding the advent of aromatase inhibitors (AIs) and newer SERMs, TAM remains an important member of the armamentarium against breast cancer. Despite its advantages and a strong track record of efficacious use, TAM is associated with significant clinical problems. Its use is associated with a large inter-subject variability in its pharmacokinetics (PK) and resulting therapeutic activity/side effects. It may affect the clearance of co-administered drugs by inducing and/or inhibiting CYP3A. CYP3A plays a central role in TAM metabolism to its major metabolite, N-desmethyl tamoxifen (N-DMT), and minor metabolites including α-hydroxy tamoxifen (α-OHT), which is implicated in endometrial toxicity. The role of CYP3A5 and the impact of its polymorphic expression on the formation of α-OHT and N-DMT are not known.

Furthermore, previous work in our laboratory indicated that TAM induces CYP3A in human hepatocytes by activating pregnane X receptor (PXR). Therefore, we investigated the genetic influence on TAM metabolism in vitro and CYP3A induction in breast cancer patients undergoing TAM therapy. Using cDNA expressed CYP3A4, CYP3A5, CYP3A5*8, CYP3A5*9 microsomes and a panel of human liver microsomes genotyped for CYP3A4/5 variants, enzyme kinetics for the formation of α-OHT and N-DMT were determined. Our in vitro findings suggest that the formation of α-OHT is primarily mediated by CYP3A4, and is not affected by CYP3A5 genotype. This is further supported by the lack of association between CYP3A5 genotypes and plasma concentrations of α-OHT in our clinical study. On the other hand, both CYP3A4 and
CYP3A5 contribute to the formation of N-DMT. The intrinsic clearance for N-DMT formation by cDNA expressed CYP3A4 and CYP3A5 were 0.57 and 0.35 ml/min/pmol P450, respectively. Furthermore, there was a significant difference in the Vmax of N-DMT formation between CYP3A5*1/*1 and CYP3A5*3/*3 microsomes (267.63 Vs 101.03 pmol/min/mg).

Employing midazolam (MDZ) as a CYP3A probe, we assessed the induction of CYP3A by TAM in breast cancer patients. Apparent oral clearance of midazolam was determined at the baseline (prior to TAM administration), Day 1 and Day 42 after administration of a TAM (20 mg/day). In 6 out of 13 patients, we observed an increase in the clearance of MDZ (mean: 70%, range: 26 to 161%) indicative of CYP3A induction. Furthermore, there was a marked inter-subject variability in the extent of CYP3A induction. Blood samples were genotyped for polymorphisms in CYP3A (CYP3A4*1B and CYP3A5*3) and PXR (-25385C>T, -24381 A>C and 63396 C>T) to investigate their association with CYP3A induction. We observed a trend towards higher induction in subjects with CYP3A5 *1/*3 than CYP3A5*3/*3 genotypes. One patient with maximum induction (2.5 fold) was homozygous to TT and CC variants of PXR (-25385C>T and -24381 A>C). No association was found with CYP3A4*1B, PXR 63396 C>T and CYP3A induction. Overall, our studies provide novel insights into genetic contributions to the formation of CYP3A derived metabolites such as N-DMT and α-OHT, and to the inter-subject variability in CYP3A inductive effect of TAM.
To my (late) father, mother and brother, for their love, support and encouragement

To my wife for her love, patience, sacrifice and unconditional support in standing by me during all these years

To my son for bringing a new meaning to my life and for all the love and joy
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# Table of Contents

ABSTRACT .................................................................................................................................I

ACKNOWLEDGEMENTS.............................................................................................................V

LIST OF FIGURES.........................................................................................................................X

LIST OF TABLES ............................................................................................................................XIV

LIST OF ABBREVIATIONS ........................................................................................................... XVI

1. CHAPTER ONE: LITERATURE REVIEW .............................................................................. 1
   1.1. INTRODUCTION ............................................................................................................. 1
       1.1.1. Breast Cancer and Endocrine Therapy .................................................................. 1
       1.1.2. Tamoxifen ............................................................................................................. 2
       1.1.3. Pharmacokinetics of Tamoxifen .......................................................................... 4
   1.2. VARIABILITY IN PHARMACOKINETICS/PHARMACODYNAMICS OF ANTI-CANCER DRUGS ............................................................ 7
   1.3. OVERVIEW OF CYTOCHROME P450 (CYP) 3A ENZYMES .............................................. 10
       1.3.1. Variability in Cytochrome P450 (CYP) 3A Enzymes ........................................... 12
       1.3.2. Induction of CYP3A ............................................................................................. 13
             1.3.2.1. Mechanism of CYP3A Induction – Role of PXR ......................................... 16
             1.3.2.2. Variability in CYP3A Induction ..................................................................... 18
       1.3.3. Inhibition of CYP3A ............................................................................................. 19
             1.3.3.1. Mechanism of CYP3A Inhibition ............................................................... 21
             1.3.3.2. Variability in CYP3A Inhibition ..................................................................... 23
       1.3.4. Implications of TAM Mediated CYP3A Induction/Inhibition ................................. 24
             1.3.4.1. CYP3A Induction by TAM ........................................................................... 24
             1.3.4.2. CYP3A Inhibition by TAM ........................................................................... 26
   1.4. IMPACT OF PHARMACOGENETICS ON PK/PD OF ANTI-CANCER DRUGS ......................... 26
   1.5. CYP3A POLYMORPHISMS ............................................................................................ 29
       1.5.1. Pharmacogenetics of CYP3A4 ............................................................................. 31
       1.5.2. Pharmacogenetics of CYP3A5 ............................................................................. 32
       1.5.3. Pharmacogenetics of CYP3A7 ............................................................................. 33
       1.5.4. Pharmacogenetics of CYP3A43 ........................................................................... 34
   1.6. ROLE OF PXR POLYMORPHISMS ............................................................................. 34
   1.7. TAM AND CYP2D6 POLYMORPHISMS .................................................................... 35
   1.8. METHODS TO ASSESS CYP3A INDUCTION ................................................................ 36
       1.8.1. IN VITRO METHODS .......................................................................................... 36
       1.8.2. CLINICAL ASSESSMENT OF INDUCTION ......................................................... 38

2. CHAPTER TWO: HYPOTHESIS AND SPECIFIC AIMS ..................................................... 41
   2.1. HYPOTHESIS ............................................................................................................... 41
   2.2. SPECIFIC AIM 1 ......................................................................................................... 41
   2.3. SPECIFIC AIM 2 ......................................................................................................... 42
   2.4. SPECIFIC AIM 3 ......................................................................................................... 44

3. CHAPTER THREE: EXPERIMENTAL DESIGN AND METHODS ....................................... 45
   3.1. SPECIFIC AIM 1: ASSESSMENT OF IMPACT OF CYP3A5 ON TAMOXIFEN CLEARANCE IN VITRO ......................................................... 45
       3.1.1. Materials ............................................................................................................. 45
       3.1.2. Incubation of Testosterone with CYP3A Genotyped Microsomes ..................... 46
       3.1.3. Incubation of Midazolam with CYP3A Genotyped Microsomes ....................... 46
       3.1.4. Incubation of Tamoxifen with CYP3A Genotyped Microsomes ........................ 47
       3.1.5. Incubation of Tamoxifen with recombinant CYP3A Supersomes .................... 48
       3.1.6. HPLC Analysis of 6-β-Hydroxy Testosterone ..................................................... 48
       3.1.7. LC-MS/MS Analysis of α-OHT and N-DMT ....................................................... 49
       3.1.8. Data Analysis ...................................................................................................... 50
       3.1.9. Statistical Analysis ............................................................................................. 51
List of Figures

Figure 1: Major pathways involved in Tamoxifen metabolism ............................................ 6
Figure 2: Inter-individual variability in clearance of anti-cancer compounds .................... 9
Figure 3: Potential sources of inter-individual variability in PK of drugs ............................. 9
Figure 4: Relative abundance of CYPs in liver microsomes. ........................................... 11
Figure 5: Relative contribution of CYPs to drug metabolism. ........................................ 12
Figure 6: Schematic representation of ligand mediated PXR activation and CYP3A induction ........................................................................................................... 17
Figure 7: Schematic representation of CYP3A locus on chromosome 7. The ................. 29
Figure 8: Schematic of hepatocyte treatment for CYP3A induction studies ..................... 37
Figure 9: Metabolism of Midazolam to 1-OH and 4-OH midazolam by CYP3A4/5 ......... 39
Figure 10: A typical HPLC chromatogram of 6-β-hydroxy testosterone and 11 α-hydroxy progesterone (internal standard) in the microsomal incubates .......................... 49
Figure 11: Study schematics of the pharmacokinetic trial ................................................. 56
Figure 12: A typical allelic discrimination plot indicating the three genotypes ............... 59
Figure 13. A typical LC-MS/MS chromatogram of α-OHT (A) and N-DMT (B) in the microsomal incubates ...................................................................................... 61
Figure 14. The enzyme kinetics of α-hydroxy tamoxifen formation from TAM by the recombinant CYP3A4 (A) and CYP3A5 (B) isoforms. Each data point represents the mean of two 10 minutes incubations of TAM (0-50 µM) with 50 pmol of CYP3A4 or CYP3A5 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from Michaelis-Menten model using WinNonlin 5.2 .............................................................. 62
Figure 15. The enzyme kinetics of N-desmethyl tamoxifen (N-DMT) formation from TAM by the recombinant CYP3A4 (A) and CYP3A5 (B) isoforms. Each data point represents the mean of two 10 minutes incubations of TAM (0-50 µM) with 50 pmol of CYP3A4 or CYP3A5 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from Michaelis-Menten model using WinNonlin 5.2 .............................................................. 63
Figure 16. Comparison of Vmax of α-OHT formation between the three CYP3A5*1/*1, *1/*3 and *3/*3 genotypes. Each point represents the Vmax estimated by fitting the data to Michaelis-Menten kinetics using nonlinear regression analysis. TAM (0-50 µM) was incubated with genotyped human liver microsomes as described in the methodology section. There was no significant difference between the three genotypes. .......................................................... 64

Figure 17. Correlations between Vmax of α-OHT formation and (A) CYP3A4 protein, (B) CYP3A5 protein in a panel of genotyped human liver microsomes. ....... 65

Figure 18. Correlations between Vmax of α-OHT formation and (A) testosterone-6-β-hydroxylation, (B) midazolam-1-hydroxylation in genotyped human liver microsomes. ..................................................................................................... 65

Figure 19. Comparison of Vmax of N-DMT formation between the three CYP3A5*1/*1, *1/*3 and *3/*3 genotypes. Each point represents the Vmax estimated by fitting the data to Michaelis Menten kinetics using nonlinear regression analysis. TAM (0-50 µM) was incubated with genotyped human liver microsomes as described in the methodology section. There was a significant difference between CYP3A5*1/*1 and CYP3A5 *1/*3 or CYP3A5*3/*3 (p<0.05). ........................................................................................................... 68

Figure 20. Correlations between Vmax of N-DMT formation and (A) CYP3A4 protein, (B) CYP3A5 protein in a panel of genotyped human liver microsomes. ....... 68

Figure 21. Correlations between Vmax of α-OHT formation and (A) testosterone-6-β-hydroxylation, (B) midazolam-1-hydroxylation in genotyped human liver microsomes. ..................................................................................................... 69

Figure 22. The enzyme kinetics of N-desmethyl tamoxifen (N-DMT) formation from TAM by the recombinant CYP3A5, CYP3A5*8 and CYP3A5*9 microsomes. Each data point represents the mean of two 10 minute incubations of TAM (0-50 µM) with 50 pmol of CYP3A5 or 3A5*8 or 3A5*9 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from Michaelis-Menten model using WinNonlin 5.2. ................. 71
Figure 23. The enzyme kinetics of 1-hydroxy-midazolam formation from midazolam by the recombinant CYP3A5, CYP3A5*8 and CYP3A5*9 microsomes. Each data point represents the mean of two 5 minutes incubations of midazolam (0-400 µM) with 50 pmol of CYP3A5 or 3A5*8 or 3A5*9 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from substrate inhibition model using WinNonlin 5.2. ............................................ 71

Figure 24. A typical chromatogram of a blank plasma spiked with MDZ, labeled ^15_N MDZ (IS), 4-OH MDZ and 1-OH MDZ. ......................................................... 75

Figure 25. Representative calibration plasma standard curves for Midazolam (a), and 1-OH-MDZ in human plasma samples. ................................................................. 77

Figure 26. Plasma concentration time profile of MDZ in breast cancer patients on Day 0 (Blue), Day 1 (Green) and Day 42 (Red) (A). Profile of patient ID= 06 showing induction of CYP3A on Day 42 as seen by decrease in the AUC and Cmax (B). ......................................................................................................... 79

Figure 27. Changes in oral clearance (CL/F, L/hr) of MDZ between Day 0 and Day 42, and Day 1 and Day 42 (A). Changes in AUC of MDZ between Day 0 and Day 42, and Day 1 and Day 42 (B). Changes in half life of MDZ between Day 0 and Day 42, and Day 1 and Day 42 (C). CL/F and AUC estimates were determined by noncompartmental analysis using WinNonlin 5.2. .................. 82

Figure 28. Association of CYP3A5 genotype with CYP3A induction determined as fold change in midazolam oral clearance. DNA was isolated from fresh blood collected in EDTA vacutainers using Puregene kit and genotyped using TaqMan SNP genotyping assay. ................................................................. 84

Figure 29. Association of PXR -25385C>T genotype with CYP3A induction determined as fold change in midazolam oral clearance. DNA was isolated from fresh blood collected in EDTA vacutainers using Puregene kit and genotyped using TaqMan SNP genotyping assay. ................................................................. 85

Figure 30. Association of PXR -24381 A>C genotype with CYP3A induction determined as fold change in midazolam oral clearance. DNA was isolated from fresh
blood collected in EDTA vacutainers using Puregene kit and genotyped using TaqMan SNP genotyping assay. ......................................................... 85

Figure 31. Association of mean plasma $\alpha$-OHT (ng/ml) between CYP3A5*1/*3 vs CYP3A5*3/*3 genotypes on Day 1 and Day 42....................................................... 86
List of Tables

Table 1. List of known CYP3A inducers ............................................................... 15
Table 2. Factors influencing the variability in CYP3A induction by drugs .......... 19
Table 3. List of common reversible and mechanism based inhibitors ............... 22
Table 4. Classification of CYP3A inhibitors based on their potency (Adapted from US FDA Drug Interaction guidance) ................................................. 23
Table 5. Distribution of important CYP3A polymorphisms in various ethnic groups. ... 30
Table 6. Enzyme kinetic parameter estimates for the formation of α-OHT and N-DMT by recombinant CYP3A4 and CYP3A5. Each incubation contained TAM (0-50 µM), 50 pmol of rCYP3A4 or rCYP3A5 and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis Menten model using non linear regression analysis. Results are Mean ± SE from duplicate incubations. ........................................................................................................... 62
Table 7. Enzyme kinetic estimates for the formation of α-OHT and N-DMT in a panel of genotyped human liver microsomes......................................................... 66
Table 8. Enzyme kinetic parameter estimates for the formation of N-DMT and 1-hydroxy midazolam by recombinant CYP3A5, CYP3A5*8 and CYP3A5*9 from TAM and midazolam, respectively. Each incubation contained TAM (0-50 µM) or Midazolam (0-400 µM), 50 pmol of rCYP3A5 or rCYP3A5*8 or rCYP3A5*9 and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis Menten model for N-DMT formation and substrate inhibition kinetics for 1-hydroxy midazolam formation, using non linear regression analysis. Results are Mean ± SE from duplicate incubations. .......... 70
Table 9. Enzyme kinetic parameter estimates for the formation of α-OHT and N-DMT by CYP3A4 intronic variant from TAM. Each incubation contained TAM (0-50 µM), 0.1mg/ml of protein and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis Menten model for α-OHT and N-DMT formation using non linear regression analysis. Each incubation was performed in duplicates. ................................................................................... 72
Table 10. Enzyme kinetic parameter estimates for the formation of 6-β-hydroxy testosterone by CYP3A4 intronic variant from testosterone. Each incubation contained testosterone (0-400 µM), 0.25mg/ml of protein and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis Menten model for 6-β-hydroxy testosterone formation using non linear regression analysis. Each incubation was performed in duplicates. 73

Table 11. Demographics of patients enrolled in the PK study 78

Table 12. Changes (%) in Midazolam Pharmacokinetics in Patients (N=6) 80

Table 13. Pharmacokinetic estimates of midazolam after administration of 2 mg oral midazolam syrup in 13 breast cancer patients on three visits. 81

Table 14. Percent change in midazolam oral clearance due to CYP3A induction by 94
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>α-OHT</td>
<td>Alpha hydroxy tamoxifen</td>
</tr>
<tr>
<td>1-OH MDZ</td>
<td>1-hydroxy midazolam</td>
</tr>
<tr>
<td>4-OH MDZ</td>
<td>4-hydroxy midazolam</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4-hydroxy tamoxifen</td>
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<td>6-MP</td>
<td>6-mercaptopurine</td>
</tr>
<tr>
<td>ACS</td>
<td>American Cancer Society</td>
</tr>
<tr>
<td>AI</td>
<td>Aromatase Inhibitor</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike Information Criteria</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
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<tr>
<td>BIC</td>
<td>Bayesian Information Criteria</td>
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<tr>
<td>BRCA-1</td>
<td>Breast Cancer-1</td>
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<tr>
<td>CAR</td>
<td>Constitutive Androstane Receptor</td>
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<tr>
<td>CL/F</td>
<td>Oral clearance</td>
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<td>CLint</td>
<td>Intrinsic Clearance</td>
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<tr>
<td>Cmax</td>
<td>Maximum plasma drug concentration</td>
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<td>Cmin</td>
<td>Minimum plasma drug concentration</td>
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<td>Cytochrome P450</td>
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<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier Transform-Ion Cyclotron Resonance</td>
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<tr>
<td>FXR</td>
<td>Farnesoid X Receptor</td>
</tr>
<tr>
<td>GCRC</td>
<td>General Clinical Research Center</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal growth factor Receptor 2</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>hPAR</td>
<td>Human Proteinase-Activated Receptor</td>
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<tr>
<td>HPLC</td>
<td>High performance Liquid Chromatography</td>
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<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>IdMOC</td>
<td>Integrated discrete multiple organ culture system</td>
</tr>
<tr>
<td>Km</td>
<td>Michealis Menten constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LC-MS</td>
<td>Liquid Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>LHRHa</td>
<td>Leutinizing hormone analogue</td>
</tr>
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<td>LTQ-FT</td>
<td>Linear Trap Quadruple-Fourier Transform</td>
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<tr>
<td>MDZ</td>
<td>Midazolam</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multi-Drug Resistance 1</td>
</tr>
<tr>
<td>MRP2</td>
<td>Multi-Drug Resistance Protein 2</td>
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<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
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<td>N-DMT</td>
<td>N-desmethyl tamoxifen</td>
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<td>Organic Anion-Transporting Polypeptide-C</td>
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<td>P-glycoprotein</td>
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<td>Pregnane X Receptor</td>
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<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>RXR</td>
<td>Retinoic Acid X Receptor</td>
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<tr>
<td>SE</td>
<td>Standard Error</td>
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<tr>
<td>SERM</td>
<td>Selective Estrogen Receptor Modulator</td>
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<td>Sulfotransferases</td>
</tr>
<tr>
<td>SXR</td>
<td>Steroid X Receptor</td>
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<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>Tmax</td>
<td>Time to the maximum plasma concentration</td>
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<tr>
<td>TPMT</td>
<td>Thiopurine Methyl Transferase</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP glucuronosyl transferases</td>
</tr>
<tr>
<td>UNG</td>
<td>Uracil N Glycosylase</td>
</tr>
<tr>
<td>Vd</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
</tr>
<tr>
<td>VKORC1</td>
<td>Vitamin K Epoxide Reductase Complex subunit 1</td>
</tr>
<tr>
<td>Vmax</td>
<td>Maximum reaction rate of enzymes</td>
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1. CHAPTER ONE: LITERATURE REVIEW

1.1. Introduction

1.1.1. Breast Cancer and Endocrine Therapy

Breast cancer is the second most common cancer among women and the second leading cause of cancer death in the US. During their life time, 1 in 8 (12%) women have the risk of developing breast cancer with an expected mortality rate of 1 in 35 (3%). According to the 2009 report of American Cancer Society (ACS), an estimated 192,370 new cases of invasive breast cancer will be diagnosed, and approximately 40,610 will succumb to the disease (ACS 2009 statistics).

The various treatment options for breast cancer include a combination of radiation, surgery, chemotherapy, hormonal and targeted therapy. The therapy depends on the tumor size, location, stage, age of patient, hormonal receptor and menopausal status. Endocrine (hormonal) therapy has been widely used for chemoprevention and treatment in patients expressing estrogen receptor (ER) positive tumors. The role of estrogens in the pathogenesis of breast cancer is well established. Estradiol binds to ER in the nucleus, and the ER homodimer complex then binds to the estrogen receptor elements present upstream to the promoter region of ER target genes. This results in a conformational change, increasing the transcription of genes responsible for cell proliferation leading to tumor progression (Klein-Hitpass, et al., 1989; McDonnell and Norris, 2002). Since approximately 70% of the tumors are positive for ERs, depriving the tumors of estrogen mediated stimulus remains the most effective and targeted form of therapy. Two main isoforms of ER have been identified - ERα...
and ERβ with different tissue distribution and affinities to various SERMs (Katzenellenbogen and Katzenellenbogen, 2000). ERα is widely expressed in breast cancer cells, endometrium, ovarian cells and hypothalamus, while presence of ER β has been reported in kidney, brain, bone, heart, lungs, intestinal mucosa and endothelial cells.

The first endocrine therapy developed by Dr. George Beatson in 1896 was by means of bilateral ovaricectomy (Beatson GT, 1896). Over the past decades, several forms of endocrine therapy for treatment of ER positive breast cancer have been introduced. These include a) use of a selective estrogen receptor modulator to block the estrogen receptor (SERM, eg, Tamoxifen) or down regulate estrogen receptor using estrogen receptor antagonist (eg, Fulvestrant), b) suppression of estrogen synthesis in postmenopausal women using aromatase inhibitors (eg, Letrozole), c) hormonal ablation by surgery or radiotherapy or by administration of leutinizing hormone analogue (LHRHa) eg., Goserelin in premenopausal women. Despite the availability of several treatment options, there is still a need to better understand the disposition of these drugs in order to optimize the therapy and minimize unwanted side effects.

1.1.2. Tamoxifen

Tamoxifen (TAM) is a selective estrogen receptor modulator (SERM) that is currently FDA-approved for the treatment of all stages of hormone-responsive breast cancer and for the prevention of breast cancer in high-risk women (Jordan, 2003b; Brauch and Jordan, 2009). Originally tested as a contraceptive in 1976, TAM was later found to suppress breast cancer proliferation in in vitro studies and was developed into a hormonal therapy for breast cancer. Since its introduction, TAM has been the first line therapy for patients with estrogen receptor
positive tumors for over three decades. A five-year TAM therapy in patients with invasive breast cancer results in a 31% decrease in the annual death rate and a 40-50% decrease in the risk of primary breast cancer in high risk women (Jordan, 2003a). Notwithstanding the advent of aromatase inhibitors (AIs) and newer SERMs like raloxifene, TAM remains an important member of the armamentarium against breast cancer (Bao, et al., 2006; Fisher, et al., 2005b; Ligibel and Winer, 2005). It continues to be the only drug available for use in premenopausal women for chemoprevention or treatment of ER-positive breast cancer (White, 1999). TAM acts by competitively binding to ER and blocks the action of estradiol. TAM also has a mixed ER agonist and antagonist properties depending on the target tissues. By way of its agonist action, TAM helps to maintain bone mineral density and lower lipid levels thereby ameliorating bone and cardiovascular diseases in women (Osborne, 1998; Kristensen, et al., 1994). In the uterus, TAM’s agonist action has been reported to increase the risk of developing endometrial cancer in postmenopausal women (Jordan and Morrow, 1994).

Despite its advantages and a strong track record of efficacious use, TAM is associated with significant clinical problems. These include extensive inter-individual variability in its pharmacokinetics and therapeutic outcome, drug-drug interactions, acquired drug resistance and a 2 to 8 fold higher risk of developing endometrial cancer. Only half of the patients treated respond to adjuvant TAM (Jordan, 2000). Clinical studies suggest that plasma concentration of TAM and its metabolites vary widely among patients (Lonning, et al., 1992; Stearns, et al., 2003). Inter-individual differences in the formation of active/toxic metabolites may be an important source of variability in response to TAM. This variability
can be attributed to the factors that impact the basal and induced expression of CYP3A enzyme.

1.1.3. **Pharmacokinetics of Tamoxifen**

After an oral administration of 20 mg dose, TAM is rapidly absorbed and the peak plasma concentrations (Cmax) are achieved within 3-4 hours in healthy volunteers (Guelen, et al., 1987). Its bioavailability is approximately 100% suggesting minimal first pass metabolism (Tukker, et al., 1986; Herrlinger, et al., 1992). Due to its high lipophilicity, TAM is extensively bound (>95%) to the plasma protein albumin (Buckley and Goa, 1989).

Distribution of TAM in 14 patients undergoing long term therapy revealed the presence of higher concentrations in breast, uterus, liver, lung, ovaries, endometrium and pancreas (Lien, et al., 1991; Fromson, et al., 1973). The concentrations of TAM and N-DMT were 20-400 fold higher in endometrium and 5-11 higher in breast tissue when compared to plasma levels (Kisanga, et al., 2004; Giorda, et al., 2000). The apparent volume of distribution of TAM is 50-60L (Lonning, et al., 1992).

TAM is extensively metabolized in humans to form a large number of metabolites. Several of these metabolites are biologically active contributing to the therapeutic efficacy and/or toxic side effects. The biotransformation of TAM is primarily mediated by cytochrome-P450 enzymes, mainly through N-demethylation and hydroxylation to form three primary metabolites, α-hydroxy-TAM (α-OHT), N-desmethyl-TAM (N-DMT), and 4-hydroxy-TAM (4-OHT) [Fig. 1].
Several studies have reported that CYP3A is the major enzyme involved in N-demethylation, whereas CYP2D6 is the primary catalyst for 4-hydroxylation (Desta, et al., 2004; Williams, et al., 2002). The plasma levels of N-DMT are twofold higher than TAM, since it has a half life twice that of TAM (14 days vs. 7 days). N-DMT and 4-OHT are further metabolized to a recently characterized active metabolite 4-hydroxy-N-desmethyl tamoxifen (endoxifen). Endoxifen and 4-OHT are the active therapeutic moieties and are formed via 4-hydroxylation of N-DMT and TAM, respectively by CYP2D6. When compared with TAM, these metabolites are at least 100 times more potent in binding to estrogen.
Figure 1: Major pathways involved in Tamoxifen metabolism
receptor and suppressing breast cancer cell proliferation \textit{in vitro} (Jordan, 1982). Endoxifen has a similar affinity to ER receptor as that of 4-OHT, and is present at an average six fold higher concentrations than 4-OHT (Stearns, et al., 2003; Oseni, et al., 2008). Another metabolite of significance is $\alpha$-OHT, a genotoxic compound, which upon further activation forms DNA adducts and has been proposed to cause endometrial cancer (Crewe, et al., 1997; Boocock, et al., 2002). $\alpha$-OHT is exclusively formed from TAM by CYP3A. It undergoes sulphonation by hydroxysteroid sulfotransferases and binds to the amino groups of guanine in DNA, leading to the formation of DNA adducts in the endometrium (Kim, et al., 2005; Shibutani, et al., 1998).

TAM and its metabolites (4-OHT and endoxifen) further undergo Phase II conjugation reactions by human UDP glucuronosyl transferases (UGTs) UGT1A4, UGT2B15 and sulfotransferases (SULT) SULT1A1 (Nishiyama, et al., 2002; Ogura, et al., 2006). 4-OHT was mostly found as glucuronide conjugates in the bile and urine. (Poon, et al., 1993; Sun, et al., 2006).

1.2. Variability in Pharmacokinetics/Pharmacodynamics of Anti-cancer Drugs

A very high inter-individual variability (25-70\%) in the clearance of anticancer drugs has been reported, resulting in different pharmacokinetic and pharmacodynamic profiles (Fig. 2) (Felici, et al., 2002; Mathijssen, et al., 2007). Such a wide inter-individual variability in TAM and other anti-cancer agents often results in different therapeutic outcomes in patients. The primary route of elimination for most anticancer compounds is via hepatic
clearance, and is subject to wide variability due to the differences in the expression of
CYPs and UGTs. Recognizing the underlying causes for variability in pharmacokinetics
/pharmacodynamics will help in optimizing dosage regimen and improving the benefit
/risk ratio in patients. Some of the potential sources of variation include differences in
absorption, distribution, metabolism and excretion of drugs, genetic polymorphisms in
enzymes, transporters and receptors involved in the disposition pathways, physiological
factors including age, gender, disease state and organ dysfunction, and drug interactions
due to concomitant administration of drugs/herbal remedies (Fig. 3). Furthermore,
differences in concentrations of drugs in tumors can also be a significant factor
contributing to the variability (Zucchetti, et al., 1999).

Identifying genetic polymorphisms in CYP/UGTs, drug transporters and target receptors
that are involved in the drug disposition can aid in individualizing dosage regimen by
increasing the number of responders and decreasing the number adverse drug reactions.
Pharmacogenetics has already been implemented in oncology practice to identify patients
who respond to trastuzumab (Herceptin) and gefitinib (Iressa) by testing for over
expression of HER2/neu oncogenes and mutations in epidermal growth factor,
respectively (Frueh, et al., 2008). A detailed account on the impact of pharmacogenetics
on the anticancer drugs is provided in section 1.4.
Figure 2: Inter-individual variability in clearance of anti-cancer compounds (Adapted from Felici A et al 2002)

Figure 3: Potential sources of inter-individual variability in PK of drugs
1.3. Overview of Cytochrome P450 (CYP) 3A Enzymes

CYP enzymes play an important role in the metabolism of both endogenous (steroids, bile acids, cholesterol) and exogenous (drugs, chemicals, environmental toxins) compounds by converting them into more hydrophilic products, thereby enhancing their elimination from the body (Coon, 2005; Guengerich, 2006). CYPs belong to a family of membrane bound heme containing proteins and are localized primarily in the endoplasmic reticulum of the cell. The human genome is encoded with 57 CYP proteins, and nearly 15 of them are involved in the biotransformation of xenobiotics and endogenous chemicals. CYP1, 2 and 3 are the primary enzymes involved in the metabolism of xenobiotics (Spatzenegger and Jaeger, 1995).

Among all the CYP enzymes, CYP3A is most abundantly expressed in liver and intestine, comprising almost 30-40% of the total hepatic and 80% of intestinal CYP content [Fig. 4] (Paine, et al., 2006; Shimada, et al., 1994). Furthermore, approximately 50-60% of the drugs in the market are metabolized by CYP3A enzymes [Fig. 5] (Li, et al., 1995; Evans and Relling, 1999). Hence inter-individual variability in CYP3A expression can have significant influence on systemic exposure of the substrate drugs, altering their efficacy and toxicity. There are four isoforms of CYP3A- CYP3A4, CYP3A5, CYP3A7 and CYP3A43. In adults, CYP3A4 and CYP3A5 are the major isoforms with approximately 84% amino acid sequence homology and have significant overlapping substrate specificities. The expression of CYP3A7 is predominant during fetal development and is silenced or relatively low 1 or 2 years after birth. Conversely, the expression of CYP3A4/5 is very low during fetal life and reaches 50% of adult levels within a year after
birth (Hines, 2007; de Wildt, et al., 1999). Although CYP3A4 is highly expressed in liver and intestine, the expression of CYP3A5 is higher than CYP3A4 in extrahepatic tissues like kidney, lungs and blood. CYP3A43 is a relatively new isoform and is expressed at lower levels in adult and fetal livers (Westlind, et al., 2001a). Moreover, the contribution of CYP3A43 and its functional significance to drug metabolism is not well characterized.

Figure 4: Relative abundance of CYPs in liver microsomes.
1.3.1. Variability in Cytochrome P450 (CYP) 3A Enzymes

A substantial inter-individual variation of approximately 40-60 fold, in the expression of CYP3A enzymes and up to 400 fold in CYP3A activity due to drug interactions (CYP inhibition/induction) has been reported (Lamba, et al., 2002; Thummel and Wilkinson, 1998). Using midazolam as a probe substrate, Thummel et al determined the inter- and intra-individual variability in the expression of CYP3A4 in the biopsy tissues of 21 donor livers. They reported a 37 fold variation with the CYP3A protein levels ranging from 1.1 to 40.8 pmol/mg (Thummel, et al., 1994a). In another study examining the liver and intestinal CYP3A levels by Paine et al, the CYP3A content was found to be ranging from
4.0 to 262.0, 3.0 to 90.8, 2.1 to 98, and <1.9 to 59.5 pmol/mg of protein for liver, duodenum, jejunum, and ileum, respectively accounting for up to 64 fold variation (Paine, et al., 1997).

A combination of environmental, genetic, physiological and patho-physiological factors is responsible for such an extensive variability. Environmental factors such as food intake, smoking and concomitant drug administration lead to increased or decreased CYP3A activity. Among all these factors, inhibition/induction of CYP3A and the presence of single nucleotide polymorphisms in drug metabolizing enzymes (and transporters) have been considered to be the major sources of variability (Plant, 2007).

1.3.2. Induction of CYP3A

Induction of CYP3A4/5 is an indirect, slow regulatory process occurring at the transcriptional level and resulting in increased protein expression/activity. From the evolutionary point of view, induction can be considered as the body’s defense mechanism against toxic chemicals. The net effect of drug mediated CYP3A induction is alteration in pharmacokinetics and pharmacodynamics of substrate drugs (Kolars, et al., 1991; Dilger, et al., 2000). The pharmacokinetic or clinical impact of CYP induction depends on the duration of exposure to inducer, localization of the protein, enzyme biosynthesis/degradation and the pharmacological effect of drug/metabolites.

Induction is usually observed after administration of repeated doses of drug. There are two major consequences of CYP3A induction 1) Increase in the clearance of the substrate drugs, resulting in decreased plasma concentration and reduced efficacy. 2) Increase in
toxicity due to the augmented formation of reactive metabolites or decrease in toxicity due to enhanced detoxification process (Seeff, et al., 1986). Pharmacokinetically, induction results in decrease in AUC, half-life, bioavailability and increase in the plasma clearance of the substrates. If a drug induces its own metabolism, it is known as an auto-inducer (e.g., Carbamazepine, Efavirenz).

Several known CYP3A4/5/7/43 inducers have been presented in Table.1. Rifampicin is one of the most potent CYP3A inducers and has been implicated in several drug interactions. In addition to inducing CYP3A, rifampicin also induces CYP1A2, 2A6, 2B6, 2C8, 2C18, 2C19, 3A5 and 3A7 (Niemi, et al., 2003). When co-administered with rifampicin, a significant decrease in the AUC of oral CYP3A substrates is observed rendering the drugs ineffective. For example, the AUC of the following drugs are suppressed by more than 80% significantly reducing therapeutic efficacy - midazolam (98%), triazolam (95%), tamoxifen (86%), tormifene (87%), sirolimus (82%), verapamil (91%), simvastatin (81%), nifedipine (92%), warfarin (85%), indinavir (92%), nelfinavir (82%) (Niemi et al 2003). Co-administration of rifampicin also decreased the plasma concentration of immunosuppressive agent cyclosporine in transplant patients, resulting in acute allograft rejection (Modry, et al., 1985;Riva, et al., 1996;Hebert, et al., 1992;Hebert, et al., 1999;Chenhsu, et al., 2000). Another clinically important interaction that led to undesirable consequences was unwanted pregnancies observed in women who were administering oral contraceptives with CYP3A inducers like rifampicin or St.John’s Wort (Heimark, et al., 1987). Hence, CYP3A induction has been considered to be an undesirable property and many pharmaceutical companies routinely evaluate CYP
induction potential of new drugs in the discovery stage as a part of selection of lead candidates (Smith, 2000; Lin, 2006).

Table 1. List of known CYP3A inducers

<table>
<thead>
<tr>
<th>CYP3A</th>
<th>Inducers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A7</td>
<td>Rifampicin, Phenytoin, Clotrimazole, Cyclosporin, Dexamethasone</td>
<td>Usui et al. 2003, Maruyama et al. 2007</td>
</tr>
<tr>
<td>CYP3A43</td>
<td>Dexamethasone</td>
<td>Krusekopf et al. 2003</td>
</tr>
</tbody>
</table>

In the case of rifampicin, the maximum CYP3A induction occurs within a day, reaching plateau after a week of rifampicin treatment and the enzyme activity returns to the baseline activity within two weeks after discontinuation of therapy (Tran, et al., 1999). Furthermore, the influence of CYP3A induction by rifampicin was found to be greater with orally administered compounds that undergo extensive first pass metabolism than intravenous administration. Understanding the time course of induction and de induction will help the clinician in modifying the dosage of CYP3A substrates.
1.3.2.1. Mechanism of CYP3A Induction – Role of PXR

The expression of CYP3A is regulated by an orphan nuclear receptor Pregnane X receptor (PXR) (Lamba, et al., 2002). In 1998, three different groups identified the same nuclear receptor responsible for regulating the expression of CYP3A4 and named it PXR, Steroid X receptor (SXR) and human proteinase-activated receptor (hPAR) (Kliewer, et al., 1998;Blumberg, et al., 1998;Bertilsson, et al., 1998). PXR is highly expressed in liver and intestine. Notably, CYP3A is also found at higher levels in the same tissues and is induced by several exogenous and endogenous compounds. When a ligand binds to PXR in cytoplasm, this ligand-PXR complex translocates to the nucleus of the cell and heterodimerizes with 9-cis retinoic acid receptor (RXR) (Kliewer, et al., 2002b). The PXR RXR heterodimer then binds to a PXR response element upstream to the promoter region of CYP3A leading to a change in conformation, thereby increasing the transcription of CYP3A4 and enhancing the metabolism of several drugs [Fig. 6] (Willson and Kliewer, 2002;Baes, et al., 1994). The response elements to which PXR binds can be a direct repeat with 3 or 4 nucleotide spacer (DR3 or DR4) or an everted repeat with a 6 nucleotide spacer (ER6). Some of the well known ligands for PXR include rifampicin, paclitaxel, nifedipine, phenobarbital, ritonavir, St. John’s Wort, troglitazone, endogenous compounds like estradiol, corticosterone and bile acids. (Kliewer, et al., 2002a). In addition, PXR is known to regulate the expression of CYP3A5, CYP3A7, CYP2B6, CYP2C9, UDP-glucuronyltransferases (UGT) UGT1A1, 1A3, 1A4, 1A6, glutathione S-transferase A2 (GSTA2) and sulfotransferase (SULT) 1A1 and drug transporters (P-glycoprotein (MDR1), multidrug-resistance-associated protein 2 (MRP2) and organic anion transporter polypeptide 2 (OATP2)) (Burk, et al., 2004;Gardner-Stephen, et al., 2004;Gerbal-Chaloin,
et al., 2001; Xie, et al., 2003; Duanmu, et al., 2002; Goodwin, et al., 2001). Hence PXR has been known as “Master Regulator” of drug metabolizing enzymes and transporters. (Dussault and Forman, 2002)

Though PXR activation is mainly responsible for the induction of CYP3A enzymes, other nuclear receptors known to modulate CYP3A expression include constitutive androstane receptor (CAR), vitamin D receptor (VDR) and Farnesoid X receptor (FXR).

Other mechanisms that can result in enhanced expression of CYP3A are increased mRNA stability or translational efficiency and protein stabilization induced by posttranslational modifications (Lin and Lu, 2001). For example troleandomycin does not result in increased production of CYP3A4 protein, but decreases its rate of degradation. (Watkins, et al., 1986; Danan, et al., 1981).

Figure 6: Schematic representation of ligand mediated PXR activation and CYP3A induction

PXR- Pregnane X Receptor, PXR – Retinoic Acid X Receptor, PXR- RE- PXR Response Elements
Inhibition of PXR activity has been considered as a strategy to overcome drug resistance by attenuating the expression of drug metabolizing enzymes in tumor tissues (Masuyama, et al., 2007). Some of the compounds that are known to inhibit PXR include sulforaphane, ketoconazole, antineoplastic agent trabectedin and HIV protease inhibitor A-792611 (Wang, et al., 2007; Synold, et al., 2001; Healan-Greenberg, et al., 2008). However, the clinical utility of PXR antagonists \textit{in vivo} is not known and requires further research (Biswas, et al., 2009).

1.3.2.2. \textbf{Variability in CYP3A Induction}

A large variability has been observed in the levels of CYP3A protein or mRNA and its activity post induction. Using 6-\(\beta\)-hydroxycortisol/cortisol ratio as a marker, Ged et al evaluated the CYP3A induction potential by rifampicin (600 mg/day for 4 days) in 18 patients. A 2-30 fold change was observed in the induction with the extent of increase in the protein levels ranging from 160\% to 2900\% (Ged, et al., 1989; Lin and Lu, 2001). Similarly, Kolars et al reported a 0-12 fold change in intestinal CYP3A4 mRNA levels after treatment of rifampicin 600mg/7days (Kolars, et al., 1992).
Table 2. Factors influencing the variability in CYP3A induction by drugs (Adapted from (Tang, et al., 2005))

<table>
<thead>
<tr>
<th>Factors</th>
<th>Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable transport and metabolism of inducers in vivo</td>
<td>Variable tissue and intracellular inducer concentrations resulting in different extent of induction</td>
</tr>
<tr>
<td>Genetic variations of P450 genes and their regulatory regions</td>
<td>Altered pattern of induction</td>
</tr>
<tr>
<td>Genetic variations of receptors and regulatory proteins required for induction</td>
<td>Altered pattern of induction</td>
</tr>
<tr>
<td>Physiological factors: disease states (inflammation, infection, and liver dysfunction), gender, hormonal homeostasis</td>
<td>Altered response to inducers</td>
</tr>
<tr>
<td>Environmental factors: dietary components, environmental contaminants</td>
<td>Altered response to inducers</td>
</tr>
</tbody>
</table>

The extent of decrease in AUC of verapamil and midazolam caused by CYP3A induction due to rifampicin ranged from 5 to 60 fold and 11.5 to 55 fold, respectively. (Fromm, et al., 1998; Backman, et al., 1996). Various factors contributing to variability in CYP3A induction have been tabulated in Table.2 above. Identifying the factors that result in induction of an enzyme and determining their contribution will result in modifying the treatment regimen accordingly.

1.3.3. Inhibition of CYP3A

Inhibition of CYP3A enzyme is the most common cause of observed clinical drug interactions and has led to withdrawal of several drugs from the market eg., terfenadine, mibebradil and cisapride (Lasser, et al., 2002). Unlike induction, CYP3A inhibition is an instantaneous process and usually occurs after a single dose administration of the
inhibitor. As a result of inhibition, there is an increase in plasma concentration of the substrate drugs and their bioavailability, leading to undesirable prolongation of pharmacological effects (Lin and Lu, 1998; Lin and Lu, 2001). For example, torsades de pointes or fatal ventricular arrhythmia have been reported to occur with co-administration of non-sedating anti-histamines like terfenadine or astemizole with potent CYP3A inhibitors like ketoconazole, clarithromycin, erythromycin and grapefruit juice (Dresser, et al., 2000). Other examples of inhibition mediated clinical drug interaction resulting due to co-administration of CYP3A inhibitors includes severe rhabdomyolysis with statins (cerivastatin, atorvastatin and lovastatin), symptomatic hypotension with calcium channel antagonists (nifedipine, felodipine, mibebradil) and excessive sedation with benzodiazepines (midazolam, diazepam), (Neuvonen and Suhonen, 1995; Madsen, et al., 1996; Olkkola, et al., 1993; Ozdemir, et al., 1998; Wysowski and Swartz, 2005)

Although most of the interactions have negative consequences, CYP3A inhibition has also been found to be beneficial for certain compounds. Ritonavir, a potent CYP3A4 inhibitor has been employed successfully to boost the bioavailability of other protease inhibitors used widely for treatment of HIV infection (Cooper, et al., 2003). Addition of ritonavir to patients undergoing saquinavir therapy resulted in 33 and 58 fold greater Cmax and AUC, respectively (Merry, et al., 1998; Merry, et al., 1997). Another good example is cyclosporin, a calcineurin inhibitor and an immunosuppressive agent, is a substrate of CYP3A4. Similar to other immunosuppressive agents, the high cost associated with the use of cyclosporin has been a barrier to its use (Evans and Manninen, 1988). By inhibiting CYP3A4 and increasing the bioavailability of cyclosporin, it has
been demonstrated that ketoconazole substantially (approx. 60 to 80%) reduced the dosage required to maintain adequate immunosuppression and therefore its cost (Keogh, et al., 1995;Gomez, et al., 1995;Martin, et al., 1999).

1.3.3.1. Mechanism of CYP3A Inhibition

The mechanisms involved in CYP3A inhibition include reversible, quasi-reversible and irreversible (mechanism based) inhibition (Vanden, et al., 1995;Murray, 1997;Ortiz de Montellano, 1995). Reversible inhibition has been the most common cause of drug-drug interaction. It is transient and occurs when an inhibitor forms weak bonds with CYP enzymes without inactivating it permanently. The enzyme activity usually returns to normal after removal of the inhibitor (Lin and Lu, 2001). Reversible inhibition can be further classified into competitive (substrate and inhibitor bind to the same active site at the enzyme), non-competitive (substrate and inhibitor bind to the different sites at the enzyme) and mixed type of inhibition (Rodriguez-Antona, et al., 2002). Some of the compounds that are known to exhibit reversible CYP3A inhibition are listed in Table 3. Based on the potency and their ability to increase the AUC of co-administered compounds in vivo, USFDA has classified inhibitors as strong, moderate and weak inhibitors (Table 4).

In the case of irreversible mechanism based inhibition (suicide inhibition), the drug gets converted into a metabolic intermediate complex that results in a covalent modification of the catalytic site and leads to permanent inactivation of enzyme activity (eg., ritonavir,
verapamil, tamoxifen, clarithromycon, erythromycin) (Zhou, et al., 2005; Wang, et al., 2005; Galetin and Houston, 2006; Polasek and Miners, 2006).

Table 3. List of common reversible and mechanism based inhibitors

<table>
<thead>
<tr>
<th>Mechanism of Inhibition</th>
<th>Inhibitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversible</td>
<td>Fluconazole, Itraconazole, Ketoconazole, Quinidine, Indinavir</td>
<td>Dresser et al 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pelkonen et al 2008</td>
</tr>
<tr>
<td>Mechanism Based (Suicide)</td>
<td>Ritonavir, Nelfinavir, Irinotecan Bergamottin and Dihydroxy bergamottin (Grape fruit juice), Erythromycin, Clarithromycin, Verapamil, Mibefradil, Isoniazid, Diltiazem, Saquinavir</td>
<td>Zhou S et al 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dresser et al 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pelkonen et al 2008</td>
</tr>
</tbody>
</table>

The covalent binding can occur either with the haem or the protein. The functional CYP activity is not restored by the removal of inhibitor, but only by de novo synthesis of enzymes (Lin and Lu, 2001). Some of the salient features of mechanism based inhibition are a) Time dependent inhibition b) Dose/concentration dependent inhibition c) NADPH dependent inhibition in vitro.
Table 4. Classification of CYP3A inhibitors based on their potency (Adapted from US FDA Drug Interaction guidance)

<table>
<thead>
<tr>
<th>Strong CYP3A inhibitors</th>
<th>Moderate CYP3A inhibitors</th>
<th>Weak CYP3A inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 5-fold increase in AUC</td>
<td>≥ 2 but &lt;5-fold increase in AUC</td>
<td>≥ 1.25 but &lt;2-fold increase in AUC</td>
</tr>
<tr>
<td>Atazanavir, Clarithromycin, Indinavir, Itraconazole, Ketoconazole, Nefazodone, Nelfinavir, Ritonavir, Saquinavir, Telithromycin</td>
<td>Amprenavir, Aprepitant, Diltiazem, Erythromycin, Fluconazole, Fosamprenavir, Grapefruit juice, Verapamil</td>
<td>Cimetidine</td>
</tr>
</tbody>
</table>

1.3.3.2. Variability in CYP3A Inhibition

Since the pharmacokinetic and clinical consequences are different for reversible and irreversible inhibitors, there is considerable emphasis on the need to understand the mechanism of CYP3A inhibition (Lin, et al., 2000). Furthermore, significant inter-individual variability has been reported in drug-drug and food-drug interactions due to CYP3A inhibition. With ketoconazole terfenadine interaction, a five-fold variation was observed in response to ketoconazole inhibition resulting in 1500% to 7200% increase in AUC of terfenadine (Honig, et al., 1993). Similarly, a 20-fold difference was reported in the extent of increase in AUC of venlafaxine when co-administered with quinidine (Lessard, et al., 1999). Bergamottin and dihydroxy bergamottin, components of grapefruit juice are inhibitors of intestinal CYP3A4 and have been reported to increase the AUC of midazolam and cyclosporine by 26 to 100% and -16 to 200%, respectively (Yee, et al., 1995;Kupferschmidt, et al., 1995). The reasons for this variability can be attributed
to the differences in in vivo inhibitor concentrations, Ki values (inhibition constant), substrate pharmacokinetics (high or low clearance compounds), basal level of enzymes and the presence of genetic polymorphisms (Lin and Lu, 2001).

1.3.4. Implications of TAM Mediated CYP3A Induction/Inhibition

1.3.4.1. CYP3A Induction by TAM

Co-administration of TAM has been shown to markedly reduce the plasma concentration and the area under the plasma concentration curve (AUC) of several drugs. The most striking effects have been observed in clinical trials with TAM and aromatase inhibitors, letrozole and anastrozole, wherein a mean reduction of 37% and 27% was observed in the plasma levels of letrozole and anastrozole, respectively (Dowsett, et al., 1999; Dowsett, et al., 2001). In case of TAM and letrozole interaction, AUC decreased in some subjects by more than 60%. Such a decrease in concentration may have resulted in the overall lack of therapeutic benefits. Since both TAM and letrozole are substrates of CYP3A, induction of CYP3A by TAM could be one of the possible reasons to the decreased plasma levels of letrozole. In addition, TAM can also induce its own metabolism, leading to different plasma levels of active/toxic metabolites further contributing to the variability observed in TAM therapy. The fact that CYP3A4 induction may have a profound influence on TAM activity is supported by earlier studies where pre-administration of rifampicin, one of the most potent CYP3A4 inducer known, resulted in reduced plasma levels and efficacy of anti-estrogens TAM and toremifene (Kivisto, et al., 1998).
The potential of TAM to induce CYP3A in animal models and *in vitro* cell lines has been well established. In female rats treated with TAM, White et al observed up-regulation of CYP3A activity as measured by the increase in 6- and 16 alpha hydroxylation of testosterone (White, et al., 1993). In another study, Nuwaysir et al observed dose-dependent increase in the expression of CYP2B1, CYP2B2, CYP3A after 7 days of administration of TAM to male and female rats (Nuwaysir, et al., 1995). A trend towards increase in clearance of midazolam (a probe substrate for CYP3A) in preclinical rat studies further supported the induction of CYP3A by TAM (Cotreau, et al., 2001). In previous studies from our laboratory, it was observed that TAM and 4-OHT up regulate the expression of CYP3A4 transcripts and activity in primary cultures of human hepatocytes, (Desai, et al., 2002). Furthermore, silencing hPXR employing siRNA sequence was found to abrogate hPXR activation and CYP3A4 induction by TAM, indicating the critical involvement of hPXR in mediating the inductive effect of TAM (Sane, et al., 2008). In addition, TAM is also known to augment the expression of Phase II enzymes (sulfotransferase, glutathione transferases) and drug transporters (P-glycoprotein, multidrug resistance protein2) (Hellriegel, et al., 1996;Nuwaysir, et al., 1996;Nuwaysir, et al., 1998;Gant, et al., 1995;Riley, et al., 2000).

Despite the confirmation from *in vitro* and preclinical studies, clinical evidence showing induction of CYP3A by TAM in breast cancer patients is lacking. Furthermore, genetic variability in the expression of PXR is likely to impact the extent of CYP3A4 induction by TAM, which may result in alteration in its own metabolism to N-DMT and α-OHT (auto-induction, also known as time-dependent changes) or that of other co-administered
drugs. Auto-induction can result in higher levels of N-DMT and α-OHT producing different outcomes in patients.

1.3.4.2. CYP3A Inhibition by TAM

In human liver microsomes and cDNA expressed human CYP3A4/5 enzymes, TAM and its metabolites N-DMT, 4-OHT and 3-OHT inhibited CYP3A activity measured as midazolam-1-hydroxylation and testosterone-6β-hydroxylation (Zhao, et al., 2002). Though TAM and N-DMT have been reported to be mechanism based CYP3A inhibitors in vitro, so far there is paucity in data on inhibition mediated clinical drug - drug interactions with other CYP3A substrates (Zhou, et al., 2005).

1.4. Impact of Pharmacogenetics on PK/PD of Anti-Cancer Drugs

The word “Pharmacogenetics” was first coined in 1959 and is defined as the study of genetic influence on drug response (Kalow W 1962; Meyer UA 2004). Variability occurs because of variations in DNA such as polymorphisms in a single gene, or a limited set of multiple genes, sequences that influence enzyme or receptor activity. In the past few decades, there have been several investigations documenting the influence of genetic polymorphisms in drug metabolism enzymes to the variability in pharmacokinetics and drug response. (Huang RS and Ratain MJ 2009; Gardiner SJ and Begg EJ 2006; Marsh S and Liu G 2009; Tan SH et al 2008). Pharmacogenetics has already been implemented in guiding dosage regimens for 6 mercaptopurine (6-MP), irinotecan and warfarin by genotyping for thiopurine methyl transferase (TPMT), UGT1A1, CYP2C9 and vitamin k epoxide reductase subunit 1 (VKORC1) polymorphisms, respectively. For example, 6-MP
is a prodrug and is metabolized to form thioguanine nucleotides, the active metabolites that kill tumor cells by inhibiting DNA and RNA synthesis. Thiopurine methyl transferase (TPMT) is an enzyme that inactivates 6-MP preventing the formation of active metabolites. A trimodal distribution is observed with TPMT activity, with 90% individuals exhibiting high, 10% intermediate and 0.3% low enzyme activity (Maitland ML et al 2006, Smith NF et al 2004; Innocenti F et al 2000). Individuals with low levels of TPMT require a modified dose of 6-MP to avoid the toxicity (severe myelosuppression) associated with higher levels of 6-MP (Maitland ML et al 2006; Huang SM et al 2006).

Recently dosage reduction has been recommended for individuals undergoing irinotecan therapy and expressing UGT1A1*28 allele (Frueh, et al., 2008). Presence of this allele leads to decreased glucuronidation of irinotecan leading to accumulation of the active metabolite SN-38 and resulting in grade 4 neutropenia (Innocenti and Ratain, 2002). Thus, there is increasing evidence that simple genetic tests may be applied to minimize side effects that may be more deleterious than the health complaint, and identify patients with intrinsic resistance to commonly prescribed drugs so that an alternative drug can be selected (Blackhall, et al., 2006). Two pharmacogenetic tests have been recently approved by US FDA for clinical use. One is Roche’s Amplichip™ that permits detecting of CYP2D6 and 2C19 polymorphisms (29 CYP2D6 and 2 CYP2C19 SNPs) when using drugs that are substrates or inhibitors of these enzymes. The other test involves assessment of UGT1A1*28 polymorphism for safer delivery of irinotecan using Invader Assay™ (Vizirianakis, 2007).

In addition to polymorphisms in drug metabolism enzymes, SNPs in drug targets, receptors and transporters (P-glycoprotein, human organic anion transporting polypeptide-
C (OATP-C)) can also result in variable response to drug therapy. In fact, genotyping has been suggested for determining the choice of treatment agents (HLA-B*1502 for carbamazepine, Herceptin for HER2/neu expressors) and for identification of disease risk (BRCA1 mutation for testing breast cancer risk) (Huang and Ratain, 2009). Thus understanding how genetic variations in individuals contribute to differences in therapeutic response can assist in personalizing therapy based on an individual’s genotype. Towards achieving this goal, Huang and Ratain suggest five stages of pharmacogenetics in cancer therapy starting from 1) determining the role of genetics in drug response, 2) screening and identifying genetic markers, 3) validating genetic markers, 4) clinical utility assessment and 5) pharmacoeconomic impact. Screening and identifying genetic markers can be accomplished by either a candidate gene approach, which focuses on the genetic variations in enzymes/transporters/targets involved in the disposition of drugs or a genome wide approach, where genome wide association studies (GWAS) are conducted scanning markers across genomes in a large population to find genetic variations associated with a disease or drug treatment. For anticancer drugs like 6-MP and irinotecan, a candidate gene approach has provided valid markers that have been validated and successfully used to personalize treatment. A recent example of a genome wide approach includes identification of variants in SLCO1B1 (solute carrier organic anion transporter family, member 1B1) that were strongly associated with increased risk of statin-induced myopathy. SLCO1B1 encodes the organic anion–transporting polypeptide OATP1B1, which is known to regulate the hepatic uptake of statins. (Link, et al., 2008)
1.5. **CYP3A Polymorphisms**

All the four CYP3A isoforms are located in tandem CYP3A43-CYP3A4-CYP3A7-CYP3A5 on chromosome 7q21.1-q22.2, consisting of 13 exons each encoding an open reading frame of 503 amino acids ([**Fig. 7**](#), Adapted from (Wojnowski, 2004)). Here CYP3A43 is in head to head orientation with CYP3A4, while the other three genes are in head to tail orientation. Single nucleotide polymorphisms in CYP3A can result in variants with higher, lower or lack of enzyme activity, thereby requiring dose modifications for substrate drugs. Several investigators have suggested that genetic polymorphisms in CYP3A genes can contribute to at least 90% of the variability observed in CYP3A expression. A list of important SNPs in CYP3A with their reported frequencies in different populations is presented in Table 5.

![Figure 7: Schematic representation of CYP3A locus on chromosome 7. The vertical bars indicate boundaries of the individual gene cassettes.](#)
**Table 5.** Distribution of important CYP3A polymorphisms in various ethnic groups. (Adapted from Burk et al 2004)

<table>
<thead>
<tr>
<th>CYP3A Gene</th>
<th>Expression: Level/ Distribution</th>
<th>Genetic Contribution to Variability</th>
<th>Variants</th>
<th>Mechanism</th>
<th>Allele Frequency (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Caucasians</td>
<td>Africans</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Strong / Unimodal</td>
<td>Strong</td>
<td>CYP3A4*1B</td>
<td>-392A&gt;G (increased activity)</td>
<td>2-9.6%</td>
<td>35-67%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A4*6</td>
<td>Frame Shift</td>
<td>NK</td>
<td>NK</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A4*17</td>
<td>Phe189Ser alteration.</td>
<td>2.1%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A4*20</td>
<td>Frame Shift</td>
<td>6%</td>
<td>26%</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>Medium / Bimodal</td>
<td>Strong</td>
<td>CYP3A5*3</td>
<td>Splice Defect (loss of activity)</td>
<td>90%</td>
<td>27-50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A5*6</td>
<td>Splice Defect (loss of activity)</td>
<td>0</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A5*7</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A5*8</td>
<td>R28C</td>
<td>NK</td>
<td>NK</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A5*9</td>
<td>A337T</td>
<td>NK</td>
<td>NK</td>
</tr>
<tr>
<td>CYP3A7</td>
<td>Medium / Bimodal</td>
<td>Strong</td>
<td>CYP3A7*1B</td>
<td>NA</td>
<td>0%</td>
<td>1%</td>
</tr>
<tr>
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<td>CYP3A7*1C</td>
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<td>3%</td>
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<td>0%</td>
<td>1%</td>
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<td></td>
<td></td>
<td></td>
<td>CYP3A7*1E</td>
<td>NA</td>
<td>8%</td>
<td>0%</td>
</tr>
</tbody>
</table>

NK- Not Known
1.5.1. Pharmacogenetics of CYP3A4

To date, more than 39 SNPs have been published on the home page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.cypalleles.ki.se/). Most of the identified SNPs have been reported to occur at low frequencies (<2%) and only a few are known to alter the activity. The most common variant A-392G transition (CYP3A4*1B) located in the promoter region has been associated with an increased transcriptional activity in *in vitro* studies (Amirimani, et al., 2003). However, conflicting results regarding its *in vivo* effect have been reported in clinical studies. This variant has been associated with increased clearance in patients undergoing docetaxel treatment (Amirimani, et al., 2003; Tran, et al., 2006; Amirimani, et al., 2003). Rodriguez-Antona et al have shown that individuals expressing CYP3A4*1B (−392A>G) had significantly lower enzyme activity than CYP3A4*1A (Rodriguez-Antona, et al., 2005). Some investigators have reported significant association of CYP3A4*1B to decreased clearance of thiotepa and cyclosporine, while others have shown no effect (Ekhart, et al., 2009; Min and Ellingrod, 2003; He, et al., 2005; Spurdle, et al., 2002). In addition, several studies have implicated CYP3A4*1B to greater risk of prostate cancer, lung cancer and reduced risk for leukemia (Rebbeck, et al., 1998; Felix, et al., 1998). The allelic frequency varies among different ethnic groups – Caucasian (2-9%), African Americans (35-67%) and Hispanic Americans (9-11%) (Lamba, et al., 2002). Other alleles with decreased activity include CYP3A4*5, *6, *11, *16 and *17 but are expressed in relatively lower frequencies (Westlind-Johnsson, et al., 2006b; Zeigler-Johnson, et al., 2004; Murayama, et al., 2002). Recently, CYP3A4*20 the first null allele in CYP3A4 was identified and was found to be devoid of any enzyme activity (Westlind-Johnsson, et al., 2006a).
Furthermore, a novel intronic variant identified by Dr. Wolfgang Sadee’ group at the University of Ohio was associated with reduced mRNA expression and lower statin dose requirement (personal communication). Information on the frequency of this polymorphism is not yet available. The presence of lower frequency non functional polymorphisms identified so far suggest that the variability in CYP3A expression cannot be explained by these polymorphisms alone, and requires further studies to determine the contribution of these polymorphisms to metabolism of drugs.

1.5.2. Pharmacogenetics of CYP3A5

Considerable effort has been made to identify the mutations in the CYP3A genes that might affect the expression and function of the CYP3A enzymes. The expression of CYP3A5 has been found to be polymorphic and can account for up to 50% of the total CYP3A content especially in individuals expressing the wild type CYP3A5*1/*1 alleles. A number of genetic polymorphisms with functional significance have been identified in CYP3A5 that include CYP3A5*3, CYP3A5*5, CYP3A5*6, CYP3A5*7 and CYP3A5*11 (Lamba, et al., 2002; Lee, et al., 2003). The most frequent and functionally important polymorphism consists of an 6986A>G transition within intron 3 is CYP3A5*3. This polymorphism creates an alternative splice site resulting in a frame shift, and truncation of the protein, leading to loss of CYP3A5 activity (Kuehl, et al., 2001; Hustert, et al., 2001a). As a result, the expression of CYP3A5 is found to exhibit bimodal distribution, with individuals being categorized as “high expressors” or “low expressors”, depending on the ethnic distribution. Significant association has been reported between CYP3A5*3 genotype and decreased clearance of tacrolimus, sirolimus,
cyclosporine and saquinavir (Dai, et al., 2006; Mouly, et al., 2005). The variant CYP3A5*3 (G6986) allele is very common in all the ethnic groups studied and is actually more prevalent than the wild-type allele for most populations. The allelic frequency is as follows - Caucasians 91%, African Americans 40-60%, Asians 75% and Europeans 70% (Lamba, et al., 2002; Roy, et al., 2005). CYP3A5*6 is also a splice variant resulting in the deletion of exon 7, further leading to lower CYP3A5 expression and activity (Kuehl, et al., 2001). This variant is found in 13% of Africans and has not been detected in other population. Other variants reported to have decreased activity include CYP3A5*7, CYP3A5*8 and CYP3A5*9.

1.5.3. Pharmacogenetics of CYP3A7

CYP3A7 was originally identified to be predominantly expressed in the fetal liver and its levels were reported to be either low or undetectable in adults. However, recent reports indicate the expression of CYP3A7 in 54-78% of adult livers (Schuetz, et al., 1994; Greuet, et al., 1996). To date, 6 CYP3A7 alleles have been identified and listed on the home page of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.cypalleles.ki.se/). The functional significance of all these polymorphisms is not known yet. Most of the individuals genotyped for CYP3A7 carry either CYP3A7*1C or CYP3A7*1B promoter allele (Kuehl, et al., 2001; Sim, et al., 2005). In fact CYP3A7*1C has been found to be a marker for higher expression of CYP3A7 in the intestine (Burk, et al., 2002). Furthermore, there is limited data on the substrates of CYP3A7. All-trans and cis-retinoic acid that are used in the treatment of promyelocytic
leukemia and acne respectively, have been reported to be metabolized by CYP3A7 (Marill, et al., 2002; Niles, 2004; Jimenez-Lara, et al., 2004; Cooper, 2003). In a recent investigation, Crettol S et al reported that CYP3A7*1C carriers require 1.4-1.6 fold higher cyclosporine dose post transplantation than the wild type carriers (Crettol, et al., 2008). Given the fact that the role of CYP3A7 in the metabolism of compounds has not been studied well in comparison to CYP3A4 or CYP3A5, its importance to the variability in CYP3A expression needs further examination.

1.5.4. Pharmacogenetics of CYP3A43

CYP3A43 is the newest member of CYP3A family and was identified, cloned and characterized only in the last decade (Domanski, et al., 2001; Gellner, et al., 2001; Westlind, et al., 2001b). It is expressed in liver, kidney, prostate and pancreas. CYP3A43 also has an amino acid sequence similar to 75% of CYP3A4/5 and 71% to that of CYP3A7 (Domanski, et al., 2001). Screening for sequence variations resulted in the identification of three variants in the coding region, one silent, one missense and a frameshift mutation (Cauffiez, et al., 2004). Recently, CYP3A43*3 a missense variant was found to be associated with increased prostate cancer risk (Stone, et al., 2005). The significance of the CYP3A43 and its polymorphisms on the disposition of drugs in not known.

1.6. Role of PXR Polymorphisms

Since CYP3A expression is largely regulated by hPXR, there is a strong likelihood that polymorphisms in human PXR will contribute to CYP3A variability (Zhang, et al., 2001; Hustert, et al., 2001b; Koyano, et al., 2002). Attempts to delineate polymorphic
expressions in these nuclear receptors were initiated in the past few years (Zhang, et al., 2001; Lamba, et al., 2008; Koyano, et al., 2004). Thus far, more than 90 SNPs have been identified in hPXR (www.pharmgkb.org). Several of these have been associated with reduced ligand activation in transient transfection experiments. Basal CYP3A4 activity was related to SNP 25385C>T and 24381 A>C in the PXR promoter region, 63396 C>T in putative HNF (human necrosis factor) binding site (Lamba, et al., 2008). Moreover, the T allele of 63396 was associated with higher level of CYP3A induction by rifampicin and increased CYP3A4 activity in primary human hepatocytes. In fact, in clinical studies PXR 63396C>T has been associated with reduced concentrations of unboosted atazanavir, a CYP3A substrate used in treatment of HIV infection (Siccardi, et al., 2008). The clinical impact of other polymorphisms is not known and is being investigated by several researchers.

1.7. TAM and CYP2D6 Polymorphisms

CYP2D6 is another cytochrome P450 isoform that is responsible for catalyzing the conversion of TAM to 4-OHT and N-DMT to endoxifen. In a study evaluating the effect of paroxetine, a CYP2D6 inhibitor on the disposition of TAM in 12 patients, Stearns et al report that women expressing CYP2D6 variants (*4, *6 and *8) had lower levels of endoxifen (one of the active metabolites) than who had the wild type variant (Stearns, et al., 2003). In another study, Jin et al. observed that patients who carried either the homozygous (*4/*4) or the heterozygous (wt/*4) variant genotype of CYP2D6 had statistically significantly lower mean plasma endoxifen levels (20.0 and 43.1 nM, respectively) than those who carried the homozygous wild-type (wt/wt) genotype (78.0 nM) (Jin, et al., 2005). These patients also had fewer incidences of hot flashes. Recently,
further evidence was provided on the association between CYP2D6 genotype and endoxifen concentrations in 158 patients in various ethnic groups, emphasizing the relationship between CYP2D6 genotype, CYP2D6 inhibitor and endoxifen plasma levels (Borges, et al., 2006). However, mechanistic studies showing the differences in clinical outcome due to the impact of CYP2D6 genotype or CYP2D6 inhibitors are lacking.

1.8. Methods to Assess CYP3A Induction

1.8.1. In vitro Methods

CYP3A inductive effect of compounds can be assessed by both in vitro and in vivo methodology. Previously, animal models were treated with the test compounds and expression of CYP enzymes ex vivo was determined to assess the fold induction. However, the major drawback with the animal models was the inter-species differences in the CYP expression making the extrapolation to humans unreliable. To overcome this difficulty, primary culture of human hepatocyte that mimic in vivo conditions have been used extensively to identify compounds that induce CYP3A, and is considered to be the “gold standard” method. Some of its limitations include non-availability of hepatocytes, high inter individual variation, rapid decline of enzyme expression and high cost (Brandon, et al., 2003). An illustration of a typical CYP induction study schema using hepatocytes is presented in [Fig. 8]. This method entails determining CYP3A activity using a probe substrate (testosterone), quantifying mRNA levels by real time PCR (RT-PCR) and protein levels after 72 hours of drug treatment. A positive control such as rifampicin is usually included to account for any variability between the hepatocyte preparations. As per the FDA drug interaction guidance, a drug that produces changes in CYP3A activity
equal to or greater than 40% of the positive control (rifampicin) is considered to be inducer \textit{in vitro} and requires further \textit{in vivo} induction studies.

Figure 8: Schematic of hepatocyte treatment for CYP3A induction studies.

In the last decade, there has been a significant improvement in our understanding of the molecular mechanisms of CYP3A induction. The discovery of PXR led to the development of rapid and high throughput receptor assays to determine the potential of a compound to activate PXR and induce CYP3A4. However, the results of PXR activation assays may not be reliable for compounds that exhibit complex CYP3A inhibition and induction eg. Ritonavir. Other methods to assess CYP3A induction include in silico
assays, alternative human hepatoma cell lines like HepG2, HepaRG and integrated discrete multiple organ co-culture (IdMOC) system.

1.8.2. Clinical Assessment of Induction

Determination of CYP3A activity \textit{in vivo} entails the use of marker substrates that are specifically metabolized by CYP3A. The test compound is administered daily to achieve steady state, when the maximum impact on CYP3A induction can be determined. CYP3A activity is then ascertained by administering the probe substrate at the baseline prior to test drug administration and at the steady state. Changes in the pharmacokinetic parameter estimates (eg, increase in CL/F or decrease in AUC) of the probe provides indication of CYP3A induction.

Several compounds have been indentified and validated as a marker for CYP3A activity including midazolam, testosterone, urinary 6-hydroxy cortisol/cortisol ratio, $^{14}$C-erythromycin, triazolam, alfentanil, nifedipine, dextromethorphan and lidocaine (Streetman, et al., 2000; Liu, et al., 2007). Midazolam is the most commonly used probe for the determination of CYP3A activity both \textit{in vitro} and \textit{in vivo}. Midazolam has several advantages over other probe substrates. It is a chemically stable, readily measurable analyte in plasma, absorbed completely from the gastrointestinal tract, and eliminated rapidly after intravascular or oral administration.
Figure 9: Metabolism of Midazolam to 1-OH and 4-OH midazolam by CYP3A4/5

It is exclusively metabolized by CYP3A4/5 to its primary metabolite 1-OH-Midazolam and to a lesser extent to 4–OH-Midazolam [Fig. 9]. Furthermore, midazolam undergoes significant first-pass intestinal metabolism. Therefore, any changes in the CYP3A activity would be reflected in the pharmacokinetics of midazolam. Apparent oral clearance of midazolam has been used as a standard criterion for assessing in vivo CYP3A activity, and provides a measure of both hepatic and intestinal CYP3A function. Whereas, systemic clearance after IV administration of MDZ reflects only the hepatic activity (Thummel, et
MDZ is not a substrate of the efflux transporter P-glycoprotein and hence is considered to be a “pure” CYP3A probe (Kim, et al., 1999). The major drawback associated with midazolam is the sedative effect observed in patients, when used in the dose range of 2-10 mg.
2. CHAPTER TWO: HYPOTHESIS AND SPECIFIC AIMS

2.1. Hypothesis

The central hypothesis of this thesis is “Tamoxifen metabolism as well as the extent of CYP3A induction by Tamoxifen is impacted by pharmacogenetic factors”. To test this hypothesis we pursued the following three specific aims

2.2. Specific Aim 1

_To evaluate the effect of CYP3A polymorphisms on tamoxifen metabolic clearance in vitro._

**Rationale:** The role of CYP3A5 in the formation of $\alpha$-OHT, the genotoxic metabolite has not been specifically addressed. Given the extensive substrate overlap of CYP3A4 and 3A5, it is likely that the later is also involved in the conversion of TAM to $\alpha$-OHT. Furthermore, CYP3A also plays an important role in the demethylation of TAM to N-DMT, which is the precursor for the active metabolite endoxifen. Inheritance of CYP3A5*1 has been strongly correlated with enhanced CYP3A5 dependent metabolism in the liver _in vitro_, while presence of CYP3A5*3 accounts for markedly reduced level of CYP3A5 protein expression and function in approximately 85% of Caucasians and 55% of African Americans (Kuehl, et al., 2001). Other CYP3A5 alleles that are reported to result in decreased enzyme activity include CYP3A5*8 and CYP3A5*9 (Lee, et al., 2003; Lee, et al., 2003). Recently, a novel CYP3A4 intronic variant was identified by Wolfgang Sadee, Ph.D (personal communication) at Ohio State University, and was associated with decreased statin maintenance dose. The effect of all these variants on TAM metabolism is not known. Individuals with CYP3A polymorphisms have the ability to produce different levels of $\alpha$-OHT and N-DMT
contributing to inter-individual variability with TAM therapy. Hence we pursued the following aims employing human liver microsomes as an *in vitro* model.

a) To determine the role of CYP3A5 to the formation of α-OHT and N-DMT using recombinant CYP3A4 and CYP3A5.

b) To evaluate the impact of intronic CYP3A4 and CYP3A5 variants (CYP3A5*1/*1, *1/*3 and *3/*3) on the formation of α-OHT and N-DMT using a panel of pre-genotyped human liver microsomes.

c) To assess the effect of recombinant CYP3A5*8 and CYP3A5*9 on the formation of α-OHT and N-DMT.

This aim also involves LC-MS/MS and HPLC method development for quantitation of α-OHT and N-DMT, 1-hydroxy midazolam and 6-β-hydroxy testosterone, respectively.

2.3. Specific Aim 2

*To assess the extent of CYP3A induction in breast cancer patients using midazolam (MDZ) as a probe substrate.*

**Rationale:** Co-administration of TAM has been shown to markedly reduce the plasma concentrations and AUC of several co-administered drugs. The most striking effects have been observed during clinical trials of TAM and aromatase inhibitors, letrozole and anastrozole, where TAM administration caused a mean 37 and 27% reduction in the levels of letrozole and anastrozole, respectively (Dowsett, et al., 1999; Dowsett, et al., 2001). An important point to note here is that the inductive response varied markedly and in some cases the net reduction in AUC was much higher. For instance, in case of TAM and letrozole interaction, AUC decreased in some subjects by more than 60%. Such a decrease in
concentration may have resulted in the overall lack of therapeutic benefits. The mechanism(s) of this interaction have not been explored. The lack of an understanding of these interactions has been cited as a formidable barrier to the successful use of TAM with newer agents. As discussed earlier, since anastrozole and letrozole are both metabolized extensively by CYP3A4, we hypothesized that TAM induces CYP3A4. Previous findings from our laboratory suggest that TAM and 4-OHT induce CYP3A4 \textit{in vitro} in primary cultures of human hepatocytes (Desai, et al., 2002). On one hand, individuals with higher CYP3A4 activity will have increased capacity for TAM conversion to N-DMT. On the other hand, increased CYP3A4 levels may lead to greater production of the genotoxic $\alpha$-OHT, which may ultimately lead to higher risk of endometrial carcinogenesis. Therefore, increase in the production of $\alpha$-OHT and N-DMT upon sustained TAM use, as a result of CYP3A4 auto-induction may be an important contributory factor to endometrial carcinogenesis and variability in TAM therapy. Hence we pursued the following sub aims.

a) To assess the potential of TAM to induce CYP3A in breast cancer patients \textit{in vivo} using midazolam (2 mg, oral syrup) as a probe substrate. Single dose pharmacokinetics of MDZ and 1’-OH-MDZ was evaluated following MDZ administration before and after initiation of TAM regimen (one day versus steady state).

b) To develop a LC-MS/MS methodology for the analysis of MDZ and 1-OH MDZ in plasma samples.
2.4. Specific Aim 3

To conduct preliminary investigation on the impact of CYP3A and PXR polymorphisms on the CYP3A induction by TAM

Rationale: The impact of polymorphic expression on the induction of CYP3A genes is poorly understood. CYP3A5 is generally considered to be a non-inducible CYP3A isoform. A recent study does suggest that CYP3A5 may also be regulated by hPXR and is inducible by hPXR ligands (Burk et al 2004). Even so, the induction appears to be mainly at the intestinal CYP3A5 expression. However, it is feasible that the extent of induction may be associated with the CYP3A4 and CYP3A5 polymorphisms. Furthermore, since CYP3A expression is largely controlled by PXR, there is a strong likelihood that polymorphisms in PXR may also contribute to the CYP3A variability. Several of the identified SNPs have been associated with reduced ligand activation in transient transfection experiments.

Polymorphisms in PXR (-25385C>T, -24381 A>C and 63396 C>T) have been associated with basal CYP3A4 activity (Lamba, et al., 2008; Siccardi, et al., 2008). Thus, genotypes with lower basal activity will have higher induction and vice versa. CYP3A4*1B, a promoter variant has been associated with an increased transcription in vitro. We assessed the in vivo effect of these polymorphisms on the extent of CYP3A induction by TAM. Taken together, this aim will provide us preliminary evidence on the clinical impact of various polymorphisms on the pharmacokinetics of TAM.
3. CHAPTER THREE: EXPERIMENTAL DESIGN AND METHODS

3.1. Specific Aim 1: Assessment of impact of CYP3A5 on Tamoxifen Clearance in vitro

3.1.1. Materials

3.1.1.1. Chemicals

TAM, N-DMT and α-OHT were obtained from Sigma Chemical Co. (St. Louis, MO) and Toronto Research Chemicals (Toronto, ON, Canada). NADPH regenerating system Solution A (1.3 mM -β NADP+, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂) and Solution B (0.4 U/ml glucose 6-phosphate dehydrogenase) were obtained from BD-Biosciences.

3.1.1.2. Biologicals

Pooled human liver microsomes were obtained from BD Biosciences (Woburn, MA). CYP3A5 genotyped microsomes and heterologous baculovirus-insect cell expressing human CYP3A4, CYP3A5, CYP3A5*8 and CYP3A5*9 supplemented with P450 reductase and cytochrome b5 were obtained through a collaboration with Eli Lilly (Indianapolis, IN) and partly procured from BD Biosciences (Woburn, MA). The HPLC-grade methanol, acetonitrile, hexane and butanol were purchased from Fisher Scientitics (Santa Clara, CA). All other chemicals and reagents used were of the highest commercially available quality.
3.1.2. Incubation of Testosterone with CYP3A Genotyped Microsomes

The linearity of formation of 6-β-hydroxy testosterone in human liver microsomes was established by incubating testosterone with varying incubation times (5-45 minutes) and microsomal protein concentrations (0.01-1 mg/ml). A protein concentration of 0.25 mg/ml and an incubation time of 10 minutes were chosen based on the preliminary experiments. For microsomal incubations, methanolic solution of testosterone (0-400 μM) in a polypropylene tube was evaporated under a gentle stream of nitrogen gas. The vials were then preincubated with NADPH regenerating system (1.3 mM -β NADP+, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 0.4 U/ml glucose 6-phosphate dehydrogenase) and 100 mM phosphate buffer for 5 minutes in a shaking water bath maintained at 37°C. The reaction was initiated by addition of 0.25 mg/ml of microsomes and allowed to proceed for 10 minutes. The total volume of incubation was 100 μl. Ice cold methanol (50 μl) was added to the vials to terminate the reaction, vortexed and centrifuged at 13000 rpm for 5 minutes. 100 μl of the supernatant was injected into HPLC for quantitation of 6-β-hydroxy testosterone. Control incubations with no protein, no NADPH and/or no substrate were performed concurrently. All the incubations were performed in duplicates.

3.1.3. Incubation of Midazolam with CYP3A Genotyped Microsomes

The linearity of formation of midazolam metabolites in human liver microsomes was established with varying incubation times and protein concentrations. A protein concentration of 0.125 mg/ml and an incubation time of 5 minutes were chosen based on the
preliminary experiments. Briefly, methanolic solution of midazolam (0-400 μM) in a polypropylene tube was evaporated under a gentle stream of nitrogen gas. The vials were then preincubated with NADPH regenerating system (1.3 mM -β NADP+, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl2, and 0.4 U/ml glucose 6-phosphate dehydrogenase) and 100 mM phosphate buffer for 5 minutes in a shaking water bath maintained at 37°C. The reaction was initiated by addition of 0.125 mg/ml of microsomes and allowed to proceed for 5 minutes. The total volume of incubation was 200 μl. Ice cold methanol (100 μl) was added to the vials to terminate the reaction, vortexed and centrifuged at 13000 rpm for 5 minutes. 120 μl of the supernatant was injected into LC-MS/MS for quantitation of 1-hydroxy and 4 hydroxy midazolam. Control incubations with no protein, no NADPH and/or no substrate were performed concurrently. All the incubations were performed in duplicates.

3.1.4. Incubation of Tamoxifen with CYP3A Genotyped Microsomes

Incubation conditions were performed according to a previously reported methodology (Desta, et al., 2004). Initial conditions for linearity of metabolite formation were assessed with regards to varying protein concentration (0-1 mg/ml) and time (0-60 minutes) using human liver microsomes. Final incubations with CYP3A5 genotyped liver microsomes were performed using the following conditions. Briefly, methanolic solution of TAM (0-50 μM) was dried under nitrogen in polypropylene vials. The vials were then preincubated with NADPH regenerating system and 100 mM phosphate buffer for 5 minutes in a shaking water bath maintained at 37°C. The reaction was initiated by the addition of 0.1 mg/ml of microsomes and allowed to proceed for 10 minutes. The total volume of incubation was
250 µl. Reaction was terminated by the addition of 100 µl ice cold acetonitrile. The incubation mixture was extracted with hexane-butanol mixture; supernatant was dried under nitrogen and reconstituted with 250 µl of the mobile phase (acetonitrile-water, 80/20) for LC-MS/MS analysis. 10 µl of the sample was injected into LC-MS/MS for quantitation of α-OHT and N-DMT. Control incubations with no protein, no NADPH and/or no substrate were performed concurrently. All the incubations were performed in duplicates.

3.1.5. Incubation of Tamoxifen with recombinant CYP3A Supersomes

Incubation conditions for recombinant CYPs were similar to that of genotyped microsomes. Briefly, the reaction mixture contained 50 pmol of either rCYP3A4 or rCYP3A5 supplemented with P450 reductase and cytochrome b5, NADPH regenerating system, phosphate buffer 100 mM and TAM (0-50 µM). The mixture was incubated in a water bath for 10 minutes maintained at 37°C. The reaction was terminated by the addition of ice cold acetonitrile. The metabolites formed were extracted, dried, and reconstituted with 250 µl of the mobile phase (acetonitrile-water, 80/20) for LC-MS/MS analysis. 10 µl of the sample was injected into LC-MS/MS for quantitation of α-OHT and N-DMT.

3.1.6. HPLC Analysis of 6-β-Hydroxy Testosterone

6-β-hydroxy testosterone levels were quantitated employing a published HPLC method validated and routinely used in our laboratory (Nallani, et al., 2001; Hariparsad, et al., 2004; Desai, et al., 2002). Briefly, Waters 510 pumps were used to elute the 60:40 methanol/water mobile phase at 1 mL/min through a C18 µ-bondapak column (3.9 x 30 mm).
A Waters 2487 dual wavelength detector set at 250 nm was used for the detection of 6ß-hydroxy testosterone.

![Graph showing HPLC chromatogram](image)

Figure 10: A typical HPLC chromatogram of 6-ß-hydroxy testosterone and 11 α-hydroxy progesterone (internal standard) in the microsomal incubates.

The total run lasted for 6 minutes, with 6-ß-hydroxy testosterone and 11α-hydroxy progesterone eluting at 2.8 and 5 minutes, respectively [Fig. 10]. The standard concentrations of 6-ß-hydroxy testosterone ranged from 0.1 – 50µM. The interday and intraday variability in the HPLC analysis was <5% and the detection limit was 0.05µM for 6ß-hydroxy testosterone.

3.1.7. LC-MS/MS Analysis of α-OHT and N-DMT

The HPLC separation for the analysis of α-OHT and N-DMT was performed using a Thermo Scientific® Surveyor MST™ pump and MicroAS autosampler. Waters® XBridge™ (C₁₈ column, particle size-3.5 µm, dimensions-2.1 x 100 mm) column was used for the separation
of analytes. The mobile phase consisted of solution A (acetonitrile, 0.1% formic acid) and solution B (water, 0.1% formic acid). Separation was achieved using a gradient program of 20-95% B for 20 min, held at 100% B for 10 min, 100-20% B in 2 min and held at 20% B for 8 min. Analyses was performed using a Thermo Scientific LTQ-FT™ operated in positive ion electrospray mode. MS spectra were produced by collision-induced dissociation (CID) and recorded in a scan range of m/z 100-380 using nitrogen as the sheath and auxiliary gas. The source voltage was held at 4.70 kV with a capillary temperature of 290°C. Sheath gas was set to 30, aux gas to 5 and CID isolation widths to 1.5. High mass accuracy measurements were performed in the Fourier transform ion cyclotron resonance (FT-ICR) portion of the LTQ-FT™ with the resolution set at 25,000 for faster duty cycles. Data analysis was performed using Thermo Scientific® Xcalibur™ 2.0 software. Analytes were quantified using peak area ratios of standard curves. The retention times for α-OHT, N-DMT and TAM were 7.3, 13.55 and 14.06 minutes, respectively. Standard curves were linear over the ranges of 0.5 – 600 ng/ml.

3.1.8. Data Analysis

Data from recombinant CYP and microsomal experiments were analysed using three enzyme kinetic models – Michaelis-Menten (A), Sigmoidal (B) and Substrate inhibition kinetics (C).

\[
Rate = \frac{V_{\text{max}} \cdot C}{K_m + C} \quad (A)
\]

\[
Rate = \frac{V_{\text{max}} \cdot C^\gamma}{K_m + C^\gamma} \quad (B)
\]

\[
Rate = \frac{V_{\text{max}} \cdot C}{K_m + C \cdot (1 + C/K_i)} \quad (C)
\]
Where $V_{max}$ is the maximum rate of formation of the metabolite and reflects the maximum metabolic capacity, $Km$ is the Michaelis–Menten constant and represents the concentration of the substrate at half maximum rate, $C$ is the substrate concentration, $\gamma$ is the Hill coefficient – a measure of steepness of curve and presence of co-operativity, and $K_i$ is the dissociation constant for substrate binding to an enzyme. Final parameter estimates of $Km$ and $V_{max}$ were determined by nonlinear regression analysis using WinNonlin 5.2 (Pharsight Corporation, Inc., Mountain View, CA, USA). The best model was selected based on the distribution of residuals, standard error of the estimates and statistical criteria (AIC- Akaike Information Criteria and BIC- Bayesian Information Criteria).

The estimates of the Michaelis–Menten parameters were then used to calculate $in vitro$ intrinsic clearance using equation (Eqn. D).

$$CL\text{ int} = \frac{V_{max}}{Km} \quad (D)$$

3.1.9. Statistical Analysis

Formation of N-DMT and $\alpha$-OHT in human liver microsomes genotyped for CYP3A5*1/*1, CYP3A5*1/*3 and CYP3A5*3/*3 were analyzed using ANOVA followed by post hoc Tukey’s test. A $p$ value less than 0.05 was considered statistically significant. The formation of metabolites was calculated and expressed as picomoles per minute per mg protein for microsomes and picomoles per minute per picomole protein for recombinant CYPs. All the analyses were performed using the mean values obtained from duplicate incubations. Data has been presented as mean ± S.D (n=2).
3.2. Specific Aim 2: Clinical Assessment of CYP3A Induction by Tamoxifen

3.2.1. Materials

3.2.1.1. Chemicals

MDZ, 1-OH MDZ and 4-OH MDZ were obtained from Sigma Chemical Co. (St. Louis, MO) and Toronto Research Chemicals (Toronto, ON, Canada). The HPLC-grade methanol, acetonitrile, hexane and butanol were purchased from Fisher Scientifics (Santa Clara, CA). All other chemicals and reagents used were of the highest commercially available quality.

3.2.1.2. Biologicals

Blank plasma samples for standard curve generation were obtained from Hoxworth Blood Center (Cincinnati, OH).

3.2.2. Development and Validation of LC-MS/MS Method

3.2.2.1. Stock Solution and Calibration Standards

Standard solutions of MDZ, 1-OH-MDZ, 4-OH-MDZ were prepared by dissolving 1 mg of each compound in 1 ml of methanol. The internal standard (IS) stock solution of $^{15}$N$_3$-MDZ was also prepared similarly in methanol. The calibration standards were prepared by spiking appropriate volumes in plasma to yield final concentrations ranging from 0.08 to 50 ng/ml for MDZ and its metabolites. All stock solutions and plasma standards were stored at $-20^\circ$C until analysis.
3.2.2.2. Plasma Sample Processing

All plasma samples were allowed to thaw at room temperature. One ml of plasma was placed into a clean polypropylene tube, and IS solution of $^{15}$N$_3$-MDZ (4 ng/ml) was added to each tube and mixed. To this mixture, 200 $\mu$l of NaOH solution (NaOH in methanol) with a concentration of 1 mm was added, vortex-mixed for 30 seconds, and allowed to stand for at least 5 min before extraction with 5 ml mixture of hexane–butanol (98:2, v/v). The tubes were shaken for 30 minutes and the supernatant was collected. A second extraction was performed with 5 ml of hexane–chloroform (70:30, v/v) following the same procedure. The organic phase was collected and evaporated under dry nitrogen. The dry residue was re-dissolved in 250 $\mu$l of acetonitrile: water (80:20) and 10 $\mu$l was injected onto the column.

3.2.2.3. LC-MS/MS Conditions

The HPLC separation for the analysis was performed using a Thermo Scientific® Surveyor MSTM pump and MicroAS autosampler. The details of chromatography conditions were similar to that described in section 3.1.7. The retention times for 4-OH-MDZ, 1-OH-MDZ and MDZ were 4.27, 4.88 and 5.36 minutes, respectively. The linear regressions of ratios versus concentrations were fitted over the concentration range of 0.08 to 50 ng/ml for MDZ & its metabolites in human plasma.
3.2.2.4. Data Analysis: Calibration Curves and Weighting

Data analysis was performed using Thermo Scientific® Xcalibur™ 2.0 software. The spiked calibration standards were extracted and analysed in duplicates. Calibration curves were generated daily based on the peak area ratios of the MDZ or 1-OH MDZ to IS versus the concentration of MDZ or 1-OH MDZ, respectively. A quadratic fit with $1/x^2$ weighting best fit the data.

3.2.3. CYP3A Induction by Tamoxifen in Breast Cancer Patients

3.2.3.1. Study Design

The protocol for this study was approved by University of Cincinnati (UC) and Veteran Affairs Institutional Review Boards (IRB). All participating volunteers had signed an informed consent before starting any study related procedures. Each subject stayed at Veteran Affairs General Clinical Research Center (VA-GCRC) for at least 8 hours during PK sample collection on three different days. TAM therapy (20 mg, oral tablet, once daily) was initiated on day 0 and the volunteers made visits to the GCRC as per the following schedule.

[Fig. 11]

Visit 1 - One week prior to initiating TAM therapy (day – 7) for MDZ samples

Visit 2 – One day after starting TAM therapy (day 1) – sample collection for MDZ

Visit 3 - 42 days after starting TAM therapy (this will ensure that TAM and its metabolites reached steady state drug levels).
On each visit, MDZ 2 mg oral syrup was administered for assessing CYP3A activity. Patients were instructed to avoid ingesting any CYP3A4 modulating agents (grapefruit juice, alcohol) at least 24 hours before taking MDZ. Patients were also restricted from consuming products containing St. John’s Wort (CYP3A inducer) for the entire duration of study since this may obscure the data collected. Smoking and drinking alcohol were also restricted for at least one day prior to their hospital visit and until last blood sample collection.

For pharmacokinetic analysis, blood samples (10 ml) were collected in heparin/EDTA-containing vacutainers through an intravenous catheter, before and at 5, 15, 30 min, 1, 2, 3, 4, 5, 6, 7 and 8 hr after MDZ administration. For visits 2 and 3, samples at 3, 4, 5, 6, 7 and 8 hrs were utilized for TAM assays. Additionally, samples were collected at 24 hrs post TAM administration (i.e., immediately before consumption of the next TAM dose). Immediately after withdrawal, blood samples were centrifuged at 4000 rpm for 10 minutes in a refrigerated centrifuge. Plasma was aliquoted in labeled polypropylene tubes and stored at -80°C until analysis by LC-MS/MS method.
3.2.3.2. Pharmacokinetic Analysis

Peak plasma levels (Cmax), time to Cmax (Tmax), oral clearance (CL/F; where F is the bioavailability of MDZ), AUC₀-last: area under the plasma concentration curve from time 0 to last time point, AUC₀-∞ was calculated by dividing the last measured concentration by λ (elimination rate constant, obtained from slope of terminal log-linear portion of concentration versus time curve), volume of distribution (Vd) and elimination half-life (t₁/₂) for Midazolam and 1-OH- Midazolam were determined for each treatment day by non compartmental analysis using WinNonlin 5.2 (Pharsight Inc.).
3.3. Specific Aim 3: Impact of CYP3A and PXR Variants on CYP3A Induction

3.3.1. Materials

TaqMan SNP assay components for each of the SNPs – CYP3A4*1B, CYP3A5*3, PXR variants (25385C>T, 24381 A>C and 63396 C>T) were procured from Applied Biosystems (Foster City, USA). TaqMan Universal PCR Master Mix, and 384 well plates for PCR were procured from Applied Biosystems (Foster City, USA).

3.3.2. DNA Isolation and Quantitation

A single blood sample (10 ml) was collected from each subject for genotyping polymorphisms in CYP3A4, CYP3A5 and PXR. DNA was extracted from whole blood samples using the Gentra Pure Gene Kit, as per the manufacturer’s instructions. The purity of DNA sample was quantitated using the NanoDrop spectrophotometer (NanoDrop Technologies). The 260/280 nm ratio calculated by the NanoDrop spectrophotometer was used to evaluate the DNA purity. The ratio of 260/280 was between 1.8-2.0 for all the samples.

3.3.3. PCR

For PCR, 2 μl of DNA sample was mixed with 2.5 μl 2x TaqMan® Universal PCR Master Mix, 0.125 μl of 20x TaqMan SNP genotyping Assay Mix and 0.375 μl of water (Total
volume - 5 μl). DNA amplification was performed in a Geneamp 9700 thermal cycler using the following conditions:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature/Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG Incubation</td>
<td>50°C – 2 min</td>
</tr>
<tr>
<td>Amplitaq DNA Activation</td>
<td>95°C - 10 min</td>
</tr>
<tr>
<td>Denature</td>
<td>92°C - 15 sec</td>
</tr>
<tr>
<td>Anneal/Extension</td>
<td>60°C - 1 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
</tr>
</tbody>
</table>

UNG-Uracil N Gycosylase

### 3.3.4. Allelic Discrimination

ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) was used for allelic discrimination and determining the genotypes. A typical allelic discrimination plot indicating the ratio of fluorescence intensities of VIC or FAM dye to the passive reference is presented on the x and y axis [Fig. 12]. Each dot represents a patient and the color distinguishes each patient of different genotypes (blue – homozygous wild type, green - heterozygous and red-homozygous mutant).
Figure 12: A typical allelic discrimination plot indicating the three genotypes.
4. CHAPTER FOUR: RESULTS

4.1. Specific Aim 1: Assessment of impact of CYP3A5 on Tamoxifen Clearance in vitro

As discussed earlier, there is a paucity of data on the role of CYP3A4/5 and the impact of its genetic polymorphisms on the formation of α-OHT, a genotoxic metabolite implicated in endometrial cancer and N-DMT, an active metabolite. Inheritance of CYP3A5*1 is associated with enhanced CYP3A5 dependent metabolism in the liver and has been reported to influence the disposition of CYP3A substrates (Kuehl, et al., 2001). Using cDNA expressing specific CYP3A isoforms and human liver microsomes genotyped for CYP3A5*1/*1, CYP3A5*1/*3 and CYP3A5*3/*3 variants, we elucidated the relative contribution of CYP3A4 and CYP3A5, and assessed the impact of polymorphic expression of CYP3A5 on the formation of α-OHT and N-DMT.

4.1.1. Contribution of CYP3A4/5 to the formation of α-OHT and N-DMT

The kinetic parameters for the formation of α-OHT and the impact of polymorphic CYP3A5 expression on its formation have not been reported. Therefore, we investigated the formation of α-OHT and N-DMT using baculovirus cell line expressing CYP3A4 and CYP3A5. Both rCYP3A5 and rCYP3A5 were supplemented with cytochrome b5 and oxido-reductase. The concentrations of TAM used were in the range of 0-50 µM. The metabolites in the microsome samples were extracted and quantitated using LC-MS/MS [Fig. 13]. The Vmax and Km values were calculated by fitting the data to Michaelis-Menten equation using WinNonlin 5.2. The mean estimates for Vmax and Km with the standard errors are listed in Table 6. In the case of α-OHT, the intrinsic formation clearance (CLint) with rCYP3A4 was
approximately 6-fold higher than with rCYP3A5, indicating a relatively minor role for CYP3A5 (3.32 vs 0.65 µl/min/pmol P450). Representative kinetic plots for the formation of α-OHT by rCYP3A4 and rCYP3A5 are shown in [Fig. 14].

For N-DMT, the CLint values for rCYP3A4 and rCYP3A5 were 0.57 and 0.35 ml/min/pmol P450 respectively. This corroborates to the previously reported observation that CYP3A5 contributes to the formation of N-DMT (Desta, et al., 2004;Williams, et al., 2002). The Km values estimated were similar for both the enzymes (7.18 vs 6.87µM, Table 6, [Fig. 15]).

Figure 13. A typical LC-MS/MS chromatogram of α-OHT (A) and N-DMT (B) in the microsomal incubates.
Table 6. Enzyme kinetic parameter estimates for the formation of α-OHT and N-DMT by recombinant CYP3A4 and CYP3A5. Each incubation contained TAM (0-50 µM), 50 pmol of rCYP3A4 or rCYP3A5 and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis-Menten model using non linear regression analysis. Results are Mean ± SE from duplicate incubations.

<table>
<thead>
<tr>
<th>Recombinant CYP</th>
<th>Vmax (pmol/min/pmol P450)</th>
<th>Km (µM)</th>
<th>CLint (µl/min/pmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-OHT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rCYP3A4</td>
<td>62.36 ± 12.3</td>
<td>19.28 ± 4.2</td>
<td>3.23</td>
</tr>
<tr>
<td>rCYP3A5</td>
<td>8.66 ± 0.97</td>
<td>13.4 ± 1.9</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>N-DMT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rCYP3A4</td>
<td>4.06 ± 0.28</td>
<td>7.18 ± 1.5</td>
<td>0.57</td>
</tr>
<tr>
<td>rCYP3A5</td>
<td>2.14 ± 0.06</td>
<td>6.87 ± 0.7</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Vmax, maximum velocity; Km, substrate concentration at which the reaction velocity is 50% of Vmax; CLint, intrinsic clearance. Estimates are Mean±SE from duplicate incubations.

Figure 14. The enzyme kinetics of α-hydroxy tamoxifen formation from TAM by the recombinant CYP3A4 (A) and CYP3A5 (B) isoforms. Each data point represents the mean of two 10 minutes incubations of TAM (0-50 µM) with 50 pmol of CYP3A4 or CYP3A5 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from Michaelis-Menten model using WinNonlin 5.2.
Figure 15. The enzyme kinetics of N-desmethyl tamoxifen (N-DMT) formation from TAM by the recombinant CYP3A4 (A) and CYP3A5 (B) isoforms. Each data point represents the mean of two 10 minutes incubations of TAM (0-50 µM) with 50 pmol of CYP3A4 or CYP3A5 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from Michaelis-Menten model using WinNonlin 5.2.

4.1.2. Effect of CYP3A5*3 genotyped microsomes on the formation of α-OHT

We next assessed the impact of CYP3A5 polymorphisms on the formation of α-OHT using a panel of 20 human liver microsomes. The linearity of the formation of α-OHT with respect to varying protein concentration and time of incubation was established in preliminary experiments. The Vmax and Km values were estimated by fitting the data to Michaelis-Menten model using non linear regression analysis (Table 7). The data on CYP3A4/5 protein content, testosterone 6-β-hydroxylation and midazolam 1-hydroxylation in same set of microsomes are also presented in Table 7.

In the case of α-OHT, there was no significant difference in the Vmax values between the three CYP3A5 genotypes [Fig. 16]. We observed a 28-fold variation in the Vmax values
ranging from 1.8 to 49.6 pmol/min/mg and a 9-fold variation in the Km values ranging from 0.7 to 29.2 µM. Furthermore, the Vmax values from genotyped microsomes were compared with the protein contents of CYP3A4, CYP3A5 and the CYP3A activity determined as testosterone 6-β-hydroxylation and midazolam 1-hydroxylation in the same set of microsomes. The correlation between Vmax of TAM α-hydroxylation and CYP3A4 protein (r=0.80, p=0.0002) was significant and was greater than the correlation with CYP3A5 protein (r=0.47, p=0.062) [Fig. 17]. In addition, we also observed a significant correlation between Vmax and testosterone-6β hydroxylation activity (r=0.79, p=0.0002), a marker specific to CYP3A4 than CYP3A5. The correlation with 1- hydroxy midazolam was less significant with r=0.44 and p = 0.0314 [Fig. 18].

Figure 16. Comparison of Vmax of α- OHT formation between the three CYP3A5*1/*1, *1/*3 and *3/*3 genotypes. Each point represents the Vmax estimated by fitting the data to Michaelis-Menten kinetics using nonlinear regression analysis. TAM (0-50 µM) was incubated with genotyped human liver microsomes as described in the methodology section. There was no significant difference between the three genotypes.
Figure 17. Correlations between Vmax of α-OHT formation and (A) CYP3A4 protein, (B) CYP3A5 protein in a panel of genotyped human liver microsomes.

Figure 18. Correlations between Vmax of α-OHT formation and (A) testosterone-6-β-hydroxylation, (B) midazolam-1-hydroxylation in genotyped human liver microsomes.
Table 7. Enzyme kinetic estimates for the formation of α-OHT and N-DMT in a panel of genotyped human liver microsomes.

<table>
<thead>
<tr>
<th>Liver ID</th>
<th>CYP3A5 Genotype</th>
<th>N-DMT</th>
<th>α-OHT</th>
<th>1-hydroxy midazolam (nmol/min/mg)</th>
<th>6-β-hydroxy testosterone (nmol/min/mg)</th>
<th>CYP3A4 Protein (pmol/mg)</th>
<th>CYP3A5 Protein (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH-1019  *1/*1</td>
<td>408.98</td>
<td>6.94</td>
<td>58.91</td>
<td>49.55</td>
<td>14.21</td>
<td>3.49</td>
<td>8.15</td>
</tr>
<tr>
<td>HH-860   *1/*1</td>
<td>227.14</td>
<td>10.58</td>
<td>21.48</td>
<td>5.65</td>
<td>7.65</td>
<td>0.74</td>
<td>2.245</td>
</tr>
<tr>
<td>MCV-36   *1/*1</td>
<td>244.59</td>
<td>7.86</td>
<td>31.13</td>
<td>17.69</td>
<td>10.45</td>
<td>1.69</td>
<td>1.74</td>
</tr>
<tr>
<td>CD-8002  *1/*1</td>
<td>236.19</td>
<td>10.66</td>
<td>22.15</td>
<td>1.76</td>
<td>12.65</td>
<td>0.14</td>
<td>0.9</td>
</tr>
<tr>
<td>HH 739   *1/*1</td>
<td>223.41</td>
<td>4.15</td>
<td>53.83</td>
<td>2.71</td>
<td>5.97</td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>HH 785   *1/*1</td>
<td>253.76</td>
<td>3.32</td>
<td>76.53</td>
<td>4.2</td>
<td>6</td>
<td>0.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Mean</td>
<td>267.63</td>
<td>7.80</td>
<td>41.32</td>
<td>12.43</td>
<td>9.23</td>
<td>1.12</td>
<td>2.78</td>
</tr>
<tr>
<td>SD</td>
<td>65.09</td>
<td>3.18</td>
<td>21.72</td>
<td>17.21</td>
<td>3.27</td>
<td>1.15</td>
<td>2.61</td>
</tr>
<tr>
<td>HH-1044  *1/*3</td>
<td>107.41</td>
<td>19.42</td>
<td>5.53</td>
<td>8.49</td>
<td>25.82</td>
<td>0.33</td>
<td>0.841</td>
</tr>
<tr>
<td>HH-525   *1/*3</td>
<td>135.96</td>
<td>16.00</td>
<td>8.50</td>
<td>4.23</td>
<td>22.91</td>
<td>0.18</td>
<td>0.499</td>
</tr>
<tr>
<td>HL-G     *1/*3</td>
<td>112.92</td>
<td>21.52</td>
<td>5.25</td>
<td>3.20</td>
<td>16.52</td>
<td>0.19</td>
<td>0.7945</td>
</tr>
<tr>
<td>HL-W     *1/*3</td>
<td>92.35</td>
<td>7.86</td>
<td>11.74</td>
<td>3.89</td>
<td>16.76</td>
<td>0.23</td>
<td>1.15</td>
</tr>
<tr>
<td>HH-776   *1/*3</td>
<td>69.90</td>
<td>17.53</td>
<td>3.99</td>
<td>5.26</td>
<td>29.24</td>
<td>0.18</td>
<td>0.8745</td>
</tr>
<tr>
<td>Mean</td>
<td>103.71</td>
<td>16.47</td>
<td>7.00</td>
<td>5.02</td>
<td>22.25</td>
<td>0.22</td>
<td>0.75</td>
</tr>
<tr>
<td>SD</td>
<td>24.55</td>
<td>5.24</td>
<td>3.12</td>
<td>2.08</td>
<td>5.59</td>
<td>0.06</td>
<td>0.17</td>
</tr>
<tr>
<td>HH189    *3/*3</td>
<td>112.42</td>
<td>3.89</td>
<td>28.87</td>
<td>2.07</td>
<td>0.71</td>
<td>2.81</td>
<td>2.8</td>
</tr>
<tr>
<td>HH507    *3/*3</td>
<td>89.09</td>
<td>5.2</td>
<td>17.13</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
<td>2.08</td>
</tr>
<tr>
<td>XENO     *3/*3</td>
<td>52.99</td>
<td>4.4</td>
<td>12.03</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
<td>0.6</td>
</tr>
<tr>
<td>HL-D     *3/*3</td>
<td>97.32</td>
<td>17.44</td>
<td>5.58</td>
<td>1.80</td>
<td>9.62</td>
<td>0.19</td>
<td>0.253</td>
</tr>
<tr>
<td>HL-J     *3/*3</td>
<td>122.67</td>
<td>9.84</td>
<td>12.47</td>
<td>5.90</td>
<td>8.16</td>
<td>0.72</td>
<td>0.7975</td>
</tr>
<tr>
<td>HL-P     *3/*3</td>
<td>106.09</td>
<td>7.64</td>
<td>13.88</td>
<td>4.39</td>
<td>7.96</td>
<td>0.55</td>
<td>0.945</td>
</tr>
<tr>
<td>HH-689   *3/*3</td>
<td>73.99</td>
<td>9.30</td>
<td>7.96</td>
<td>ND</td>
<td>ND</td>
<td>--</td>
<td>0.135</td>
</tr>
<tr>
<td>HL-R     *3/*3</td>
<td>153.66</td>
<td>13.72</td>
<td>11.20</td>
<td>7.09</td>
<td>12.99</td>
<td>0.55</td>
<td>0.8355</td>
</tr>
<tr>
<td>Mean</td>
<td>101.03</td>
<td>8.93</td>
<td>13.64</td>
<td>4.25</td>
<td>7.89</td>
<td>0.96</td>
<td>0.91</td>
</tr>
<tr>
<td>SD</td>
<td>30.68</td>
<td>4.75</td>
<td>7.08</td>
<td>2.32</td>
<td>4.49</td>
<td>1.05</td>
<td>0.89</td>
</tr>
</tbody>
</table>

* pmol/min/mg, ** μM, ND – Not Detected, a – Data from Lilly
4.1.3. Effect of CYP3A5*3 genotyped microsomes on the formation of N-DMT

In the case of N-DMT formation, there was a significant difference in the Vmax values between CYP3A5*1/*1 (267.63 pmol/min/mg) and CYP3A5*1/*3 (103.71 pmol/min/mg) or CYP3A5*3/*3 (101.03 pmol/min/mg) microsomes (p<0.05) [Fig. 19]. A 7- and 5- fold variation in the Vmax values (53 to 408 pmol/min/mg) and Km values (3.3 to 17.4 µM) were observed. We then compared Vmax of N-DMT formation with the protein content of CYP3A4 (r=0.56, p=0.04) and CYP3A5 (r=0.81, p<0.0001), and the correlations were significant [Fig. 20]. Moreover, the correlation between Vmax and midazolam hydroxylation (r=0.79, p=0.0002), Vmax and testosterone-6-β-hydroxylation (r=0.68, p=0.002) were also significant further suggesting the role of CYP3A5 in N-DMT formation [Fig. 21]. This observed difference in intrinsic clearance between the wild type and the mutant CYP3A5 variant may be an important contributor to the variability observed with TAM pharmacokinetics.
Figure 19. Comparison of Vmax of N-DMT formation between the three CYP3A5*1/*1, *1/*3 and *3/*3 genotypes. Each point represents the Vmax estimated by fitting the data to Michaelis Menten kinetics using nonlinear regression analysis. TAM (0-50 µM) was incubated with genotyped human liver microsomes as described in the methodology section. There was a significant difference between CYP3A5*1/*1 and CYP3A5 *1/*3 or CYP3A5*3/*3 (p<0.05).

Figure 20. Correlations between Vmax of N-DMT formation and (A) CYP3A4 protein, (B) CYP3A5 protein in a panel of genotyped human liver microsomes.
4.1.4. Effect of recombinant CYP3A5*8 and CYP3A5*9 on the formation of N-DMT

CYP3A5*8 and CYP3A5*9 have been reported to have decreased enzyme activity when compared to the wild type CYP3A5 (Lee, et al., 2003). Using recombinant CYP3A5*8 and CYP3A5*9, we determined the enzyme kinetic estimates for N-DMT formation. The data was best described by Michaelis-Menten model using non-linear regression analysis. Vmax and Km estimates for N-DMT and the Michaelis-Menten plots are presented in Table 8 and [Fig. 22], respectively. The intrinsic clearance for the formation of N-DMT by rCYP3A5*8 was half of that observed for rCYP3A5 (0.28 vs 0.15 ml/min/pmol). On the other hand, the intrinsic clearance for N-DMT formation by rCYP3A5*9 was not significantly different from rCYP3A5 (0.24 vs 0.28 ml/min/pmol P450). However, the estimates of Vmax and Km were significantly higher with rCYP3A5*9 in comparison to the wild type allele (7.62 vs 2.68 nmol/min/pmol P450 and 31.39 vs 9.28 µM), respectively. We also evaluated the enzyme kinetics for 1-hydroxy midazolam formation...
from midazolam, a known substrate for CYP3A4 and 3A5. A similar trend was observed in the formation clearance of 1-hydroxy midazolam. The intrinsic formation clearance was lower with rCYP3A5*8 than with rCYP3A5 (1.84 vs 3.31 ml/min/pmol P450). The Vmax and Km values estimated were higher with rCYP3A5*9 than that of rCYP3A5 (69.24 vs 36.06 nmol/min/pmol P450 and 20.42 vs 10.89 µM) as seen in Table 8 and [Fig. 23].

**Table 8.** Enzyme kinetic parameter estimates for the formation of N-DMT and 1-hydroxy midazolam by recombinant CYP3A5, CYP3A5*8 and CYP3A5*9 from TAM and midazolam, respectively. Each incubation contained TAM (0-50 µM) or Midazolam (0-400 µM), 50 pmol of rCYP3A5 or rCYP3A5*8 or rCYP3A5*9 and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis-Menten model for N-DMT formation and substrate inhibition kinetics for 1-hydroxy midazolam formation, using non linear regression analysis. Results are Mean ± SE from duplicate incubations.

<table>
<thead>
<tr>
<th>Recombinant CYP3A5</th>
<th>Vmax (nmol/min/pmol P450)</th>
<th>Km (µM)</th>
<th>CLint (ml/min/pmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-DMT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rCYP3A5</td>
<td>2.68 ± 0.16</td>
<td>9.28 ± 1.5</td>
<td>0.28</td>
</tr>
<tr>
<td>rCYP3A5*8</td>
<td>1.53 ± 0.21</td>
<td>10.25 ± 2.1</td>
<td>0.15</td>
</tr>
<tr>
<td>rCYP3A5*9</td>
<td>7.62 ± 1.4</td>
<td>31.39 ± 6.1</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>1-Hydroxy midazolam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rCYP3A5</td>
<td>36.06 ± 4.6</td>
<td>10.89</td>
<td>3.31</td>
</tr>
<tr>
<td>rCYP3A5*8</td>
<td>9.8 ± 2.5</td>
<td>5.3 ± 0.8</td>
<td>1.84</td>
</tr>
<tr>
<td>rCYP3A5*9</td>
<td>69.24 ± 6.1</td>
<td>20.42 ± 4.3</td>
<td>3.39</td>
</tr>
</tbody>
</table>

Vmax, maximum velocity; Km, substrate concentration at which the reaction velocity is 50% of Vmax; CLint, intrinsic clearance.
Figure 22. The enzyme kinetics of N-desmethyl tamoxifen (N-DMT) formation from TAM by the recombinant CYP3A5, CYP3A5*8 and CYP3A5*9 microsomes. Each data point represents the mean of two 10 minute incubations of TAM (0-50 µM) with 50 pmol of CYP3A5 or 3A5*8 or 3A5*9 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from Michaelis-Menten model using WinNonlin 5.2.

Figure 23. The enzyme kinetics of 1-hydroxy-midazolam formation from midazolam by the recombinant CYP3A5, CYP3A5*8 and CYP3A5*9 microsomes. Each data point represents the mean of two 5 minutes incubations of midazolam (0-400 µM) with 50 pmol of CYP3A5 or 3A5*8 or 3A5*9 in the presence of NADPH regenerating system. Solid line represents the simulated curve generated from substrate inhibition model using WinNonlin 5.2.
4.1.5. Effect of novel CYP3A4 intronic variant on the formation of α-OHT and N-DMT

We then investigated the effect of a novel intronic variant in CYP3A4 on the formation of α-OHT and N-DMT. Enzyme kinetic estimates were determined by incubating TAM with the genotyped microsomes as described in the experimental section. The three sets of genotyped human liver microsomes (HL-Q, HH-639, HH-1123) available were of CYP3A5*3/*3 and CYP3A4*1 (intronic SNP). Hence, we compared the data on α-OHT and N-DMT formation by these microsomes with the data from the microsomes that were of CYP3A5*3/*3 and CYP3A4*1 wild type genotype. The Vmax and Km estimates for the formation of α-OHT in the wild type (4.17 pmol/min/mg and 9.68 µM) and mutant variant (3.13 pmol/min/mg and 10.79 µM) were not significantly different (Table 9).

However, the Vmax of N-DMT was lower in the mutant variant (73.16 pmol/min/mg)

Table 9. Enzyme kinetic parameter estimates for the formation of α-OHT and N-DMT by CYP3A4 intronic variant from TAM. Each incubation contained TAM (0-50 µM), 0.1mg/ml of protein and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis-Menten model for α-OHT and N-DMT formation using non-linear regression analysis. Each incubation was performed in duplicates.
Table 10. Enzyme kinetic parameter estimates for the formation of 6-β-hydroxy testosterone by CYP3A4 intronic variant from testosterone. Each incubation contained testosterone (0-400 µM), 0.25mg/ml of protein and a NADPH-regenerating system in 100 mM phosphate buffer. Data was fitted to Michaelis Menten model for 6-β-hydroxy testosterone formation using non linear regression analysis. Each incubation was performed in duplicates.

<table>
<thead>
<tr>
<th>Liver ID</th>
<th>CYP3A5 Genotype</th>
<th>CYP3A4 Genotype</th>
<th>Vmax (nmol/min/mg)</th>
<th>Km (µM)</th>
<th>CLint (ml/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-D</td>
<td>*3/*3</td>
<td>*1/*1</td>
<td>0.59</td>
<td>10.42</td>
<td>0.06</td>
</tr>
<tr>
<td>HL-J</td>
<td>*3/*3</td>
<td>*1/*1</td>
<td>2.23</td>
<td>58.75</td>
<td>0.04</td>
</tr>
<tr>
<td>HL-P</td>
<td>*3/*3</td>
<td>*1/*1</td>
<td>1.78</td>
<td>72.52</td>
<td>0.02</td>
</tr>
<tr>
<td>HH-689</td>
<td>*3/*3</td>
<td>*1/*1</td>
<td>0.23</td>
<td>36.18</td>
<td>0.01</td>
</tr>
<tr>
<td>HL-R</td>
<td>*3/*3</td>
<td>*1/*1</td>
<td>2.83</td>
<td>69.95</td>
<td>0.04</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>1.53</td>
<td>49.56</td>
<td>0.03</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td>1.10</td>
<td>26.16</td>
<td>0.02</td>
</tr>
<tr>
<td>HL-Q</td>
<td>*3/*3</td>
<td>*1/*1 (intronic)</td>
<td>0.39</td>
<td>26.58</td>
<td>0.01</td>
</tr>
<tr>
<td>HH-639</td>
<td>*3/*3</td>
<td>*1/*1 (intronic)</td>
<td>0.37</td>
<td>22.81</td>
<td>0.02</td>
</tr>
<tr>
<td>HH-1123</td>
<td>*3/*3</td>
<td>*1/*1 (intronic)</td>
<td>0.52</td>
<td>13.92</td>
<td>0.04</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>0.43</td>
<td>21.10</td>
<td>0.02</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td>0.08</td>
<td>6.50</td>
<td>0.01</td>
</tr>
</tbody>
</table>

expressors than the wild type CYP3A4 (110.7 pmol/min/mg), but was not statistically significant (p=0.055).

Furthermore, we evaluated the formation of 6-β-hydroxy testosterone in the same set of microsomes. There was a clear trend towards lower Vmax and Km values for the formation of 6-β-hydroxy testosterone in the microsomes expressing the intronic variant. However, the differences were not statistically significant as seen from the CLint (0.03 vs 0.02 ml/min/mg) (Table 10).
4.2. Specific Aim 2: Clinical Assessment of CYP3A Induction by Tamoxifen

Previous findings from our laboratory indicated that TAM and 4-OHT induce CYP3A in primary cultures of human hepatocytes via activation of PXR (Desai et al 2002). This raises the possibility that TAM enhances its own metabolism – a phenomenon known as auto-induction of clearance or time-dependent pharmacokinetics. CYP3A4 auto-induction upon prolonged TAM use may result in increased conversion of TAM to α-OHT and N-DMT, contributing to differences in TAM pharmacokinetics as well as efficacy and endometrial toxicity. Employing MDZ as a probe substrate to determine changes in CYP3A activity, we determined the potential of TAM to induce CYP3A in breast cancer patients.

4.2.1. LC-MS/MS method development for quantitation of Midazolam

4.2.1.1. Chromatography

Chromatographic conditions and the composition of mobile phase in particular, were optimized to achieve good sensitivity and peak shapes for the compounds. No interference peaks were detected for the analytes or the internal standard from the different sources of plasma. The retention times for 4-OH-MDZ, 1-OH-MDZ and MDZ were 4.27, 4.88 and 5.36 minutes, respectively. Typical chromatograms for a blank plasma spiked with MDZ, the IS $^{15}$N$_3$ MDZ and its metabolites are shown in [Fig. 24].
The mass transitions monitored for MDZ, 1-OH MDZ and 4-OH MDZ were m/z 326 → 291, m/z 342 → 324 and m/z 342 → 325, respectively. The IS $^{15}$N$_3$ MDZ was monitored at m/z 329 → 294.

Figure 24. A typical chromatogram of a blank plasma spiked with MDZ, labeled $^{15}$N$_3$-MDZ (IS), 4-OH MDZ and 1-OH MDZ.
4.2.1.2. Linearity of Calibration Curves

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range of 0.08 to 50 ng/ml for MDZ, 1-OH MDZ and 4-OH MDZ human plasma. The correlation coefficients of the $1/x^2$-weighted calibration curves for MDZ and 1-OH MDZ were $r^2 = 0.99$. Good linearity was seen in the concentration ranges of 0.1-50 ng/ml [Fig. 25].

4.2.1.3. Extraction and Recovery

Recovery of all compounds of interest was tested in quality control (QC) samples. QC samples were made in plasma at three concentrations and were analyzed between each set of samples. The present method produced a satisfactory recovery of 87–105%, thus implying that extraction of the plasma did not result in any substantial loss of the chemical constituents.
Figure 25. Representative calibration plasma standard curves for Midazolam (a), and 1-OH-MDZ in human plasma samples.
4.2.2. Induction of CYP3A by TAM in Breast Cancer Patients

CYP3A induction by TAM was assessed using oral MDZ (2 mg, oral syrup) as a probe substrate. As discussed before, MDZ is exclusively metabolized by CYP3A4/5 to its primary metabolite 1-OH MDZ. Any changes in CYP3A activity will be reflected in oral clearance, a criterion widely used to assess CYP3A activity. The demographic details of the 13 patients enrolled in the pharmacokinetic study are presented in Table 11. The mean age and weights of the patients enrolled were 49 ± 7 yrs and 75 ± 17 kg, respectively. All the 13 patients completed the three visits and provided approximately a total of 35 blood samples for MDZ pharmacokinetic analysis as per the protocol. No major adverse events were reported during the course of the trial. The plasma samples collected for pharmacokinetic analysis were subject to double extraction procedure and quantitated for MDZ and 1-OH MDZ using the validated LC-MS/MS methodology (detailed in methodology section).

Table 11. Demographics of patients enrolled in the PK study

<table>
<thead>
<tr>
<th>ID</th>
<th>AGE (yrs)</th>
<th>WT (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>73</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
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</tr>
<tr>
<td>4</td>
<td>49</td>
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<tr>
<td>14</td>
<td>35</td>
<td>64</td>
</tr>
<tr>
<td>15</td>
<td>38</td>
<td>107</td>
</tr>
</tbody>
</table>

Mean 49.46 75.60
SD 7.77 17.30
Figure 26. Plasma concentration time profile of MDZ in breast cancer patients on Day 0 (Blue), Day 1 (Green) and Day 42 (Red) (A). Profile of patient ID= 06 showing induction of CYP3A on Day 42 as seen by decrease in the AUC and Cmax (B).
The concentration data of each patient was used to estimate the pharmacokinetic parameters for MDZ by non compartmental analysis employing WinNonlin 5.2. There was no statistically significant difference in mean CL/F, AUC and half life estimates between Day 0 and Day 42 or Day 1 and Day 42. However, in 6 out of the 13 patients, a mean increase of 70% (range 6 to 161%) in the oral clearance of MDZ was observed between Day 0 to Day 42. The corresponding mean decrease in AUC$_{0-\infty}$ and half life for those 6 patients were 31% (range 4 to 59%) and 30% (range 19 to 71%), respectively (Table 12). The parameter estimates of CL/F, AUC0-last, AUC$_{0-\infty}$, Vd, Tmax, Cmax and half life for all the treatment days have been tabulated in table 13. The plasma concentration Vs time profiles of MDZ for all the patients are presented in [Fig. 26A]. As an example, MDZ concentration time profile of patient ID=06 is presented in [Fig. 26B] to indicate the significant decrease in AUC and Cmax on day 42 in comparison to Day 0 or Day 1. The changes in CL/F, AUC$_{0-\infty}$ and half life of MDZ between Day 0 and Day 42, Day 1 and Day 42 are presented in [Fig. 27A, B and C]. Thus, our data clearly shows significant CYP3A induction in some patients. Furthermore, extensive inter-individual variability in CYP3A induction was observed.

**Table 12.** Changes (%) in Midazolam Pharmacokinetics in Patients (N=6)

<table>
<thead>
<tr>
<th>ID</th>
<th>% Increase in CL/F</th>
<th>% Decrease in AUC</th>
<th>% Decrease in Half life</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>76</td>
<td>36</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>132</td>
<td>56</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>10</td>
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<td>20</td>
<td>23</td>
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<tr>
<td>14</td>
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<td>59</td>
<td>71</td>
</tr>
<tr>
<td>15</td>
<td>21</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Mean</td>
<td>70</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>SD</td>
<td>64</td>
<td>23</td>
<td>20</td>
</tr>
</tbody>
</table>

80
Table 13. Pharmacokinetic estimates of midazolam after administration of 2 mg oral midazolam syrup in 13 breast cancer patients on three visits.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Visits</th>
<th>Half life (hr)</th>
<th>Tmax (hr)</th>
<th>Cmax (ng/mL)</th>
<th>AUClast (ng.hr/ml)</th>
<th>AUCinf (ng.hr/mL)</th>
<th>Vd (L)</th>
<th>CL/F (L/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Day 0</td>
<td>1.3</td>
<td>0.5</td>
<td>11.9</td>
<td>28.69</td>
<td>31.41</td>
<td>123.76</td>
<td>63.68</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>1.2</td>
<td>0.5</td>
<td>16.9</td>
<td>41.79</td>
<td>44.57</td>
<td>78.08</td>
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Figure 27. Changes in oral clearance (CL/F, L/hr) of MDZ between Day 0 and Day 42, and Day 1 and Day 42 (A). Changes in AUC of MDZ between Day 0 and Day 42, and Day 1 and Day 42 (B). Changes in half life of MDZ between Day 0 and Day 42, and Day 1 and Day 42 (C). CL/F and AUC estimates were determined by noncompartmental analysis using WinNonlin 5.2.
4.3. Specific Aim 3: Impact of CYP3A and PXR Variants on CYP3A Induction

The impact of polymorphic expression on the induction of CYP3A is poorly understood. CYP3A5 is generally considered to be a non-inducible CYP3A isoform in comparison to CYP3A4. A recent study does suggest that CYP3A5 may also be regulated by hPXR and is inducible by hPXR ligands (Burk et al 2004). Even so, the induction appears to be mainly at the intestinal CYP3A5 expression. However, it is feasible that the extent of induction may be associated with the CYP3A4 and CYP3A5 polymorphisms. Furthermore, polymorphisms in PXR have been associated with higher induction in CYP3A activity following treatment with rifampicin (Lamba et al 2008). Individuals expressing these variants are likely to exhibit higher level of CYP3A induction as a result of TAM therapy, which may be reflected in higher levels of N-DMT or α-OHT and increased propensity for drug interactions. Therefore, a preliminary assessment on the impact of CYP3A4*1B, CYP3A5*3 and PXR polymorphisms on CYP3A induction was conducted.

4.3.1. Association of CYP3A and PXR Genotypes with Fold Change in Clearance

To investigate the genetic basis for the observed differences in CYP3A induction, a 10 ml of blood sample was collected from each patient and was genotyped for polymorphisms in CYP3A and PXR using TaqMan SNP chemistry as described in the methodology section. Specifically, we genotyped for CYP3A4*1B, CYP3A5*3 and the three PXR variants (63396T>C, -24381A>C, -25385C>T) that have been previously known to modulate CYP3A activity (Lamba et al 2008, Siccardi M et al 2008). Association of
CYP3A5 genotypes with fold change in MDZ clearance revealed a trend towards higher induction in individuals with CYP3A5*1/*3 vs. CYP3A5*3/*3 genotypes [Fig. 28]. Furthermore, one individual with maximum increase (2.5 fold) in midazolam CL/F was homozygous to C and T alleles of PXR -24381A>C and -25385C>T variants, respectively [Fig. 29 and 30]. No association was observed between CYP3A4*1B and fold change in midazolam clearance. In addition, there was no significant difference in α-OHT mean plasma concentrations between individuals expressing CYP3A5*1/*3 vs CYP3A5*3/*3 [Fig. 31].

Figure 28. Association of CYP3A5 genotype with CYP3A induction determined as fold change in midazolam oral clearance. DNA was isolated from fresh blood collected in EDTA vacutainers using Puregene kit and genotyped using TaqMan SNP genotyping assay.
Figure 29. Association of PXR -25385C>T genotype with CYP3A induction determined as fold change in midazolam oral clearance. DNA was isolated from fresh blood collected in EDTA vacutainers using Puregene kit and genotyped using TaqMan SNP genotyping assay.

Figure 30. Association of PXR -24381 A>C genotype with CYP3A induction determined as fold change in midazolam oral clearance. DNA was isolated from fresh blood collected in EDTA vacutainers using Puregene kit and genotyped using TaqMan SNP genotyping assay.
Figure 31. Association of mean plasma α-OHT (ng/ml) between CYP3A5*1/*3 vs CYP3A5*3/*3 genotypes on Day 1 and Day 42.
5. CHAPTER FIVE: DISCUSSION

TAM is a widely used and highly effective endocrine agent for the treatment of breast cancer in the adjuvant and metastatic settings and for chemoprevention. Originally tested as an oral contraceptive, TAM was later found to be an effective “antiestrogen”. Since its introduction in 1970, TAM has been the first line of therapy in treating patients with ER positive tumors. TAM has a mixed ER agonist and antagonist properties depending on the target tissues. By way of its agonist action, TAM helps to maintain bone mineral density and lower lipid levels thereby ameliorating bone and cardiovascular diseases in women (Osborne, 1998; Kristensen, et al., 1994). In the uterus, TAM’s agonist action has been reported to increase the risk of developing endometrial cancer in postmenopausal women (Jordan and Morrow, 1994). It was in the pursuit of understanding these estrogenic effects like uterine lining growth, α and β isoforms of ER were identified.

Notwithstanding the advent of aromatase inhibitors (AIs) and newer selective estrogen receptor modulators (SERMs), TAM remains an important member of the armamentarium against breast cancer. TAM continues to be the single drug available for pre-menopausal women who wish to pursue chemoprevention or in the treatment of pre-menopausal women with estrogen receptor-positive breast cancer (Bao, et al., 2006; Fisher, et al., 2005a). Many physicians prefer TAM due to its record of effectiveness and in instances where patients do not favorably respond to other endocrine agents including aromatase inhibitors or present with osteoporosis. Despite being successfully used for more than 30 years, some of the clinical problems associated with the use of TAM include high inter-individual variability in its pharmacokinetics and therapeutic outcome, drug-drug interactions, acquired drug
resistance and increased risk of endometrial cancer. Other commonly observed side effects of TAM therapy are hot flushes, vaginal bleeding and discharge, and thromboembolism (Perez, 2007).

The ability of TAM to induce endometrial cancer has been a great concern for the past few decades. Patients on TAM therapy have a 2-6 fold higher risk of developing endometrial cancer, with a even greater risk in women older than 50 (Bergman, et al., 2000; Reddy and Chow, 2000). This poses a huge dilemma to the patients and to the physicians. It is important to indicate that the exact mechanism of TAM-induced carcinogenesis remains unresolved, and in fact, somewhat controversial. Endometriosis may be linked to the estrogenic activity of TAM in the uterus. The other major proposed hypothesis suggests that TAM is metabolically activated to genotoxic species (Brown, 2009). \(\alpha\)-OHT, one of the primary metabolites formed by hydroxylation of TAM undergoes activation to form DNA adducts resulting in endometrial cancer. Infact, several investigators have identified TAM- DNA adducts in rats, monkeys and women treated with TAM (Carthew, et al., 2000; Shibutani, et al., 2000; Hemminki, et al., 1996; Orton and Topham, 1997; Shibutani, et al., 2003; Martin, et al., 2003). Inspite of several other treatment modalities for breast cancer in postmenopausal patients, TAM (20 mg/day for 5 years) is still being recommended as the drug of choice to reduce the risk of invasive ER-positive tumors, with benefits for at least 10 years (Visvanathan, et al., 2009). Furthermore, its use has been more cost effective than other drugs. Therefore, attempts to better understand some of the unresolved issues and to optimize the use of TAM continues to be a high priority.
The impact of CYP2D6 polymorphisms on the disposition of TAM has been investigated by several investigators over the last few years. CYP2D6 is the cytochrome P450 isoform that is mainly responsible for catalyzing the conversion of TAM to 4-OHT and N-DMT to endoxifen, the active metabolite. Plasma levels of endoxifen have been linked to CYP2D6 genotype and higher incidences of hot flushes (Goetz, et al., 2005; Jin, et al., 2005; Stearns, et al., 2003; Borges, et al., 2006). Jin et al. observed that patients who carried either the homozygous (*4/*4) or the heterozygous (wt/*4) variant genotype of CYP2D6 had statistically significantly lower mean plasma endoxifen levels (20.0 and 43.1 nM, respectively) than those who carried the homozygous wild-type (wt/wt) genotype (78.0 nM). These patients also had fewer incidences of hot flashes. Furthermore, individuals with CYP2D6 *4/*4 genotype have been associated with poor patient outcome, worse relapse-free and disease-free survival (Goetz, et al., 2008; Goetz and Ingle, 2007; Brauch and Jordan, 2009).

However, there is paucity of data on the impact of CYP3A activity and its polymorphic expression on the disposition of TAM. The major metabolite, N-DMT and the genotoxic product, α-OHT are exclusively formed by CYP3A. On one hand, individuals with higher CYP3A activity will have increased capacity for TAM conversion to N-DMT, contributing to variability in the plasma levels of endoxifen. On the other hand, increased CYP3A levels, may lead to increased production of the genotoxic α-OHT, which may ultimately lead to increased chances of endometrial carcinogenesis (Shibutani, et al., 2000). In addition, co-administration of TAM has been shown to markedly reduce the plasma concentrations and AUC of letrozole and anastrozole, causing a mean 37 and 27 % reduction in the levels of
letrozole and anastrozole, respectively (Dowsett, et al., 1999; Dowsett, et al., 2001). Indeed, previous investigations from our laboratory indicated that TAM and 4-OHT induced CYP3A4 activity in primary human hepatocytes via activation of PXR (Desai, et al., 2002; Sane, et al., 2008). Furthermore, there is increasing evidence that contribution of CYP3A5 activity is equal to that of CYP3A4 for the metabolism of several compounds including tacrolimus, cyclosporine, vincristine, ethylmorphine, lidocaine, testosterone, sildenafil and alfentanil (Klees, et al., 2005; Gillam, et al., 1995; Huang, et al., 2004; Dai, et al., 2006; Dennison, et al., 2007). For tacrolimus, vincristine and sildenafil, polymorphic expression in CYP3A5 have been associated with lower metabolism in individuals expressing CYP3A5*3/*3 (Dennison, et al., 2007; Chandel, et al., 2009; Tirelli, et al., 2008; Ferraresso, et al., 2007; Ku, et al., 2008).

Based on the current literature, and given the major role played by CYP3A in the metabolism of TAM, there is a strong likelihood that changes in CYP3A activity due to genetics or induction has the potential to impact TAM activation/detoxification and its efficacy/toxicity in vivo. Therefore, the current study was undertaken a) to determine the relative contribution of CYP3A4/5 and to assess the impact of CYP3A5 polymorphic expression to the formation of α-OHT and N-DMT, b) to evaluate the extent to which TAM induces CYP3A in women initiating TAM therapy using MDZ as probe substrate, and c) to conduct preliminary assessment on the impact of genetic variability on CYP3A induction by TAM.
5.1. Contribution of CYP3A4/5 and the impact of its polymorphic expression to the formation of α-OHT and N-DMT

Employing cDNA expressed CYP3A4 and CYP3A5, we demonstrated that the formation of α-OHT is primarily mediated by CYP3A4. The intrinsic formation clearance (Vmax/Km) of α-OHT was 6 fold higher with rCYP3A4 than rCYP3A5 (3.23 vs 0.65 µl/min/pmol P450) indicating the limited contribution by CYP3A5. This is further substantiated by the lack of difference in Vmax of α-OHT between microsomes genotyped for CYP3A5*1/*1, CYP3A5*1/*3 and CYP3A5*3/*3. Furthermore, we observed significant correlations between Vmax and CYP3A4 protein content and, Vmax and CYP3A4 activity (testosterone 6-β-hydroxylation) implying that the formation of α-OHT is principally catalyzed by CYP3A4. This is also supported by the poor correlation between Vmax and CYP3A5 protein content. Previous studies have investigated the involvement of CYP3A4 in α-OHT of TAM (Crewe, et al., 1997; Notley, et al., 2005; Desta, et al., 2004). However, the kinetic parameters for the formation of α-OHT and the role of polymorphic CYP3A5 on α-OHT have not been reported. Moreover, the TAM concentrations (18 and 250 µM) used by Notley et al are significantly higher than the reported plasma concentration of TAM (0.1-1µM). In our studies, the concentration of TAM used ranges from 0-50 µM simulating the plasma and hepatic levels.

Since CYP3A4 is found to be the major enzyme involved in the formation of α-OHT, polymorphisms in CYP3A4 may provide an explanation to why only some patients develop endometrial cancer. Recently, a novel intronic variant was identified in CYP3A4 by Wolfgang Sadee, Ph.D, at the Ohio State University (personal communication). This
variant results in decreased mRNA expression and has been associated with reduced statin maintenance dose. Using human liver microsomes genotyped for the novel variant, we investigated its impact on the intrinsic formation clearance of α-OHT. Though there was no significant difference in CLint between the wild type and variant microsomes, a trend towards lower Vmax was observed in the microsomes genotyped for the intronic variant. A similar observation was seen for N-DMT. Given the small sample size (n=3), we did not see a statistically significant difference between the genotypes and warrants further investigation.

In the case of N-DMT, the formation intrinsic clearance (CLint) by recombinant CYP3A4 and CYP3A5 were similar (0.57 vs 0.35 ml/min/pmol P450). This implies that both CYP3A4 and CYP3A5 play an equal role in the formation of N-DMT. In addition, a threefold difference was observed in the Vmax of N-DMT between CYP3A5*1/*1 and CYP3A5*1/*3 or CYP3A5*3/*3 genotyped microsomes suggesting that CYP3A5 variants may contribute to the variability observed in TAM therapy. Furthermore, a significant correlation was observed between Vmax and CYP3A4 (r=0.56, p=0.04), and CYP3A5 (r=0.81, p<0.0001) protein content. This data, taken together with the significant association between Vmax and CYP3A activity suggests that polymorphisms in CYP3A5 may contribute to the variability in plasma N-DMT levels. Since N-DMT is present in higher concentrations in plasma and is the precursor to the active metabolite endoxifen, polymorphic expression of CYP3A5 may play an important role in TAM therapy. We then assessed the effect of other variants, CYP3A5*8 and CYP3A5*9 on the formation of N-DMT using cDNA expressed isoforms. A 50% reduction in CLint was
observed with rCYP3A5*8 in comparison to the wildtype rCYP3A5 allele. In fact, Lee et al had reported ~90% reduction in CLint of testosterone and nifedipine oxidation with this particular variant (Lee et al 2003). Overall, there have been some conflicting results on the influence of CYP3A5 polymorphisms on the pharmacokinetics of TAM. Jin et al., reported that individuals with CYP3A5*1/*1 alleles had higher plasma levels of endoxifen than those with CYP3A5*3/*3 alleles (Jin, et al., 2005). On the other hand, Tucker et al and Goetz et al., showed that there was no influence of CYP3A5 polymorphisms on TAM metabolism or overall survival (Tucker, et al., 2005). In another study, Wegman et al., reported that patients with CYP3A5*3/*3 polymorphism had increased risk of recurrence after two years of TAM treatment (Wegman, et al., 2007). Using recombinant CYP3A and a panel of genotyped microsomes, our systematic in vitro study provides evidence to the impact of CYP3A5 polymorphic expression on the formation of N-DMT.

5.2. Clinical Assessment of CYP3A induction by Tamoxifen

Based on our in vitro findings that TAM and 4-OHT induce CYP3A4 in primary human hepatocytes, we undertook clinical assessment of CYP3A induction by TAM in breast cancer patients using MDZ as the probe substrate. Our findings are the first report on the induction of CYP3A by TAM in clinical settings. In 6 out of 13 patients, we observed a mean increase in oral midazolam CL/F of 70% (range 21-161%) between Day 0 and Day 42. This increase in CL/F observed in 6 patients is similar to the reported percent increase seen with induction of CYP3A by St. Johns Wort as (see table 14 below). A corresponding decrease in AUC and half life by ~30% was observed in these patients.
Such an induction would result in higher levels of α-OHT, increasing the risk of endometrial toxicity in patients undergoing TAM therapy.

Table 14. Percent change in midazolam oral clearance due to CYP3A induction by St. John’s Wort

<table>
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<th>Inducer</th>
<th>Midazolam Dose</th>
<th>Population</th>
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<td>St. John’s Wort</td>
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<td>N=12, Men, 20-33 yrs</td>
<td>33 % ↑</td>
<td>(Imai, et al., 2008)</td>
</tr>
<tr>
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<td>4 mg</td>
<td>N=21, 52% Female, 20-55 yrs</td>
<td>168 % ↑</td>
<td>(Dresser, et al., 2003)</td>
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<tr>
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<td>5 mg</td>
<td>N=12, Female, 20-34 yrs</td>
<td>53% ↑</td>
<td>(Hall, et al., 2003)</td>
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<td>5 mg</td>
<td>N=12, 42% Female, 23-35 yrs</td>
<td>109% ↑/50%↓</td>
<td>(Wang, et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>5 mg</td>
<td>N=30, 26% Female, 19-51 yrs</td>
<td>56 % ↑</td>
<td>(Xie, et al., 2005)</td>
</tr>
</tbody>
</table>

Clearly, our data strongly underscores the hypothesis that TAM therapy leads to induction of CYP3A in some patients, and it also suggests that there is extensive variability in the extent of induction. Other factors that may confound the observed induction include diet, disease condition, CYP3A inhibitory effect of TAM and the fact that midazolam may be a P-glycoprotein substrate. The dietary effect was minimized by restricting patients from consuming products containing St. John’s Wort (CYP3A inducer) for the entire duration of study. Smoking and drinking alcohol were also restricted for at least one day prior to their hospital visit and until last blood sample collection. TAM is reported to inhibit CYP3A in human liver microsomes, but there are no reports on clinical drug - drug interactions with other CYP3A substrates (Zhou, et al., 2005; Zhao, et al., 2002). In our study, we observed a marginal increase in Cmax and AUC of MDZ on Day 1 after a single dose of TAM. As seen in table 12, this does not change our interpretation because the mean percent changes in MDZ pharmacokinetics are similar. For eg, mean percent increase in CL/F from Day1 to Day 42 (71%) is similar to that observed between Day 0 to
Day 42 (70%). Another confounding issue may be induction of P-glycoprotein and/or CYP3A in the gut, which may influence MDZ pharmacokinetics. However, based on our preliminary studies of TAM effects of intestinal CYP3A or P-gp and known mechanism of MDZ absorption, this is unlikely.

The major limitation of our clinical study is the small sample size of 13 patients. Over the last few years, there has been a shift in the standard of care with many patients opting for AIs like letrozole and anastrozole. This has resulted in a slow accrual rate and a decline in availability of the patients willing to volunteer for the study. The study is still ongoing with the objective of meeting the estimated sample size of 18.

5.3. **Influence of CYP3A and PXR polymorphisms on CYP3A induction by Tamoxifen**

We then investigated the genetic basis for the above indicated differences in CYP3A induction by genotyping the blood samples for polymorphisms in CYP3A and PXR using TaqMan SNP genotyping assays. Association of genotypes with fold change in midazolam clearance suggested a trend towards higher induction in individuals with CYP3A5*1/*3 vs. CYP3A5*3/*3 genotypes. One individual with maximum fold increase in midazolam CL/F was also homozygous to C and T alleles of PXR -24381A>C and -25385C>T variants, respectively. This is in agreement with the previously reported observation that individuals with -24381CC variant have higher CYP3A4 mRNA levels (King, et al., 2007). No association was observed between CYP3A4*1B, PXR 63396T>C variants and fold increase in midazolam clearance. Furthermore, we observed no relationship between plasma α-OHT (ng/ml) and CYP3A5 genotypes, supporting our *in vitro* findings that
CYP3A5 has a limited role in the formation of α-OHT. As mentioned previously, the main drawback for our analysis is the small sample size.
6. CHAPTER SIX: CONCLUSIONS AND FUTURE DIRECTIONS

This doctoral dissertation research has provided novel insights on genetic contribution to the formation of CYP3A derived metabolites such as N-DMT and α-OHT, and to the inter-subject variability in CYP3A inductive effects of TAM. Firstly, employing cDNA expressed CYP3A and human liver microsomes genotyped for CYP3A5, we have shown that the formation of α-OHT is primarily mediated by CYP3A4 with a limited contribution by CYP3A5. Furthermore, with the growing recognition on the importance of CYP3A5 polymorphisms in the metabolism of CYP3A substrates, our in vitro data demonstrates that individuals expressing CYP3A5*1/*1 alleles may have higher levels of N-DMT than the non carriers (CYP3A5*3/*3). This result supports the observation that polymorphic expression of CYP3A5 may contribute to the observed variability in CYP3A expression (Huang, et al., 2004; Dai, et al., 2006; Kamdem, et al., 2004).

Though the plasma concentrations of N-DMT at steady state are 1.5-2 fold higher than the parent compound, its ER binding affinity is lower than TAM (Morello, et al., 2003). Furthermore, as N-DMT is metabolized by CYP2D6 to a recently characterized active metabolite endoxifen, inter-individual variability in plasma levels of N-DMT may contribute to the differences in efficacy and safety observed after TAM therapy. A prospective controlled clinical study to assess the impact of CYP3A5 polymorphisms on TAM pharmacokinetics will aid in translating our in vitro findings to clinical situation. A positive association in clinical studies may facilitate recommendations to prospectively genotype patients for CYP3A5 polymorphisms, in addition to the current proposal for identifying individuals based on CYP2D6 genotype prior to initiation of TAM therapy.
In the clinical pharmacology study, we observed a significant CYP3A induction by TAM in 6 patients with an average of 70% increase in CL/F and approximately 30% decrease in AUC and half life of MDZ. Our data provides evidence that TAM induces CYP3A supporting the previously observed drug interactions of TAM with AIs like letrozole and anastrozole. Furthermore, this may aid in devising strategies to circumvent such interactions. For instance, aromatase inhibitors or other co-administered drugs that are not CYP3A substrates may be used in preference to those that are metabolized by CYP3A. In addition, based on the observations by Kisanga et al., that TAM treatment may be improved by administering lower doses (1 mg or 5 mg daily instead of the current regimen of 20 mg) and that dose dependent CYP3A induction was noted in primary human hepatocytes, smaller doses may be advocated to reduce side effects and induction mediated drug interactions (Kisanga, et al., 2004;Sane, et al., 2008).

In light of our evidence that TAM induces CYP3A, the possibility that increased levels of genotoxic TAM metabolites can contribute to endometrial toxicity should be considered. In fact, endometrial tissues have been reported to express CYP3A4/5/7 and PXR, which participate in the biotransformation of TAM to α-OHT and N-DMT (Hukkanen, et al., 1998;Sharma, et al., 2003;Masuyama, et al., 2003). Besides, the concentrations of TAM and N-DMT have been reported to be 22-400 fold, and 5-11 folds higher in endometrium, and breast tissue, respectively than in plasma (Giorda, et al., 2000;Kisanga, et al., 2004). Such high concentrations of TAM may lead to greater production of α-OHT and increase the risk of toxicity in endometrium and breast tissues. The next steps based on the results of our studies would be to assess the potential of TAM to activate PXR and induce CYP3A in
endometrial and breast tissues. Such mechanistic studies will aid in confirming the role of genotoxic α-OHT in mediating endometrial cancer.

Moreover, preliminary examination on the impact of genetic polymorphisms revealed that CYP3A5 carriers may have higher propensity to undergo TAM mediated CYP3A induction than individuals expressing CYP3A5*3/*3. Based on the findings of genetic influence of CYP3A/PXR on the CYP3A induction, prospectively designed larger studies can be undertaken to establish a confirmatory link between these factors and the risk for drug interactions. Alternatively, in vitro studies employing hepatocytes genotyped for PXR variants can be used to delineate the genetic influence on induction of CYP3A by TAM, in place of a clinical trial. Such in vitro studies will provide insights on the effect of PXR polymorphisms, prior to embarking on a larger scale clinical study. Furthermore, such studies will aid in identifying individuals such as those that have higher CYP3A/PXR activity and are likely to have higher exposure to α-OHT or N-DMT.

Overall, our in vitro and clinical studies provide insights on genetic contribution to the formation of CYP3A derived metabolites such as α-OHT and N-DMT, and to the inter-subject variability in CYP3A inductive effects of TAM. Using genotyped human liver microsomes and cDNA expressing CYP3A isoforms, we demonstrated that a) formation of α-OHT is not impacted by CYP3A5 genotypes and, b) formation of N-DMT was dependent on CYP3A5 genotype, with a threefold higher Vmax in individuals expressing CYP3A5*1/*1 vs CYP3A5*3/*3 variants. Clinical assessment of CYP3A induction by TAM demonstrated that 6 out of 13 patients had a mean 70% increase in MDZ clearance,
with an extensive variability observed in the induction (range: 26-161%). Furthermore, individuals expressing CYP3A5*1/*3 had a trend towards higher induction than those carrying CYP3A5*3/*3. One patient with the maximal induction (2.5 fold) was also homozygous to TT and CC variants of PXR (-25385C>T and -24381 A>C). No association was found with CYP3A4*1B, PXR 63396 C>T and CYP3A induction. These findings form a basis for future undertakings that include assessment of CYP3A induction in breast and endometrial tissues, evaluating the association of PXR variants on CYP3A induction by TAM employing hepatocytes genotyped for specific variants, and confirming these results in a prospective clinical study. Ultimately, these studies will assist in optimizing TAM therapy by minimizing the adverse events and maximizing the therapeutic benefits.
REFERENCES


