INVESTIGATIONS ON TWO MAJOR FACTORS INVOLVED IN DRUG METABOLISM: CYTOCHROME P450 2D6 AND P-GLYCOPROTEIN

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Ph.D. Thesis

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LIST OF ABBREVIATIONS

ADR	Adverse drug reaction
cDNA	Complementary dezoxyribonucleic acid
CYP	Cytochrome
CV	Coefficient of variation
Δ2D6	Delta 2D6
DNA	Dezoxyribonucleic acid
dATP	Deoxy adenosine triphosphate
dNTP	Deoxynucleotidetriphosphate
EDTA	Ethylene diamine tetraacetic acid
EM	Extensive metabolizer
GUS	Beta-glucurnidase
HIV	Human immunodeficienty virus
IM	Intermendiate metaboliser
IS	Internal standard
Km	Michaelis-Menten constant
KPi	Potassium phosphate buffer
LC-MS	Liquid chromatography mass spectrography
MDR1	Multidrug resistance 1
mRNA	Messenger ribonucleic acid
NADPH	Nicotineamide adenine dinucleotide phosphate
NASH	Non-alcoholic steatohepatitis
PCR	Polymerase chain reaction
Pgp	P-glycoprotein

PM	Poor metabolizer
Q-RT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SNP	Single nucleotide polymorphism
Vmax	Velocity at maximal concentration if substrate

1. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Drug metabolism – general aspects

The biotransformation of certain drugs is classically delineated as occurring in two distinct enzymatic phases, termed Phase I and Phase II. Phase I biotransformation, in general, leads to a loss of pharmacologic activity of a xenobiotic via an oxidative or reductive reaction. Prodrugs, drugs that first must be activated through a metabolic or biochemical event to produce pharmacologically active entities, are clearly an exception to this observation. Nonetheless, Phase I biotransformation reactions can play a crucial role in drug detoxification.

While more and more effective drugs are available on the market, the incidence of adverse drug reactions (ADRs) was found to be extremely high; over 100,000 hospital patients died in 1994 due to ADRs according to the most recent statistics available. Furthermore, over 2,000,000 hospital patients experienced a serious, though non-fatal, ADRs in that same year. The fatality statistic placed ADRs between the 4^{th} and 6^{th} leading cause of death in 1994 depending on the confidence interval utilized. Using the more conservative estimate, ADRs were ranked ahead of even pneumonia and diabetes as cause of death[1]. While these findings are in need of validation, it remains that decreasing the incidence of ADRs could have a significant impact on healthcare world-wide.

In order to prevent or decrease drug-induced adverse events from occurring, it is critical to fully understand the underlying principle and mechanisms leading to ADRs. While the exact percentage of contribution remains unknown, idiosyncratic drug reactions due to genetic causes are a significant factor contributing to the overall occurrence of ADRs. Bearing this in mind, it is the goal of this body of work to

advance the current state of knowledge of interindividual variability in drug metabolism. Specifically, the overall aim of this thesis is to elucidate underlying mechanisms and determinants in genetic variations that lead to inter-subject variability for a key drug metabolizing enzyme, the cytochrome P450 2D6, which is involved in the metabolism of drugs belonging to many therapeutic classs; and also to provide some additional information about the genetic background of another crucial drug mediator efflux pump, the P-glycoprotein (Pgp).

1.2 The cytochrome P450 enzyme superfamily

The majority of Phase I reactions are accomplished via a superfamily of enzymes termed Cytochrome P450 (CYPs). CYPs are monooxygenazes and heme-containing enzymes that are widely distributed in a large number of human tissues including liver, gastrointestinal tract, lung, brain and kidney. These membrane-bound enzymes have been further localized to the luminal surface of the smooth endoplasmic reticulum in close proximity to NADPH Cytochrome P450 reductase. So far there have been near 50 gene families identified within the Cytochrome P450 superfamily[2]. Distinction is drawn based on amino acid sequence homology such that gene families share greater than 40% identity. There are four known human CYP450 enzyme families, and they account for the vast majority of Phase I xenobiotic metabolism. The subgroups of these cytochromes are marked with further letters and numbers (e.g. CYP2E1). There is a wide and occasionally unpredictable variability in both Phase I and II drug metabolism capabilities in humans. The vast majority of the biotransformation of xenobiotics is accomplished by the CYP3A4, CYP2D6, CYP2C9, CYP2C19 cytochrome P450 enzymes[3].

Quantitatively, the CYP3A subfamily is the most abundant in humans and metabolizes over 50% of therapeutic agents[4]. In humans, the CYP3A subfamily is further subdivided into several isoforms including, but not limited to, CYP3A4, CYP3A5 and CYP3A7. These isoforms are primarily expressed hepatically and constitute approximately 30% of P450 content in the liver[5]. Furthermore, it has been suggested that CYP3A4 is responsible for the metabolism of greater than 50% of therapeutic drugs[4]. This enzyme subfamily exhibits a great deal of interindividual variability, both in expression and activity. Recently, it has become clear that the 3A4 and 3A5 isoforms exhibit genetic polymorphisms in the promoter regions that may largely contribute to the aforementioned variability[6-8].

The CYP2C subfamily is the second most abundant P450 expressed in humans and comprises approximately 20% of hepatic P450[5]. In humans, this subfamily is divided into the 2C8, 2C9, 2C18, 2C19 subfamilies. While abundant hepatically, these isoforms do not contribute significantly to overall Phase I biotransformation reactions. In fact, taken together, this family participates in less than 20% of these reactions[9].

1.3 Interindividual Variability in Drug Metabolism

It has long been recognized that humans exhibit wide, and occasionally unpredictable, variability in both Phase I and II drug metabolism capabilities. As stated earlier, the goal of clinicians and researchers alike is to both elucidate the causes of adverse drug reactions due to interindividual variability and to avoid potentially negative therapeutic outcomes. Sources of variability are diverse and numerous and can be roughly divided into three categories: environmental, physiological, and genetic.

1.3.1 Environmental Factors

Environmental sources of variability are diverse, often difficult to predict, and can include factors such as diet, chemical exposure, and ethanol consumption. For instance, there have been numerous studies investigating the effects of grapefruit juice consumption on CYP3A4. Specifically, it is now widely recognized that grapefruit juice can profoundly inhibit CYP3A4, especially in the gastrointestinal tract, resulting in enhanced absorption of CYP3A4 substrates with potentially serious clinical consequences[10]. Furthermore, cigarette smoking is included in environmental exposures known to result in interindividual variability in drug metabolism. More specifically, the chemical constituents of cigarette smoke have been extensively studied and nicotine has been implicated in the induction of hepatic metabolism[11]. Induction of CYP2E1 by nicotine was recently described by Howard *et al.* resulting in enhanced hepatic chlorzoxazone metabolism[12].

1.3.2 Physiologic Factors

Physiologic variables leading to interindividual variability in drug metabolism are likewise numerous and diverse. Factors such as age, gender, and disease status are included among these.

1.3.2.1 Age Related Changes in P450s

The ontogeny studies of both Phase I and Phase II have shown that the expression of different enzymes varies with age. For example, CYP3A7 is a human

isoform that is expressed only in the fetus and not in the adult human. Conversely, CYP3A4/5 are not expressed in the fetus and only appear after birth. Studies have also indicated that some P450 isoforms decline in content with age[13]. This is also accompanied by an age-related decline in both liver mass and blood flow. Together, these factors result in a diminished capacity for drug metabolism in the elderly that can be further complicated by a concomitant decline in renal function. Consequently, the elderly population, often required to take multiple medications, are at an even higher risk of developing ADRs due to diminished P450 related metabolism and renal clearance.

1.3.2.2 Gender Differences in Drug Metabolism

The effects of gender on drug metabolism are somewhat less well understood. Specifically, observed gender differences in drug metabolism generally do not approach clinical or statistical significance[14]. However, there are a few notable exceptions to this observation with CYP3A4 substrates appearing to undergo more rapid metabolism in females relative to males. This is thought to be due to several factors including differences in steroid hormone regulation[4]. The limited evidence on gender differences in drug metabolism suggests that males metabolize therapeutic agents more rapidly than females[14]. These data, however, should be interpreted with caution as sex-specific factors such as pregnancy, hormonal regulation of gene expression, and menopause may affect the observed differences.

1.3.2.3 Cytochromes P450 and Liver Disease

There are a number of reports in the literature describing the effect of liver disease on Cytochrome P450 expression and activity[15-18]. The overall finding from these studies is that the effect of disease on enzyme function is both disease and

isoform specific. For instance, in a study with human volunteers having mild to moderate liver disease based on Pugh score and Child class (n = 18), Addedoyin *et al.* found that CYP2C19 activity was severely impaired while CYP2D6 remained unaffected. In another study, George *et al.* analyzed the P450 content and activity in explanted livers with end-stage cirrhosis, both with and without cholestasis. These authors found that, in these diseased livers, compared to normal controls, total P450 content was significantly reduced by approximately one-half (0.45 compared to 0.23 mg/ml microsomal protein). However, the effect on individual isoforms was less well pronounced. Specifically, CYP3A content and activity was diminished in only those patients without cholestatic disease, those with cholestasis were unchanged. CYPs 2E1 and 2C, on the other hand, were diminished in those patients with cholestasis but unaffected in others. CYPs 1A1 and 1A2 were decreased in all patients. In a later review article, the authors concluded "only patients with severe liver disease, that it, cirrhosis or severe hepatitis with liver failure have significant impairment with cytochrome P450 expression"[18]. Others have shown, however, that CYP2E1 was increased in patients with non-alcoholic steatohepatitis[19]. Thus, effects of disease on Phase I drug metabolism is clearly specific to the disease and individual P450 isoforms.

1.4 The cytochrome P450 2D6 enzyme

The CYP2D subfamily comprises only 2% of human hepatic P450 but is a very important contributor with respect to Phase I drug metabolism. Indeed, cytochrome P450 2D6 (CYP2D6) metabolizes more than 25% of all currently prescribed medications which are metabolically cleared from the body[20]. These drugs

belong to a number of therapeutic classes including antiarrhythmics, tricyclic antidepressants, serotonin-selective reuptake inhibitors, and opioid analgesics. Among the cytochromes P450, CYP2D6 exhibits the greatest degree of genetic polymorphisms and has, consequently, wide inter-individual variability in both expression and function[21]. Over 75 allelic variants have been described to date; however, only a fraction of variants exhibit total loss of enzyme activity. About 3-10% of the Caucasian population exhibit low CYP2D6 activity, and are "poor metabolizers" (PMs)[22]. Individuals presenting normal CYP2D6 activity are labeled "intermediate" or "extensive" metabolizers (IMs or EMs, respectively). PMs may have unpleasant side-effects caused by inappropriate drug levels due to slow rates of metabolic clearance of CYP2D6 substrates. They also may experience therapeutic failure of CYP2D6 substrates that are pharmacologically inactive until they are converted to the active metabolites. Therefore, it is important to identify the PM subjects prior to administration of potentially toxic therapeutic agents. The genetic basis of the PM phenotype has been well studied; over 50 different mutations in the gene were found. However, more than 98% of the PM genotype can be accounted for five point-mutations and the deletion of the entire gene[23]. Stüven et al. employed a multiplex allele-specific PCR-based genotyping assay to simultaneously detect both mutant and wild-type alleles at five of these loci[24]. This assay, however, did not detect the deletion mutation, which is responsible for about 17% of the PM genotype. A rapid and comprehensive method to detect the deletion mutation and the five most common point mutations was published recently[25].

While advances in genotyping are ongoing, it is becoming clear that genotyping alone may not quantitatively predict phenotype[26]. Due to the number of mutations that commonly occur in the CYP2D6 gene and the complex nature of gene

regulation, the relationship between genotype and phenotype is not clearly established. Therefore, using genotype to predict of an individual's hepatic CYP2D6 function is hindered and the identification of IMs and PMs to prevent ADRs is restricted.

Because genotyping alone could not provide complete prediction of the CYP2D6 phenotype in humans, mRNA transcript levels have been explored as a predictive marker for enzyme activity. While the relationship between CYP2D6 mRNA transcript levels and functional enzyme activity in liver tissues has been investigated, mRNA analysis with RT-PCR designed to detect only short sequences (233 and 289 bp) of CYP2D6 have yielded inconsistent results[27, 28]. Rodriquez-Antona *et al.* found a modest correlation between the amounts of mRNA detected in 12 liver biopsy samples to dextromethorphan *O*-demethylation in the same samples, with an r value of 0.61, while the study of Andersen *et al*. with 8 human liver RNA samples found no correlation to a functional enzyme measure[28]. The contradictory findings may be related to the technical shortcoming of short fragment RT-PCR, namely that the amplicons do not represent full-length transcripts. Alternately, there may be a heretofore unrecognized post-transcriptional regulation of CYP2D6 that introduces inter-subject variability in the transcript-protein relationship.

A highly sensitive assay designed to estimate full-length CYP2D6 mRNA could allow closer estimation of tissue and cellular concentrations of CYP2D6 mRNA capable of translation into functional enzymes. This type of assay would significantly improve our ability to study the variations in expression of the highly polymorphic CYP2D6 gene in human populations. Therefore, we have developed and characterized a semi-quantitative RT-PCR assay capable of sensitively detecting the full-length 1.5 kb transcript of CYP2D6. We have designed primers that can

simultaneously reverse transcribe and produce cDNA from both tissue RNA samples and an internal standard thereby allowing estimation of CYP2D6 RNA transcript concentration. This strategy may reduce variations in amplification efficiencies that often complicate quantitative RT-PCR assays designed to detect short-sequences[28, 29]. Lastly, the resultant full-length cDNA may be subjected to further sequencing reactions to detect novel sequence variants.

To fully characterize the relationship of genotype, mRNA transcript levels, and protein expression or enzyme activity, evaluation of all three parameters in the same set of liver tissues is required. Although human lymphocytes have been proposed and evaluated as a possible surrogate tissue, detectable CYP2D6 enzyme activity has not been reported. While human liver tissues are available from several clinical sources for investigational use, including livers from organ transplant donors and remnant tissue from surgical or needle biopsies, has some limitations. Livers from organ donors are the most readily available source, but may suffer from biochemical perturbation due to pathophysiology of the brain-dead donor or tissue ischemia during organ harvest and preservation. While biopsy samples avoid many of these problems, the amount of liver tissue available is very limited. In our experience, percutaneous liver biopsies are typically on the order of a few (usually 1-5) milligrams. Total protein recovered following tissue homogenization is generally less than 20% of the biopsy mass; microsomal protein recovery is expected to be even less. Therefore, only around one mg of total protein is available from a liver needle biopsy for multiple analyses.

While recent advances in PCR-based genotyping and mRNA transcript analyses can accommodate the use of one mg of tissue, measurement of enzyme activity on such limited sample size has been a formidable challenge. While

individuals can be genotyped to distinguish poor, extensive, and ultra-rapid metabolizers, precise assessment of CYP2D6-dependent metabolic clearance, particularly within the extensive metabolizers, continues to require administration of a probe drug. Phenotyping with a validated probe drug is expensive, time consuming, and not always available or appropriate in clinical settings. Therefore, a rapid and sensitive surrogate assay that can predict the phenotype of CYP2D6 will significantly improve our ability to individualize drug management according to the CYP2D6 genetic polymorphism.

The applicability of commonly employed CYP2D6 probes, including dextromethorphan, bufuralol, and metoprolol[30-33], is limited by the relatively large amount of liver proteins required to detect product formation with these traditional substrates. For example, the well-established CYP2D6 probe, dextromethorphan which is *O*-demethylated to dextrorphan, exhibits a modest turnover of about 1 min^{-1} and intrinsic clearance of 60-250 μl/min/mg [K_m of 1-4 μM, and V_{max} ~ 250 pmol/min/mg][32, 33]. As a result, *in vitro* assays with dextromethophan require a prohibitive amount of liver homogenate or microsomal protein relative to the size of typical liver biopsy samples. A very high turnover substrate is needed to achieve sufficient sensitivity for measurement of CYP2D6 activity in liver biopsy samples.

The present series of investigations discovered that R-568, a first generation calcimimetic[34, 35], is a very high turnover, specific substrate of CYP2D6 that fulfills the above criteria. We determined the kinetics and validated the specificity and sensitivity of R-568 *O*-demethylation as a catalytic marker of CYP2D6. We observed an excellent correlation between the *O*-demethylation of dextromethorphan and R-568 (r^2 = 0.94). These data demonstrated the selectivity of CYP2D6 to catalyze R-568, and proved that this assay is significantly more sensitive than the established

dextromethophan reaction. We, therefore, employed R-568 as a CYP2D6 probe substrate for the subsequent study. The detailed data can be found in the published manuscript listed as one of the publications related to this thesis.

With the added capability to analyze full-length mRNA transcript levels and the availability of a highly sensitive probe for CYP2D6 enzyme activity, it became feasible to evaluate the quantitative relationship between CYP2D6 RNA transcript and enzyme activity in limited mass liver samples. Then we characterized the relationship between CYP2D6 genotype, full-length mRNA transcript level, and *in vitro* *O*-demethylation rate of R-568 in livers obtained from several patient populations. Our data indicate that, while the level of full-length CYP2D6 mRNA predicts *in vitro* enzymatic activity of liver tissues within a patient population, the relationship does vary greatly across patient population and/or different method of obtaining the liver.

1.5 The P-glycoprotein

P-glycoprotein (Pgp), a member of the ATP-binding cassette family of membrane transporters, is encoded by the human multidrug-resistance (*MDR1, ABCB1*) gene[36, 37]. This integral membrane protein functions as an energy-dependent drug efflux pump, and reduces the intracellular concentrations of a wide range of drugs and xenobiotics. Comprehensive reviews were published recently on this topic[38, 39]. Pgp exhibits wide substrate specificity for structurally different drugs and, consequently, mediates drug resistance to a variety of drugs, including vinca alkaloids, anthracyclines, epipodophyllotoxins, taxols, actinomycin D, cardiac glycosides, immunosuppressive agents, glucocorticoids and anti-HIV protease inhibitors[40-42]. Since many drugs are substrates of Pgp, its degree of expression and

functionality directly affects the therapeutic effectiveness of these agents. In particular, resistance to chemotherapy has become a major obstacle in anticancer treatment, but the exact regulatory mechanism of the protein expression remains elusive. One of the possible regulatory sequences was identified in the promoter region[43]. Other studies found random chromosomal rearrangements causing hybrid *MDR1* messages and consequent activation of Pgp expression[44].

The *MDR1* gene is located on the long arm of chromosome 7, and consists of a core promoter region and 29 exons. The total gene length was established and reported in 1987 to be at least 70 kilobases[45], and later in 1990, using multidrug-resistant KB-V1 cells, the *MDR1* gene size was estimated to reach more than 100 kb[46]. Although widely investigated, the exact genomic size has remained uncertain for the past 16 years, with several differing entries in the Genbank database. The online databases list *MDR1* gene length to be between 120 kb (http://www.infobiogen.fr) and 210 kb (www.ncbi.nih.gov/IEB/Research/Acembly). The NCBI database (http://www.ncbi.nlm.nih.gov) outlines a 209 kb *MDR1* genome. Another source (www.ensembl.org) enumerates 29 exons within the gene, while Aceview (www.ncbi.nih.gov/IEB/Research/Acembly) computational analysis predicts 29 exons with 18 alternative exons and 32 confirmed introns, 10 of which are alternative. In addition, alignment of the *MDR1* mRNA with the DNA sequence deposited at the Chromosome 7 Sequencing Project (www.chr7.org) reveals 29 discrete matches suggesting that there are 29 *MDR1* exons. While several changes were made in the databank during the last decade, the latest revision available online still lists the total length of *MDR1* as 209 kb (NT007933) and 6326 bp (AH002875, accessed September 2003). The *MDR1* mRNA consists of 4872 bp (NM000927). The reported gene structure is mainly based on very limited cell specimens.

Comparative and validation studies with different human tissues or cells are not yet available in the literature.

2. SPECIFIC AIMS

The aim of the study is to perform investigations on two important factors that have high influence on drug response: one of the most important human cytochrome P450 enzymes, CYP2D6, and an important cellular efflux transporter, the P-glycoprotein.

In order to achieve our goals we sought

1. To develop a new, easy-to-perform analytical method to assess CYP2D6 full-length messenger RNA levels
2. To compare this full-length mRNA method with short-fragment real-time PCR
3. To perform CYP2D6 genotyping for the most common alleles of different groups of human liver samples
4. To determine the CYP2D6 activity of different human liver tissues with a novel CYP2D6 substrate with improved sensitivity
5. To elucidate the relationship of CYP2D6 ribonucleic acid transcript levels, genotype and enzyme activity in human liver biopsy samples
6. To evaluate and validate the overall length of the human MDR1 gene with different human tissues that will be of great utility for further analyses of drug-induced resistance

3. MATERIALS AND METHODS

3.1 Tissue procurement

3.1.1 Procurement of human tissues

All procedures were carried out with approval of the Human Subjects Review Board. These liver samples were collected under four different conditions as described below and grouped accordingly as Control Subjects—Group I, Liver Biopsy Subjects—Group II, Liver Transplant Subjects—Group III, and Liver Bank Subjects—Group IV. Tissue pieces were snap frozen in liquid nitrogen and then stored at -80°C for long-term preservation. The human lymphocytes were isolated from blood obtained from the Blood Bank.

3.1.2 Procurement of human cell lines

Human liver samples used for the *MDR1* gene related experiments were obtained from the University of Washington Solid Organ Transplant Program and harvested from brain-dead donors that were rejected for transplantation mostly due to a high degree of fatty infiltration. The human lymphocytes were isolated from blood obtained from the Blood Bank at the University of Washington. Human cell lines [Messa Dox (ovarian carcinoma), CaCo2 (colon carcinoma) and UW 228 (medulloblastoma)] were purchased from American Type Culture Collection (ATCC) (Manassas, VA).

3.1.3 Preparation of liver samples for assessing enzyme activity

Homogenates of human liver were prepared by first adding ice-cold phosphate buffer (10 mM KPi, 1 mM EDTA, and 250 mM sucrose, pH 7.4), containing the Complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis,

IN), to the tissue. Homogenization was then performed in a tissue grinder with 20 strokes of the ground glass pestle. All steps of this process were conducted on ice. Protein concentration was determined by the method of Lowry et al.[47].

3.2 Determination of CYP2D6 enzyme activity

3.2.1 Procurement of CYP2D6 enzyme substrate

The parent calcimimetic compound (R)-N-(3-methoxy-α-phenylethyl)-3(2'-chloro-phenyl)-1-(propylamine hydrocholoride) (referred to as R-568 or NPS-568) and its O-desmethyl metabolite (M1) were kindly supplied by Amgen Inc (Thousand Oaks, CA). Ethylenediaminetetraacetic acid (EDTA) was obtained from JT Baker (Phillipsburg, NJ). Nicotinamide adenine dinucleotide phosphate (NADPH), dextromethorphan, ammonium acetate, capsaicin and potassium phosphate were obtained from Sigma Chemical Company (St. Louis, MO). Analytical grade methyl tert-butyl ether and acetonitrile were from Fisher Scientific (Fairlawn, NJ). Recombinant human CYPs were obtained from Gentest (Woburn, MA).

3.2.2 Incubation Conditions for CYP2D6 Activity Determination

All *in vitro* incubations with R-568 were conducted in polypropylene tubes in a total volume of 0.5 ml. Incubations were conducted in a buffer composed of 100 mM KPi and 1 mM EDTA. Protein and substrate were pre-incubated for 5 minutes in a 37°C shaking water bath and reactions were initiated by the addition of 1 mM NADPH (final concentration). Reactions were terminated by the addition of 2.0 ml ice-cold methyl tert-butyl ether and the subsequent addition of 20 μl methanol containing 5 ng of capsaicin as the internal standard (IS).

The samples were then placed in a horizontal shaker for 10 minutes followed by centrifugation at 2000 rpm for 10 minutes to separate the organic and aqueous phases. The organic layer was removed to a glass tube and evaporated to dryness under a gentle stream of air. The samples were reconstituted in 100 µl of mobile phase (60% 10 mM ammonium acetate pH 4.0, 40% acetonitrile) and transferred to injection vials for analysis by liquid chromatography-mass spectrometry (LC-MS).

The *in vitro* incubations with dextromethorphan were conducted in polypropylene tubes in a total volume of 0.5 ml. The enzymatic activity of CYP2D6 in the human liver specimens was determined by *O*-demethylation of dextromethorphan to dextrorphan. For each sample, 0.25 mg/ml total protein concentration in 0.5 ml was pre-incubated in the presence of 12 µM dextromethorphan for 5 minutes in a shaking 37°C water bath. Reactions were initiated with the addition of NADPH to a final concentration of 1 mM and terminated after 10 minutes by the addition of 2 ml acetonitrile and immediately placed on ice. Internal standard (16 ng butorphanol) was added followed by centrifugation to remove protein. After reducing the volume of the acetonitrile mixture under a gentle stream of air, samples were injected onto the liquid chromatograph-mass spectrophotometer (LC-MS).

3.2.3 LC-MS Assay conditions

For the determination of CYP2D6 activity with R-568 we used a YMC Basic C-8 column (100 x 2.1 mm) and mobile phase as stated above (60% 10 mM ammonium acetate pH 4.0, 40% acetonitrile) to achieve chromatographic separation. The flow rate was 0.2 ml/min for 5.5 minutes and 0.3 ml/min for the next 4.5 minutes, for a total run time of 10 minutes per sample. The mass spectrometer, an Agilent

Model # G1946B, was operated in the positive ion electrospray mode. The ions monitored and associated retention times were as follows: NPS-1378 (metabolite), *m/z* 290 and 2.9 minutes; R-568 (parent), *m/z* 304, and 4.6 minutes; and capsaicin (IS), *m/z* 306 and 8.7 minutes. Limit of quantitation for both R-568 and NPS-1378 were 15 femtograms per injection. All determinations were performed in triplicate.

Chromatographic separation of dextrorphan and butorphanol was accomplished with a Zorbax C-18 column, and mobile phase consisting of 70% 10 mM ammonium acetate (pH 4.0) and 30% acetonitrile at a flow rate of 0.2 ml/min. The retention times were approximately 4.4 minutes for dextrorphan and 8.3 minutes for butorphanol. The mass spectrometer (Agilent, Palo Alto, CA) was operated in the positive ion electrospray mode, and mass ions at *m/z* ratios of 258 and 328 were monitored that corresponded to the MH^+ dextrorphan and butorphanol, respectively. Again, all determinations were performed in triplicate.

We determined the Michaelis-Menten parameters, K_m and V_{max} for R-568 *O*-demethylation and compared the kinetics of R-568 and dextromethorphan oxidation by CYP2D6, the detailed data and set of results can be found in the publication listed as related to this thesis at the end of this manuscript.

3.2.4 Statistical analysis

In vitro enzymatic activity data were analyzed with a two-tailed t-test assuming unequal variances. A P-value of 0.05 or less was considered statistically significant.

3.3 Genotyping of liver samples

DNA was extracted from liver tissue (control population, liver bank, and transplant subjects) and lymphocytes (liver biopsy subjects) with the use of the

Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia CA) according to the manufacturer's instructions. Genotyping for the most common CYP2D6 variant alleles was carried out according to Scarlett *et al*. [25]. Alleles assessed were *3, *4, *5, *6, *7, and *8. All genotypes, except gene deletion variant *5, were confirmed by direct sequencing of the respective CYP2D6 region with an ABI Prism 377 DNA Sequencer.

3.4 CYP2D6 mRNA Transcript Assessment with a Semi-quantitative RT-PCR Assay

3.4.1 Isolation of messenger ribonucleic acid

Total RNA was isolated from liver samples using the Totally RNA Isolation Kit (Ambion Inc, Austin TX) and quantitated fluorimetrically. The RNA concentration was determined by measuring RNA fluorescence in 0.125 μg/ml ethidium bromide with a spectrofluorometer (Hitachi F4500) set at λ_{ex} = 287nm and λ_{em} = 618nm, using yeast RNA as a standard. RNA was verified to contain similar amounts of β-actin RNA per ng of total RNA by RT-PCR as described[48].

3.4.2 Construction of internal standard RNA

The RT-PCR standard was developed with the use of a CYP2D6 plasmid kindly supplied by Dr. Frank Gonzalez (NIH, Bethesda, MD). This pUC19 plasmid contains the full-length human CYP2D6 cDNA. A 475 bp deletion in the CYP2D6 gene internal to the start and stop codons, was created by digesting the plasmid in the presence of StuI (Promega, Madison WI) according to the manufacturer's instructions. The truncated linear DNA was then ligated to re-form the circular plasmid. The internally deleted gene fragment was subsequently amplified from the

vector via PCR, purified, and cloned into a new vector pSP73 (Promega, Madison, WI). This vector contains the T7 promoter that permits *in vitro* RNA transcription. RNA transcripts of the CYP2D6 gene and internally deleted gene (Δ2D6) were created by linearizing the full-length and truncated pSP73 plasmids followed by *in vitro* RNA transcription with the use of the Stratagene RNA Transcription Kit (Stratagene, La Jolla CA) according to the manufacturer's protocol.

The principle of the construction of internal standard is shown in Figure 1.

RNA concentrations were determined as described above.

3.4.3 RT-PCR Conditions

RT-PCR was accomplished with the use of the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis IN). The assay conditions were identical to those reported previously[49]. The primers, based on the CYP2D6 sequence deposited in Genbank (M33388), were as follows:

Forward primer 5' AGTGGCCATCTTCCTGCTCC 3'

Reverse primer 5' CGGGGCACAGCACAAAGC 3'

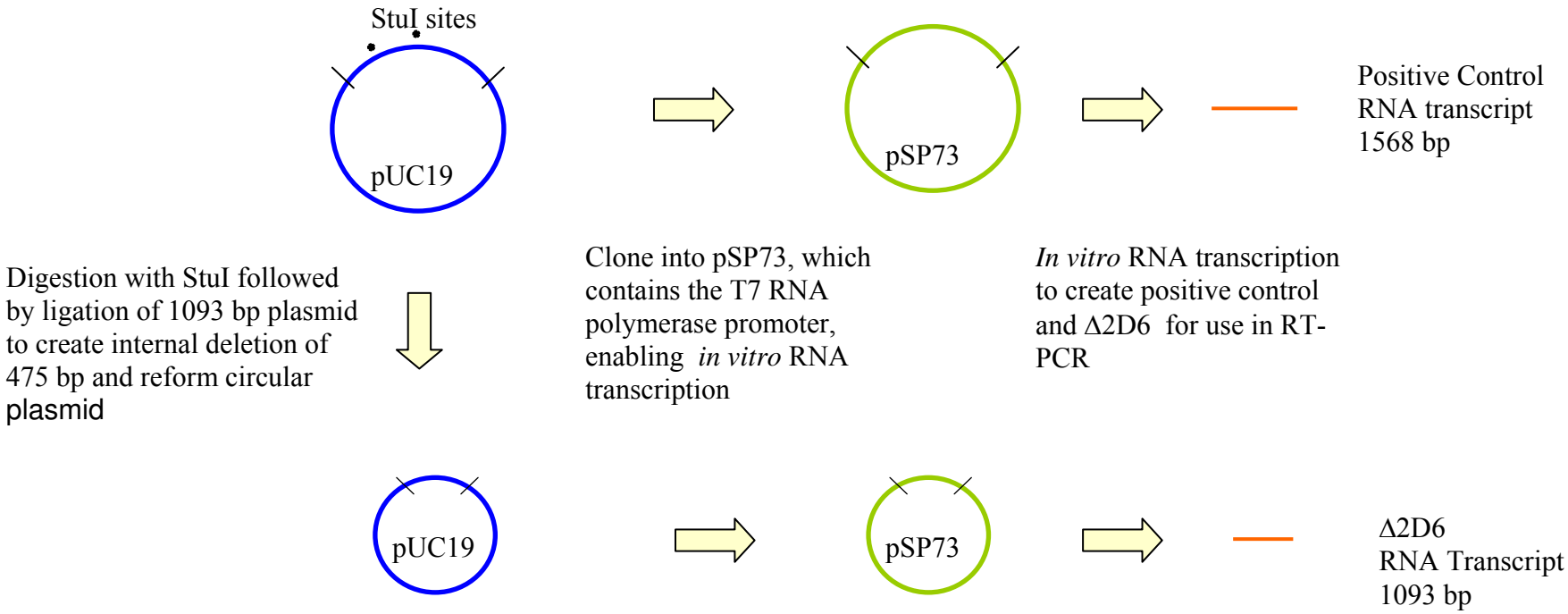

Figure 1. Schematic representation for construction of internal competitor (Δ2D6) and positive control RNA transcripts for subsequent use in RT-PCR.

The forward primer site is 36 bp downstream of the translation start site (5′ position 1655) and the reverse primer site is 1 bp upstream of the translation stop site (3′ position 5815).

To estimate CYP2D6 concentration in each liver sample, total RNA (0.5 to 2.0 μg) was used as template to perform an initial RT-PCR in the absence of the internal control. The RT-PCR products were subjected to 1% agarose gel stained with ethidium bromide, and according to the intensity of the band, we estimated and made serial (3- to 5-fold) dilutions of total RNA. We then used these dilutions in the presence of a fixed copy number of the competitor and determined CYP2D6 copy number. According to the dilution-factor and the concentration of the total RNA, the CYP2D6 RNA in liver tissue was calculated (copies/μg total RNA) using the method described by Albiero *et al*.[50] on a digital image of RT-PCR products captured with a Kodak DC120 camera. The NIH Image 1.63 software program was used to estimate the intensities of RT-PCR products. CYP2D6 RNA copy estimates were obtained from the average of duplicate RT-PCR assays for each test sample after accounting for the differences in lengths of the product and the competitor (1.56 vs. 1.09 kb), similar to that described previously[51].

3.4.4 Quantitation of short RNA transcripts of CYP2D6 with a real-time quantitative RT-PCR method

The conditions and controls for the TaqMan PCR (ABI 7900, Foster City, CA) were similar to those recently described[52]. In brief, samples of total RNA (1 μg) were converted to cDNA using Applied Biosystems Multiscribe reverse transcriptase (Foster City, CA) and full-length 2D6 gene-specific reverse primers. The cDNA synthesis was performed in parallel with random hexamers as well. Following cDNA synthesis, 50

ng of product were analyzed by quantitative PCR utilizing a TaqMan® assay on an ABI 7900HT instrument with the following primers and probe sequences:

Forward primer	5' GGACATCGTCCCCCTGGGTA 3'
Reverse primer	5' CCAGACGGCCTCATCCTTCA 3'
Probe	5'FAM-TCCCTAAGGGAACGACACTCATCACCAAC-TAMRA 3'

The amplified cDNA sequence is 128 bp long and is located 400 bp downstream the 3′ end. For quantitation of transcripts, a standard curve was generated with a full-length synthetic CYP2D6 RNA, with points ranging from 0 to 10^6 molecules. As a quality control measurement, the real-time assay was performed for the beta-glucuronidase (GUS) housekeeping gene as well for the cDNA samples obtained using random hexamers in the reverse transcriptase step. The standards were diluted in a background of yeast total RNA (Ambion, Austin, TX) at the same concentration as test samples and yielded a PCR efficiency of ~100% with a correlation coefficient of 0.99.

3.5 *MDR1* gene experiments

3.5.1 DNA isolation and PCR conditions

Genomic DNA was extracted and purified from the samples using the Puregene DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN). Primers were designed with an online primer design software program (Primer 3) and synthesized by Invitrogen Co. (Carlsbad, CA). The list of primers and their target *MDR1* products are listed in Table 1. In the case of primer sets F27-R27 and F28-R28 the reaction mixtures (50 µl total volume) contained 100 ng genomic DNA, 200 µM each dNTP, 3.0 mM $MgCl_2$, 4.0 units of Taq polymerase (Promega, Madison, WI, USA), 10

mmoles of primers, 5.0 µl Thermo DNA Poly 10X reaction buffer, and nuclease-free H_2O. Thermocycling was conducted in a Perkin Elmer DNA Thermocycler model 2400 with the following profile: initial denaturation at 94°C for 3 minutes immediately followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. In the case of the F19-R19 primer set, designed to produce over 10 kb product, the same amount of genomic DNA was amplified in 2 units of rTth DNA polymerase (Perkin Elmer, Foster City, CA), containing 10 mmoles of primers, 200 µM each dNTP, 15 µl 3.3X XL buffer, 2.4 µl $Mg(OAc)_2$ solution, and nuclease-free H_2O. The long-range PCR was conducted as following: initial denaturation at 94°C for 1 minute followed by 16 cycles of 94°C for 15 seconds, 58°C for 30 seconds and 68°C for 10 minutes. This was immediately followed by 12 cycles of 94°C for 15 seconds, 58°C for 30 seconds and 68°C for 10 minutes with an incremental increase of 15 seconds per cycle. Products were visualized in a 2.0% agarose gel stained with ethidium bromide.

Table 1. Experimental design of PCR and Southern Blot analysis, and the expected products according to the two reference sequences of the *MDR1* gene

Analytical method		Expected product and fragment according to the respective size of *MDR1*	
PCR analysis			
Primers	Primer sequence 5' to 3'	6.3 kb MDR1 length (AH_002875)	209 kb MDR1 length (NT_007933)
F 19 R 19	CCCATCATTGCAATAGCAGG GTTCAAACTTCTGCTCCTGA	190 bp	10573 bp
F 27 R27	TCCAGTTTCCTTTTGGAGGA TGTCGTTTTGTTTCAGGATCA	214 bp	2835 bp
F 28 R 28	TGGTTTCGATGATGGAGTCA AGCTTTCTGTCTTGGGCTTG	285 bp	1112 bp

Southern Blot analysis				
Restriction enzyme pairs	Radiolabeled probe	Target regions		
BamH1/Pac1	P1	Introns 8 and 9-14	6 kb	7kb and 9kb
BamH1/Kpn1	P2	Introns 15-19 and 20-21	5 kb	7kb and 14kb
BamH1/Pac1	P3	Introns 21 and 15-20	6 kb	11kb and 16kb

3.5.2 Southern Blot analysis

For these experiments 10 μg of genomic DNA was digested to completion with the restriction endonuclease pairs *BamHI/PacI* and *BamHI/KpnI* obtained from New England Biolabs (Beverly, MA). The DNA was electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled probes prepared by random primed incorporation of α^{32}P-dATP by Klenow DNA polymerase (New England Biolabs, Beverly, MA). These probes were obtained by first generating three different *MDR1* cDNA regions using primer sets F7 (CTGCTGTCTGGGCAAAGATAC)-R7 (ACAGGTTCCTGACTCACCAC), F28-R29 (CGAGCCTGGTAGTCAATGCT) and F19-R19, designated as P1, P2 and P3, respectively (Figure 2). Probe P1 is labeled as target for introns 8 and 9-14, probe P2 for introns 15-19 and 20-21, and probe P3 for introns 21 and 15-20.

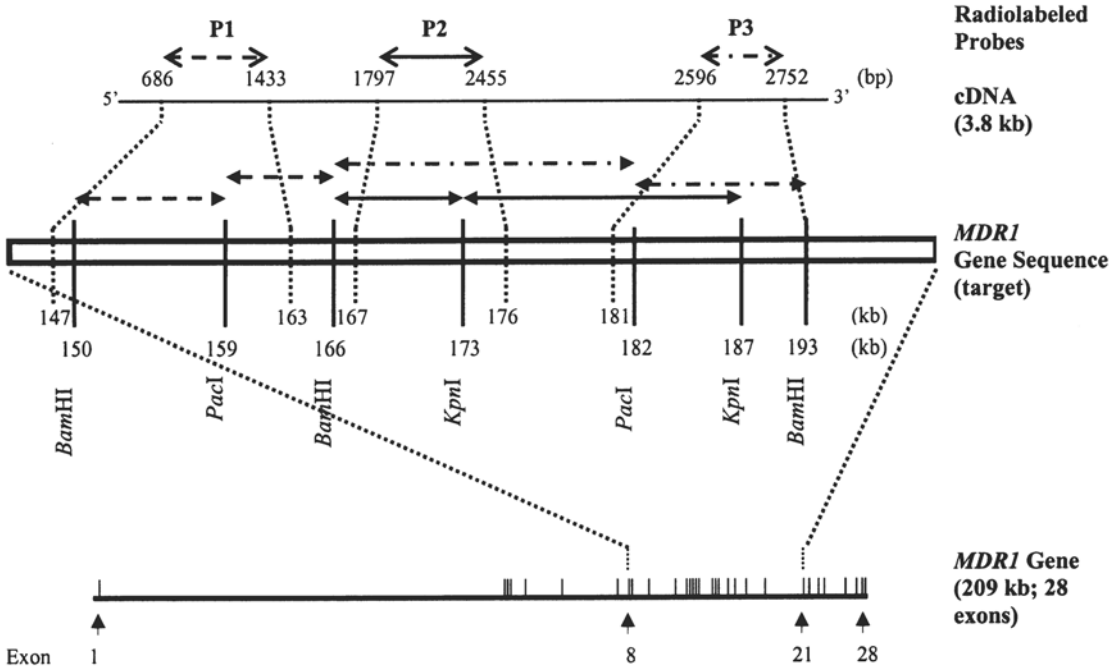

Figure 2. Schematic representation of restriction map based on the estimated 209 kb length and the radiolabeled cDNA fragments used as probes. The corresponding hybridization targets (arrows) are marked. Radiolabeled probes, P1, P2 and P3 were generated with F7-R7, F28-R29 and F19-R19 primer sets (Table 1), respectively.

4. RESULTS

4.1 CYP2D6 mRNA transcript assay

We have successfully constructed a plasmid containing an internally deleted CYP2D6 RNA transcript (Δ2D6). The purified Δ2D6 RNA served as an internal standard allowing reliable quantitation of full-length CYP2D6 mRNA transcripts. We determined the lower limit of detection for the Δ2D6 and CYP2D6 positive controls to be approximately 500 copies (Figure 3). The two RT-PCR products were readily separated by agarose gel electrophoresis due to the 475 bp deletion associated with the Δ2D6 transcript.

Total RNA isolated from human liver samples was subjected to competitive semi-quantitative RT-PCR in the presence of a known copy number of the internal standard. The CYP2D6 mRNA transcripts in these liver samples were estimated using RT-PCR conditions identical to that of the standard Δ2D6. Because the end sequences flanking the 475 bp deletion in the standard are identical to the native full-length CYP2D6 mRNA sequence, amplification efficiencies are highly similar. Comparisons of amplification efficiency of internal controls and endogenous RNA targets have been discussed by others[53, 54], and have been shown to be practically the same for internal deletions of this magnitude. Results from a typical competitive semi-quantitative RT-PCR experiment are shown in Figure 4. The dilution of total RNA which yielded equivalent signals of native CYP2D6 and Δ2D6 internal standard was used to calculate the number of CYP2D6 mRNA transcripts present in the original liver sample. The limit of detection for liver RNA is approximately 2500 copies/μg.

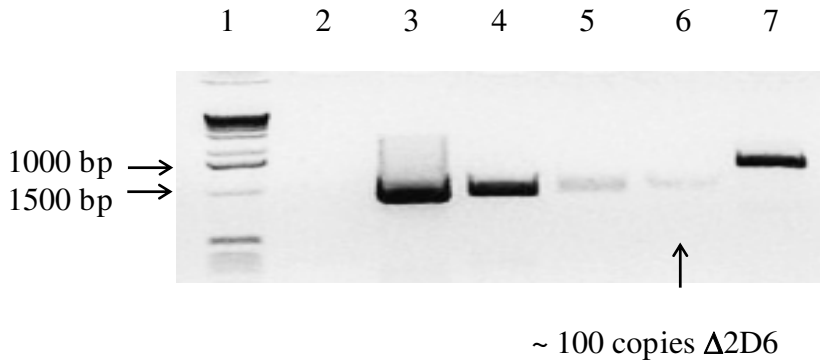

Figure 3. Determination of CYP2D6 detection limit. Serial 10 fold dilutions of the CYP2D6 RNA transcript were subjected to RT-PCR and run on a 2.0% agarose gel. Lane 1 is a 1 Kb ladder, lane 2 is the negative control in which no RNA was added to the RT-PCR reaction. Lanes 3-6 are the dilutions of Δ2D6 and lane 7 is the CYP2D6 full-length positive control.

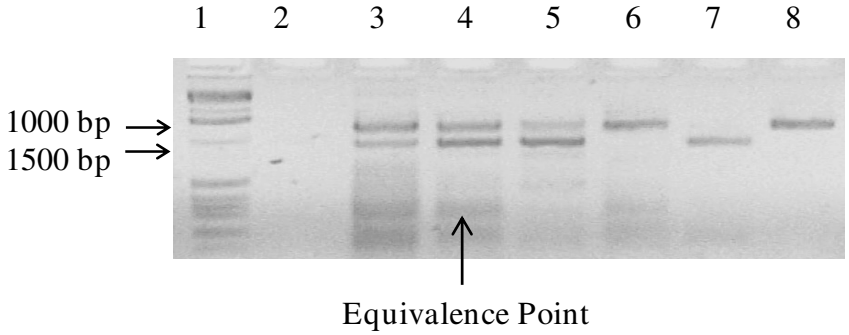

Figure 4. Representative results for RT-PCR amplification of human liver CYP2D6 mRNA, Δ2D6 construct and positive control. Lane 1 contains a 1 kb molecular weight marker, lane 2 is a negative control in which no RNA template was added to the reaction mixture, lanes 3 thru 5 contain the serial dilutions of human liver total RNA and a fixed copy number of the Δ2D6 construct (1.0 kb), lane 6 is human liver total RNA alone, lane 7 is the Δ2D6 construct alone, and lane 8 is the positive control 2D6 construct.

We first tested both the intra- and interday reproducibility in estimating CYP2D6 mRNA levels in human liver. Three different total RNA samples were each subjected to this assay on four replicate days. Results from this experiment are shown in Table 2. This semi-quantitative assay yielded reproducible results with a low average coefficient of variation (CV) of approximately 19%. Furthermore, we

repeated the assay three separate times in one day with total RNA from livers number 18 and 23; thereby testing the intraday variability of this method. Again, the assay was reproducible with an intraday CV of 10% (data not shown).

Results of mRNA copy number and genotype determinations for a small panel of human livers are given in Table 3. Analysis of the expression of the full-length mRNA transcript by RT-PCR revealed that CYP2D6 mRNA copy level in 8 livers ranged from undetectable (<2500 copies/μg total RNA) to 4.8 x 10^7 copies/μg total RNA.

Table 2. Interday variation of CYP2D6 mRNA level estimation.

	CYP2D6 mRNA per μg total RNA (x10^6)			
HL#	Copy #	Mean	S.D.	CV(%)
18	13.39			
	13.39			
	13.39			
	13.39	13.39	0.00	0.00
24	48.00			
	48.00			
	64.00			
	32.00	48.00	13.39	27.22
1	25.23			
	25.23			
	25.23			
	12.61	22.07	6.31	28.58
			average CV%	18.60

We next characterized the relationship between mRNA copy number and CYP2D6 enzymatic activity. We compared the ratio of 2D6 full-length transcript number to activity measured *in vitro* as a preliminary attempt to probe the relationship

between RNA transcript and enzyme activity. Our results presented in Table 3 showed that this ratio is relatively constant (0.59 ± 0.09) for the four wild type livers. In contrast, for the same livers the ratio for short fragment of CYP2D6 by Q-RT-PCR showed high variation (2.79 ± 1.75). The livers with wt/*4 genotype expressed significant levels of full-length mRNA transcript, but lower enzyme activity. This resulted in higher ratios, 2.91 and 1.46 for the two livers. The *4/*5 and wt/*5 livers did not contain any measurable full-length CYP2D6 transcript. However, these livers express short-fragment CYP2D6 detected with Q-RT-PCR.

Table 3. Relationship between CYP2D6 mRNA copy number and enzymatic activity

			Full-length mRNA Transcript Analysis[a]		Short-sequence RNA Transcript Analysis[b]	
Human Liver (ID #)	CYP2D6 genotype	In vitro Enzymatic Activity (pmol/min/mg)$^{\oplus}$	[mRNA]* (copies/µg)x 10^6	RNA transcripts to activity ratio (x 10^6)	[mRNA]* (copies/µg) x 10^3	RNA transcripts to activity ratio (x 10^3)
9	wt/wt	60.29 ± 24.70	36.6 (30-39.9)	0.66	53.08 (48.4-57.2)	0.88
18	wt/wt	19.58 ± 3.44	13.4 (13.4)	0.68	73.72 (58-88.1)	3.76
23	wt/wt	39.58 ± 7.13	21.5 (16.1-24.2)	0.54	186.1(178.2-190)	4.70
16	wt/wt	38.65 ± 0.38	18.7 (14.68-22.7)	0.48	70.01 (68.8-71.2)	1.81
				0.59 ± 0.09		2.79 ± 1.75
24	wt/*4	16.51 ± 3.34	48.0 (32.0-64.0)	2.91	37.15 (35.3-38.7)	2.25
1	wt/*4	15.29 ± 3.12	22.1 (12.6-25.2)	1.46	32.36 (32.1-32.7)	2.17
6	*4/*5	0.97 ± 0.48	BQL^{+}	--	56.94 (55.5-58.7)	58.70
22	wt/*5	0.56 ± 0.69	BQL^{+}	--	33.11 (32.5-33.6)	66.22

[a]Full-length transcript levels of human liver RNA samples were estimated using a semi-quantitative RT-PCR method as described in Materials and Methods.
[b]Short-sequence RNA transcript levels of the same samples were estimated with a real-time quantitative RT-PCR using identical RNA standard.
$^{\oplus}$Determined by dextromethorphan *O*-demethylation. Data represent the mean of at least triplicate measures (± SD).
*mRNA copy # data are reported as mean (range).
$^{+}$BQL indicates less than 2.5 x 10^3 copies CYP2D6 mRNA / µg total RNA under these experimental conditions.

4.2 Genotyping for CYP2D6 of human liver samples

We analyzed each of the human liver samples for its CYP2D6 genotype according to the previously described method [25]. The samples were grouped according to the patient characteristics and how the samples were obtained.

Overall, the subjects ranged in age from 7 to 76 years. There was an approximately even representation of gender across groups, and the majority of subjects were Caucasian (Table 4).

The control group (n=11) was composed mostly of patients being treated for abdominal cancer without hepatic involvement; all had histologically normal livers. These samples were collected during abdominal laparoscopy after obtaining their written consent. Their genotypes are given in Table 5. One subject was homozygous for the *4 allele, two subjects were compound heterozygous with a genotype of *4/*5, and one subject carried one copy of the *4 allele. The remainder carried only functional alleles.

The liver biopsy group was comprised mainly of patients diagnosed with chronic hepatitis C, and all subjects underwent this procedure as part of their clinical care (n=21). It should be noted that recovered biopsy masses were very small for this population with a mean mass of less than 6 mg, only less than 1-2 mg of which was available for experiments described in this report. Six of the subjects were hepatitis C-negative and their liver conditions were primary biliary cirrhosis, non-alcoholic steatohepatitis, or autoimmune cholangitis. A portion of the liver biopsies from all subjects was reserved for pathological analysis. Batts & Ludwig Scores were assigned for the purposes of grading and staging of liver disease[55] (Table 4.).

Table 4. Demographic and diagnosis of subject groups from whom liver tissues were collected

Subject Group and ID	Age	Gender	Ethnicity	Cold ischemia (time in hour)	Liver Disease State[a]		
					Diagnosis	Grade	Stage
CONTROL—Group I (n=11)							
1	57	M	NA[b]	NONE	ND[b]		
2	59	M	NA	NONE	ND		
6	56	F	NA	NONE	ND		
8	64	M	NA	NONE	ND		
13	72	F	NA	NONE	ND		
14	76	M	NA	NONE	ND		
16	59	M	NA	NONE	ND		
19	70	M	NA	NONE	ND		
20	43	M	NA	NONE	ND		
21	72	M	NA	NONE	ND		
24	54	M	NA	NONE	ND		
LIVER BIOPSY—Group II (n=21)							
1	26	M	Caucasian	NONE	NASH[b]	III	I
2	44	M	NR[b]	NONE	Hepatitis C	III	I
3	50	F	Caucasian	NONE	PBC[b]	NR	III
4	54	F	Caucasian	NONE	Hepatitis C	III	II
5	41	M	Caucasian	NONE	Hepatitis C	I	0
6	52	M	Caucasian	NONE	Hepatitis C	III	III
7	43	M	NR	NONE	Hepatitis C	III	III
8	46	M	NR	NONE	Hepatitis C	II	III
9	47	M	NR	NONE	Hepatitis C	III	III
10	52	M	Caucasian	NONE	Hepatitis C	II	II
11	43	F	Caucasian	NONE	Autoimmune cholangitis	NR	III
12	49	M	Caucasian	NONE	Hepatitis C	II	0
13	55	M	Caucasian	NONE	Hepatitis C	II	II
14	48	M	Caucasian	NONE	Hepatitis C	II	0
15	19	F	Hispanic	NONE	Autoimmune hepatitis	0	0
16	42	F	Caucasian	NONE	Autoimmune cholangitis	0	0
17	39	F	Caucasian	NONE	Hepatitis C	NR	NR
18	50	F	Caucasian	NONE	Hepatitis C	II	0
19	50	M	Caucasian	NONE	Autoimmune hepatitis	III	III
20	51	M	Caucasian	NONE	Hepatitis C	I	I
21	51	M	Caucasian	NONE	Hepatitis C	II	I

Continued

Table 4—Cont'd

Subject Group and ID	Age	Gender	Ethnicity	Cold ischemia (time in hour)	Liver Disease State[a]		
					Diagnosis	Grade	Stage
LIVER TRANSPLANT—Group III (n=11)							
1	66	M	Caucasian	6.53	ND		
2	57	F	Caucasian	5.2	ND		
3	46	M	Caucasian	6.55	ND		
4	59	F	Caucasian	6.85	ND		
5	51	M	NR	7.25	ND		
6	55	F	Caucasian	8.58	ND		
7	53	M	Caucasian	8.33	ND		
8	50	F	Caucasian	11.08	ND		
9	48	M	NR	6.57	ND		
10	51	M	Caucasian	9.01	ND		
11	46	M	Asian	9.18	ND		
LIVER BANK—Group IV (n=9)							
1	10	F	Caucasian	NA	NA		
3	51	M	Caucasian	NA	NA		
6	62	F	Caucasian	NA	NA		
9	7	M	NR	NA	NA		
16	59	M	Caucasian	NA	NA		
23	49	F	Caucasian	NA	NA		
18	68	F	Caucasian	NA	NA		
24	10	M	Caucasian	NA	NA		
22	63	F	Caucasian	NA	NA		

[a]Liver disease state were determined based on clinical diagnosis and Batts and Ludwing Histopathological Grading of Liver disases (Am J Surg Pathol 1995; 19 (12):1409-1417). 'Grade' is an indicator of the degree of lymphocytic piecemeal necrosis, lobular inflammation and necrosis. Grades range from 0 to IV with 0 exhibiting no activity and IV indicating the presence of severe necrosis and inflammation. Grade III is indicated by moderate piecemeal necrosis with moderate lobular inflammation. 'Stage' indicates the degree of fibrosis with 0 being no fibrosis present and IV representing cirrhosis. Stage III indicates the presence of septal fibrosis in the absence of obvious signs of cirrhosis.
[b]Abbreviations: NA, Not Available; ND, Not Determined; NR, Not Reported; NASH, Non-alcoholic steatohepatitis; PBC, Primary biliary cirrhosis.

The majority of subjects had moderately severe liver disease. A Batts & Ludwig Score of Grade III and Stage III, indicates the presence of moderate piecemeal necrosis, moderate lobular inflammation, and moderate fibrosis (in the absence of obvious signs of cirrhotic tissue). None of the subjects had cirrhosis. Only a very small amount of tissue, typically less than 1-2 mg (range = 0.5 to 3 mg) was available for genotype, RNA and enzyme activity analyses. Genotypes for this population are given in Table 5. Five of twenty one subjects had one copy of the *4 variant allele, one subject was compound heterozygous with a genotype of *4/*5, while one subject had one copy of the *6 and another one copy of the *7 allele. The remainder had two functional CYP2D6 alleles.

The biopsies obtained from the liver transplant population were collected immediately following re-perfusion of the newly implanted liver (n=11). These liver biopsy specimens all experienced cold-ischemia for greater than 5 hours and their recovered tissue masses were similar to the liver biopsy group. There was greater than 2-fold variation in cold-ischemic time, as the shortest duration was 5.20 hours and the longest duration was 11.1 hours. The warm-ischemia time was the time from which the donor liver was placed in the recipient's abdominal cavity until re-perfusion and did not vary appreciably between subjects. The shortest duration was 43 minutes while the longest was 50 minutes. The genotypes for this population are presented in Table 5. In this population, 4 of 11 subjects carried one copy of the *4 allele, while the remainder carried only functional alleles.

The Liver Bank samples were obtained from donor livers that were rejected for transplantation for a variety of reasons. The most common reason for rejection at the time of transplant is that the liver exhibited an unacceptable degree of fatty

infiltration. Moreover, because these livers were originally intended for transplantation, they probably experienced conditions very similar to the liver transplant population in terms of donor physiology and cold ischemia. However, these livers were not subject to warm ischemia. These livers were stored at -80º C, but did not experience any freeze-thaw cycles. Genotypes for these livers are given in Table 5. Two subjects were heterozygous carriers of the *4 allele, one was heterozygous for the *5 allele, and one subject was compound heterozygous with a *4/*5 genotype.

With respect to the genotypes we found in this study, the *4 variant had an overall allelic frequency of approximately 32.7 %, which agrees well with larger population studie [26, 56, 57].

Table 5. CYP2D6 genotype, enzyme activity, and mRNA transcripts according to subject groups

Subject group and No.	*Genotype*	*CYP2D6*			
		Enzyme activity†	*mRNA transcript‡*	*Transcript-to-activity ratio*	*Activity-to-transcript ratio*
Control: Group 1 (n = 11)					
1	wt/wt	92.72	18.17	0.20	5.1
6	wt/wt	19.9	1.51	0.08	13.18
8	wt/wt	83.03	6.56	0.08	12.66
13	wt/wt	99.54	7.33	0.07	13.58
19	wt/wt	78.41	3.56	0.05	22.03
21	wt/wt	51.14	2.88	0.06	17.57
24	wt/wt	143.17	11.77	0.08	12.16
Mean ± SD		81.13 ± 38.69	7.4 ± 5.85	0.09 ± 0.05	13.75 ± 5.20
20	wt/*4	3.76	ND	—	—
2	*4/*5	10.60	ND	—	—
14	*4/*4	26.84	ND	—	—
16	*4/*5	0.07	0.65	9.46	0.11
Liver biopsy: Group 2 (n = 21)					
6	wt/wt	54.13	21.38	0.39	2.53
8	wt/wt	27.99	21.92	0.78	1.28
10	wt/wt	59.46	4.43	0.07	13.42
12	wt/wt	17.82	5.1	0.29	3.49
13	wt/wt	10.19	4.9	0.48	2.07
14	wt/wt	12.88	5.07	0.39	2.54
15	wt/wt	20.64	14.3	0.69	1.44
16	wt/wt	20.90	2.08	0.09	10.04
17	wt/wt	8.95	1.76	0.19	5.08
18	wt/wt	6.78	4.3	0.63	1.58
19	wt/wt	13.5	1.4	0.1	9.64
11	wt/wt	52.27	NA	—	—
2	wt/wt	NA	20.43	—	—
Mean ± SD		25.46 ± 18.98	8.92 ± 8.13	0.37 ± 0.25	4.83 ± 4.23
1	wt/*4	0.08	3.98	50.74	0.02
3	wt/*4	ND	0.93	—	—
4	wt/*4	ND	1.46	—	—
5	wt/*4	23.01	3.54	0.15	6.5
7	wt/*7	1.07	7.9	7.39	0.14
9	wt/*4	2.28	ND	—	—
20	*4/*5	15.02	0.1	0.01	150.2
21	wt/*6	12.52	NA	—	—
Mean ± SD		9.0 ± 9.3	2.99 ± 2.83		

Continued

Table 5—Cont'd

Subject group and No.	Genotype	CYP2D6 Enzyme activity†	CYP2D6 mRNA transcript‡	CYP2D6 Transcript-to-activity ratio	CYP2D6 Activity-to-transcript ratio
Liver transplant: Group 3 (n = 11)					
1	wt/wt	19.35	NA	—	—
3	wt/wt	6.95	NA	—	—
4	wt/wt	4.14	NA	—	—
7	wt/wt	62.43	0.07	0.01§	891.86§
8	wt/wt	19.32	3.65	0.32	5.29
10	wt/wt	8.55	1.05	0.12	8.14
11	wt/wt	19.53	4.46	0.23	4.38
Mean ± SD		20.04 ± 19.81	2.46 ± 1.87	0.17 ± 0.13 0.22 ± 0.1§	227.4 ± 442 5.94 ± 1.96§
2	wt/*4	9.23	NA	—	
5	wt/*4	9.13	NA	—	—
6	wt/*4	10.67	ND	—	—
9	wt/*4	ND	2.21	—	—
Mean ± SD		7.26 ± 4.89			
Liver bank: Group 4 (n = 9)					
9	wt/wt	49.76	36.64	0.74	1.36
16	wt/wt	58.23	18.69	0.32	3.12
23	wt/wt	34.02	18.15	0.53	1.87
18	wt/wt	25.17	13.39	0.53	1.88
24	wt/wt	14.73	48.0	3.26§	0.3§
Mean ± SD		36.38 ± 14.09	26.97 ± 14.72	1.08 ± 1.23 0.53 ± 0.17§	1.71 ± 1.01 2.06 ± 0.75§
1	wt/*4	25.7	22.07	0.86	1.16
3	wt/*4	22.94	1.36	0.06	16.78
22	wt/*5	ND	ND	—	—
26	*4/*5	ND	ND	—	—

mRNA, Messenger ribonucleic acid; wt, wild type; NA, data unavailable; ND, below limit of assay.

†Enzyme activity data represent the mean R-568 metabolite formation velocity (in picomoles per minute per milligram) based on triplicate determination.

‡mRNA transcripts of CYP2D6 in tissues were determined in duplicates with a full-length semiquantitative reverse transcript–polymerase chain reaction assay and expressed as mRNA copies per microgram of liver RNA x 10^6 .

§Mean ± SD, where subject 24 of group 4 (liver bank sample) or subject 7 of group 3 (liver transplant sample) was excluded for determination of mean ± SD ratio analyses.

4.3 CYP2D6 mRNA Transcript Levels

We determined the copy number for the full-length CYP2D6 transcript in liver for each subject and these data are presented in Table 5. We were able to quantitate the full-length transcript for every subject having two functional alleles, but were not able to do so in some of the subjects with only one functional allele. Similar to the results obtained with CYP2D6 activity, there were approximately two orders of magnitude of variation in message level across all populations in those subjects having measurable levels of mRNA.

When comparing the mRNA copy number with the Batts & Ludwig score in the liver biopsy population, no apparent relationship was observed (Table 5). There was also no discernible relationship between ischemic time and CYP2D6 copy number within the Liver Transplant population. The regression analysis resulted in an r^2 of 0.42 for subjects with two functional alleles, and there is clearly no relationship with the wt/*4 subjects. Unfortunately, we were unable to obtain good quality RNA for 5 of the 11 transplant subjects due to the limited size of the biopsy samples.

4.4 *In vitro* CYP2D6 Enzymatic Activity

CYP2D6 activity was measured using liver homogenates instead of microsomes to conserve samples. With liver homogenates, we were able to perform all incubations in triplicate, and still have tissue left over for RNA extraction and analyses.

We have previously fully characterized the R-568, a novel and highly sensitive CYP2D6 substrate (see details in the thesis-related manuscript). We compared the R-568 and dextrometphorphan metabolism in human liver homogenates, and found an excellent correlation with an r^2 of 0.94 (Figure 5). We used R-568 for establishing the

CYP2D6 enzyme activity of our liver samples because of its 20-fold higher sensitivity compared with that of dextromethorphan, allowing us to do the LC-MS experiments in triplicates and still have enough remaining tissue samples to determine total mRNA levels.

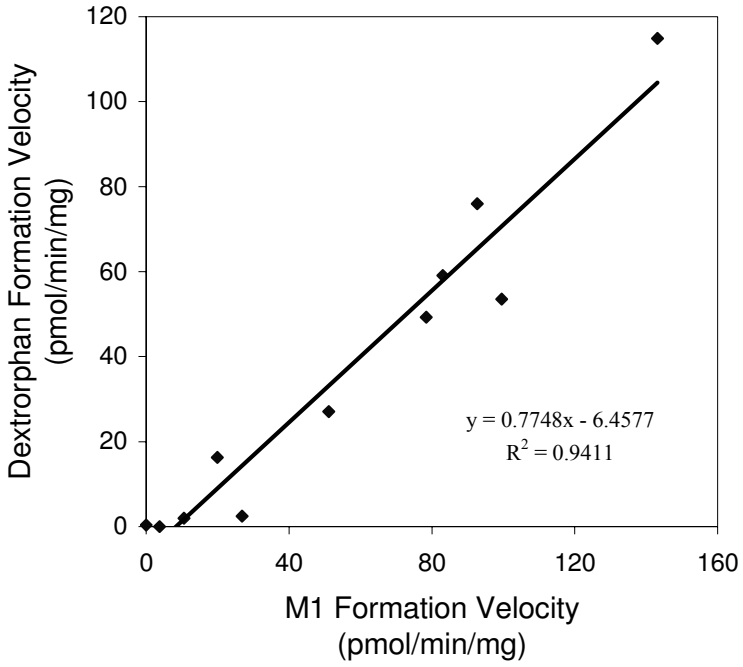

Figure 5. Correlation between R-568 and dextromethorphan metabolism in human liver homogenates. Experimental conditions were as described in Materials and Methods.

The mean CYP2D6 activities for each subject, as measured by R-568 metabolite formation velocities, are given in Table 5 and the population mean-values are depicted in Figure 6. Only data from subjects with at least one functional allele are depicted in Figure 7. The enzyme activity of the samples ranged from undetectable to 143 (pmol/min/mg) and this magnitude of variation is typical for reported CYP2D6 studies. Confined to subjects with two functional alleles, the liver biopsy, liver bank

and liver transplant groups had significantly lower mean activity relative to the Control group ($p<0.005$, $p<0.05$ and $p<0.005$ respectively, Figure 4—asterisks on the significant populations).

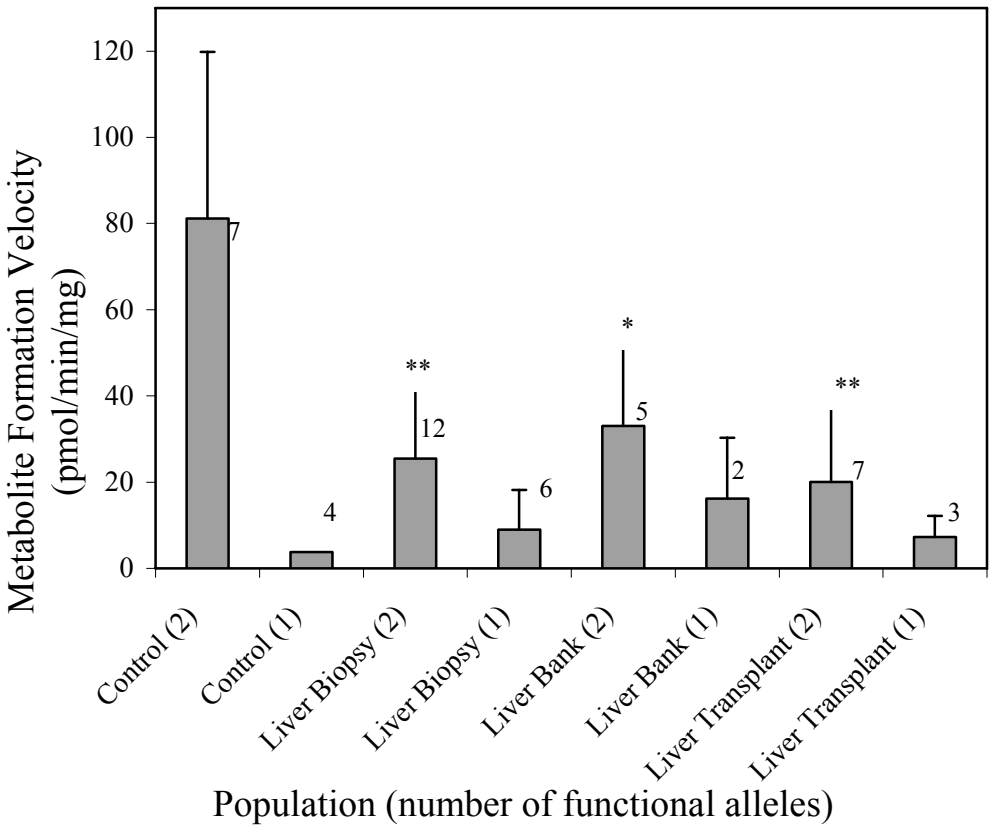

Figure 6. Comparison of hepatic CYP2D6 activity in liver homogenates between the four test population by genotype.
The data within each population are grouped by number of functional alleles, as determined by genotyping. The number of functional alleles is given in parentheses for each group. The number of subjects for each column is listed in the figure. Those with null functional alleles were excluded from this analysis. A statistical comparison of the populations (two functional alleles only) was performed using a two-tailed t-test assuming unequal variances. *$p<0.05$, **$p<0.005$ relative to control population with two functional alleles.

When comparing CYP2D6 activity among the subjects in the Liver Biopsy group, it appears CYP2D6 activity is affected by severe liver disease, though we

found no direct correlation between the Batts & Ludwig score and enzyme activity among this population. Specifically, subjects 6 and 11 had the most severe disease scores (III, III) but exhibited average activity when compared to the normal controls. The other subjects with relatively high Batts & Ludwig scores still exhibited measurable, albeit low, CYP2D6 activity. Subject 5 had one of the lowest disease score, one functional allele, and still had activity comparable to those with two functional alleles, while subjects 15 and 16 had relatively low activity compared to controls, although presented two functional alleles and no apparent histological liver alteration. However, the overall enzyme activity of this group was statistically different from the control population ($p<0.005$). Taken together, these data indicate that serious liver disease did affect CYP2D6 activity in these samples.

Furthermore, the duration of cold-ischemia does not appear to be related to CYP2D6 function. Regression analysis within this population indicated no relationship between ischemic time and CYP2D6 activity, regardless of genotype (subjects with two functional alleles, $r^2 = 0.11$; subjects with one functional allele, $r^2 = 0.36$). Liver Transplant subject 8 had the longest cold-ischemia time of 11 hours, but had CYP2D6 activity (19.53 pmol/min/mg) near the mean value for those subjects with two functional alleles in this group; although it should be noted that these formation velocities are significantly lower than that of the control population.

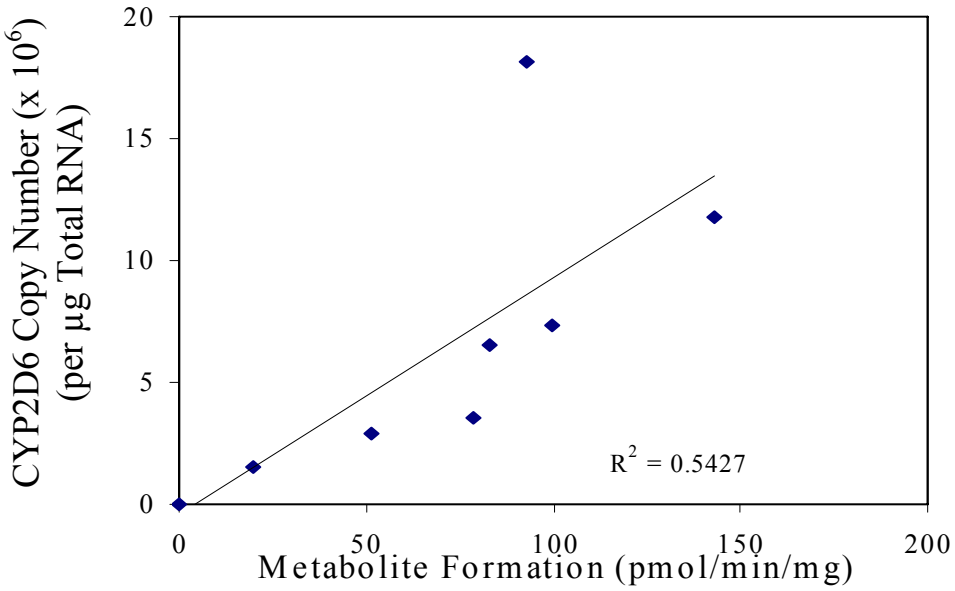

Figure 7. Correlation between CYP2D6 mRNA transcript levels and enzyme activity.
The data here are from subjects with two functional alleles in the control population. The correlation coefficient increases to 0.93, if the single outlier is omitted from the analysis.

4.5 Relationship between CYP2D6 mRNA copy number and enzyme activity

We examined the relationship between CYP2D6 mRNA copy number and *in vitro* enzymatic activity for the four sets of livers. The ratio of mRNA copy number-to- enzymatic activity (or its inverse ratio, see Table 5 last two columns) was consistent for the subjects within each group, but varies up to 10-fold across the four test groups (Table 5). Most notably, the control group exhibited a lower mean ratio compared to the other groups.

The data for the control population was subjected to regression analysis to determine the relationship between mRNA copy number and enzyme activity. For this analysis, we only included the subjects with two functional alleles (Figure 6, p value = 0.032). Interestingly, the Control group was the only population with a fair degree of

correlation (r^2 = 0.54). If we consider subject 1 as an outlier and omit it from the regression analysis, the r^2 becomes 0.93.

4.6 PCR analysis of *MDR1* gene

We have chosen a critical region of the *MDR1* gene (spanning exons 8-21; 43 kb) which would distinguish the 6.3 versus 209 kb version of *MDR1* gene for detailed analysis. To do so, we isolated DNA from liver samples, continuous cell lines expressing Pgp, and human lymphocytes. These DNA samples were analyzed with three regions of the *MDR1* gene and used three different primer sets to amplify the respective gene sequences by PCR to distinguish the two versions of *MDR1*. The predicted lengths for 6.3 kb (AH002875) versus 209 kb (NT007933) *MDR1* gene amplicons are shown in Table 1. The sequences of the primer pairs F19-R19, F27-R27 and F28-R28 were designed to yield significant differences in the product sizes according to the two proposed gene lengths. The three primer sets are designed to probe the sequences spanning introns 15, 17 and 21. Table 1 also lists the expected results of Southern Blot analysis for the two candidate sequences for *MDR1*. The schematics of restoration map of the 209 kb *MDR1* gene sequence (accession no. NT007933) is presented in Figure 6.

With DNA isolated from four human livers, three human lymphocytes and established cell lines, Messa Dox (ovarian carcinoma), CaCo2 (colon carcinoma) and UW 228 (primary human medulloblastoma cells), we performed PCR with the three sets of primers listed in Table 1 and the results are shown in Figure 7. We were not able to detect any product in the reactions corresponding to the 6.3 kb *MDR1* candidate sequence. Instead, we found for all the liver tissues (2), lymphocytes (3), and cell lines (3), the PCR products corresponded to the length predicted with a 209

kb *MDR1* sequence (NT007933). As shown in Figure 6, the products of F19/R19, F27/R27 and F28/R28 were 10.5, 2.84 and 1.1 kb, respectively. The inability to detect products of short fragments was not due to PCR conditions, as the control *MDR1* cDNA derived from Messa Dox were positive for all the reactions with the three sets of primers (Figure 8).

4.7 Southern Blot analysis of *MDR1* gene

The PCR results were verified further with Southern Blot analysis to probe introns 8 to 21 (Figure 1). We found that the radiolabeled probes P1-P3 pointed to 209 kb *MDR1* sequence candidate. With *BamHI/PacI* and P1 we found 7 and 9 kb; with *BamHI/KpnI* and P2 we found 7 and 14 kb; and finally with *BamHI/PacI* and P3 we found 11 and 16 kb fragments (Table 1 and Figures 1 and 9). The bands correspond to the estimates shown in Table 1 for the more than two hundred kb gene length. Again, there were no DNA bands corresponding to the length (5 and 6 kb) of product predicted by the 6.3 kb total gene size.

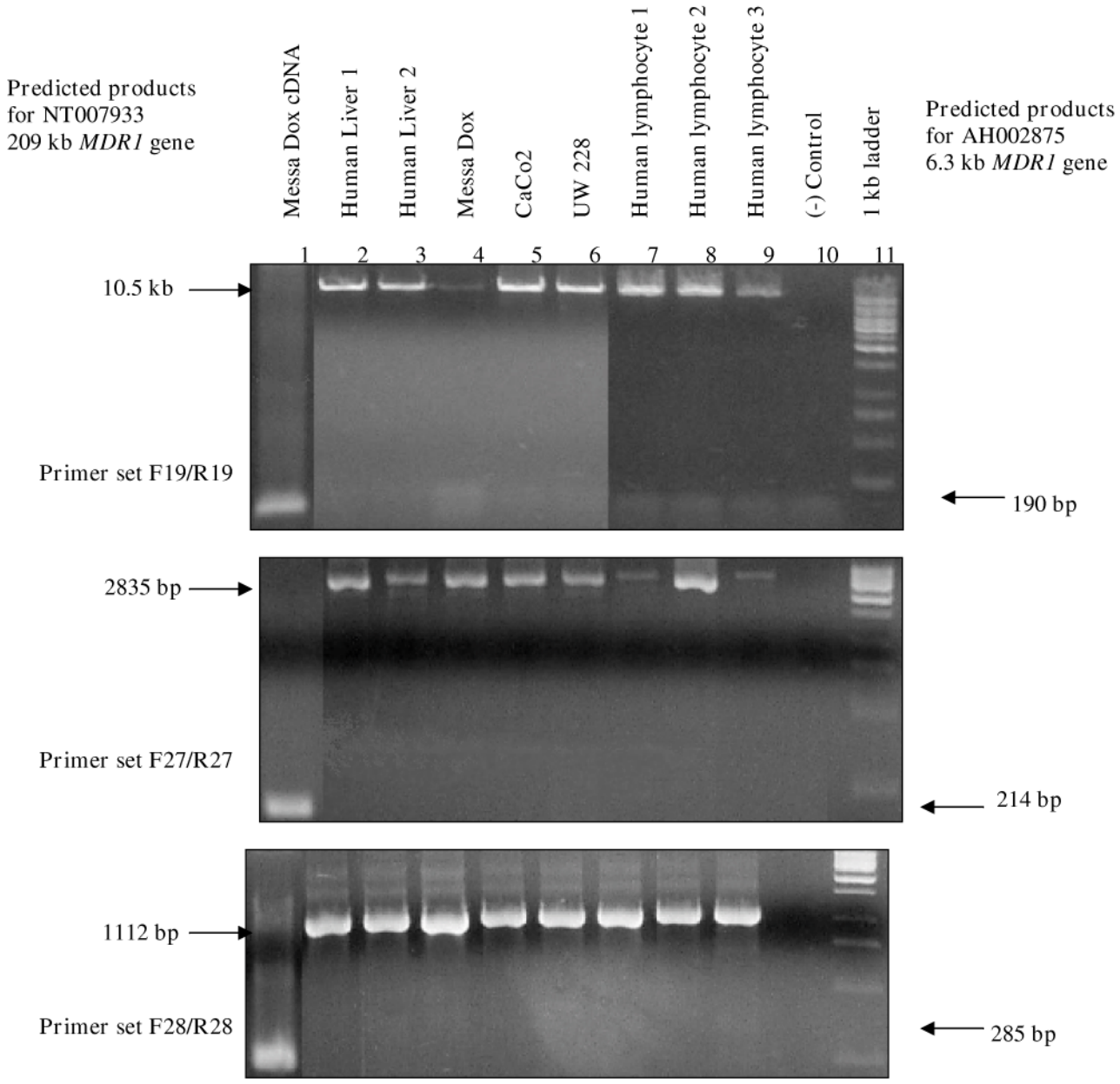

Figure 8. PCR analysis of the three MDR1 gene sequences using F19-R19, F27-R27 and F28-R28 primer set (Table 1), respectively. Lane 1 is cDNA of Messa Dox cells, lanes 2 and 3 are two different liver samples, lanes 4, 5 and 6 are Messa Dox, Caco2 and UW 228 cell lines and lanes 7, 8 and 9 are three different lymphocyte samples. Lane 10 is a negative control in which no DNA was added, and lane 11 is a 1 kb ladder. There were amplifications in all the samples according to the 209 kb gene length, having sizes according to Table 1. There were no bands showing up according to a 6.3 kb total MDR1 gene length. Results for Messa Dox cDNA were added to show reaction sensitivity for short fragments as well; the PCR products were 167 bp, 171 bp, and 249 bp for primer sets F19-R19, F27-R27 and F28-R28, respectively.

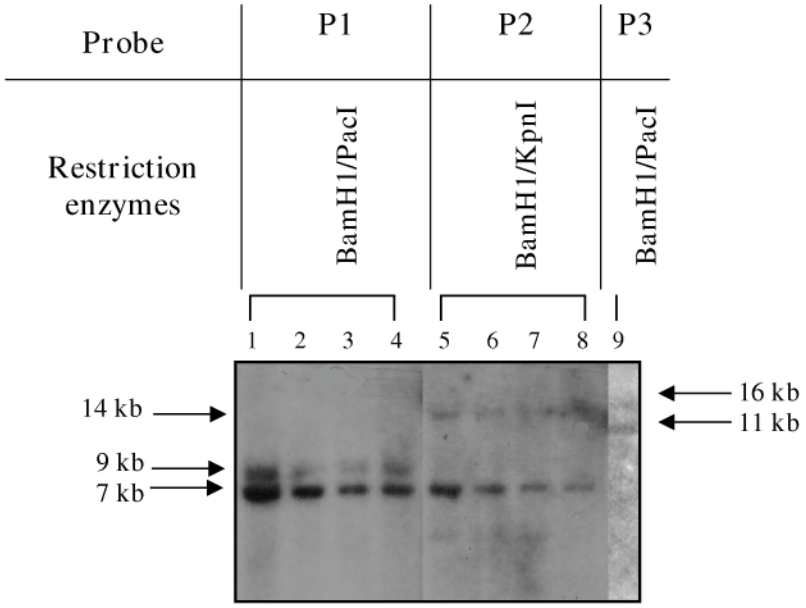

Figure 9. Southern Blot analysis of *MDR1* gene with DNA isolated from human livers. Genomic human liver DNA was subjected to BamH1/Pac1 (columns 1-4), BamH1/KpnI (columns 5-8), and BamH1/Pac1 (column 9) restrictions and P1, P2 and P3 radiolabeled probes, accordingly.

5. DISCUSSION

5.1 RT-PCR strategy to estimate CYP2D6 copy number

One of our goals of was to develop an analytical tool to assess cytochrome P450 2D6 levels in the form of full-length transcripts. While a number of strategies have been described for detecting short fragments of CYP2D6 RNA, including those designed for Q-RT-PCR, there was none reported on full-length transcript analysis. We have, for the first time, successfully developed an assay that detects a full transcript of CYP2D6 RNA. Our data suggests that it is reproducible with about 15% day-to-day and run-to-run variation (Table 2). Therefore it could be considered as a semiquantitative assay. Our CYP2D6 RNA estimates for human livers are comparable with data available in the literature; Andersen et al.[28] previously reported $3.0 \pm 4.0 \times 10^6$ copies/µg total RNA of 2D6 mRNA in human liver samples using competitive reverse transcriptase-PCR with a 220-fold variation between individuals, while others [58] found $2.1 - 5.5 \times 10^6$ copies/µg RNA in liver tissues and cultured human hepatocytes. Lind et al.[59] assessed a relative gene expression compared to 18S ribosomal RNA, and found a 100-fold variation between different white blood cell samples. Others[60] found an average of 3.2×10^6 copies/µg RNA with a variation of the same magnitude by TaqMan real-time RT-PCR, which correlates with our semiquantitative RT-PCR estimates.

Interestingly, we noticed that the short sequence CYP2D6 RNA transcript of the same livers estimated with real-time quantitative RT-PCR (Q-RT-PCR) yielded about three order of magnitude lower numbers (Table 3). We have repeated the assays many times and with both CYP2D6 gene specific primers and random hexamers. The difference was reproduced in all experiments. The disparity between

short- and long CYP2D6 mRNA estimates requires further study. We did not observe, in a limited sample size, a clear quantitative relationship between genotype and *in vitro* enzymatic activity or, by extension, phenotype; this finding is consistent with data reported in the literature[26, 57]. For livers with wt/wt genotype our RNA to activity ratio provided consistent numbers, suggesting that there might be a relationship between the two measurements.

It was interesting that liver #22 did not contain any full-length message even though it is heterozygous for the deletion mutation and not homozygous. One possible and likely explanation for this is that this liver has a mutation not included in the panel of variant alleles in the genotyping assay. Furthermore, there appears to be a complex relationship between genotype, phenotype and mRNA expression for livers with the *4 allele, the variant at the exon 3 consensus sequence. A plausible explanation for an apparently high ratio of copy number to activity, is that we are detecting defective transcripts, which exhibit a PCR product of similar size to RNA samples of individuals with the *4 allele. Such RNA products could produce non-functional proteins. Indeed, according to the mechanism described by Meyer and Zanger[57, 61], and as proposed by Hanioka e*t al.* [62] and Gough et al.[63], the *4 allele would be present in the full-length mRNA transcript.

An important aspect to the current assay design is the detection of the full-length CYP2D6 mRNA transcript. While small fragments are indeed detectable, as noted by previous studies[27-29], the detection and quantitation of the full-length message may provide a better predictor of functional CYP2D6 protein. For example, we and others were able to detect short or truncated sequences of CYP2D6 RNA in human lymphocytes but were unable to detect full-length RNA or enzyme activity in the human lymphocytes samples evaluated for CYP2D6 activity[49].

Truncated fragments of CYP2D6 mRNA may not be related to the final translated amount of protein, especially when one considers the number of mutations known to occur in the CYP2D6 DNA sequence. Furthermore, the initial reverse transcription of essentially the entire coding region of this gene permits more complete downstream sequencing reactions for detection of novel sequence variants.

While we have demonstrated the detection and quantitation of full-length CYP2D6 mRNA, it is important to note that the principle used in construction of this assay is adaptable to any number of mRNA transcripts of interest. In fact, we have direct experience with adapting this strategy to the detection and quantitation of full-length CYP2C19 mRNA transcripts[51, 64]. Furthermore, it is not necessary to restrict the assay to drug metabolizing enzymes such as the CYPs. Indeed, membrane transporters such as MDR1 and MRP would also be amenable targets of such an assay. Although the basic design may be similar, each assay must be optimized and validated to ensure reproducibility and specificity.

5.2 CYP2D6 enzymatic activity assessed with a novel substrate

Employing a novel CYP2D6 probe substrate that exhibits an exquisitely high intrinsic clearance (about 20-fold more sensitive than the overall enzyme detection limit of dextromethorphan) and an RT-PCR assay that allows estimation of the full-length transcript, we have evaluated the mRNA transcript levels, genotype and CYP2D6 activity in human livers belonging to four patient groups. Availability of R-568 to determine CYP2D6 activity has permitted elucidation of the relationship between the full-length CYP2D6 mRNA transcript levels and its *in vitro* enzyme activity in human livers collected under varying conditions. To our knowledge, this

study is the first attempt to elucidate the relationship between CYP2D6 genotype, mRNA levels and enzyme activity in human livers.

R-568 is a superior CYP2D6 substrate relative to conventional probes such as dextromethorphan due to the high V_{max}/K_m ratio observed in this study. Our data indicate a 20-fold increase in this ratio, primarily due to the significantly lower K_m for R-568 than dextromethophan demethylation (data not shown). This higher substrate affinity and improved LC-MS assay sensitivity have also contributed to our ability to measure CYP2D6 activity in biopsy samples that are available in less than a few milligrams. Such studies would not have been possible without this novel CYP2D6 probe substrate.

While dextromethorphan has been used as a standard probe for CYP2D6, the ability of CYP3A4 to metabolize this probe substrate (at high concentrations) to the *O*-desmethyl metabolite, dextrorphan, is well-documented[65]. Our data strongly suggest that, under physiological conditions, R-568 is a highly selective and sensitive CYP2D6 probe substrate.

It is noteworthy that traditional CYP2D6 probes require an order of magnitude higher protein concentrations and much longer incubation time to achieve equivalent metabolic activity[66-71]. The R-568 incubation can be accomplished within or below two minutes, as opposed to the lengthier incubation times required to detect product formation for most other assays. Therefore, the R-568 assay offers advantages with respect to the potential for rapid throughput associated with a shorter incubation time and a much lower amount of total protein necessary for detection and quantitation of enzymatic activity.

5.3 Relationship between CYP2D6 genotype, mRNA level and activity

When comparing CYP2D6 activity *across* the four sample populations, there was a statistically significant difference between the liver bank, liver transplant and liver biopsy groups relative to the control group (in subjects presumed to carry two functional alleles). Because liver bank samples were obtained from brain-dead donors whose livers were deemed not suitable for transplantation, their liver enzymes might be down-regulated for reasons including brain injury, procurement conditions, and fatty infiltration. Brain injury and brain death have been associated with massive systemic cytokine release, and severe impairment of pituitary function. Increased exposure to regulatory cytokines coupled with decreased circulating levels of growth hormone, thyroid hormone, and sex steroids may influence hepatic P450 mRNA level and enzyme activity.

The donor liver, transplanted into liver transplant subjects, could be at a healthier state than that found in the liver bank samples. However, they may also be subjected to similar conditions described above for liver bank samples, without associated fatty infiltration. While there was no relationship between ischemic time and enzyme activity *within* this group, it is likely that there was some degree of hepatic down-regulation during the procurement and ischemic periods of donor livers, similar to conditions in the liver bank population, which resulted in significantly lower overall CYP2D6 activity relative to the control population.

When considering the effects of liver disease on the liver biopsy population, it is evident that, even in livers with moderate-to-severe disease, there is no effect on CYP2D6 mRNA transcription level. However, we found about 4-fold lower CYP2D6 activity in these subjects, compared to the Control Group (Figure 6, Table 5, $p=0.0005$). This observation is consistent with a recent data collected from 14 patients

with chronic hepatitis C and 35 healthy volunteers, based on metabolism of dextromethorphan as an *in vivo* CYP2D6 probe[72]. They also found about 4-fold reduction on CYP2D6 activity in these hepatitis C patients. Subjects with severe liver disease, such as cirrhosis or severe hepatitis with liver failure were also reported to exhibit significantly diminished P450 expression[18]. On the other hand, subjects with mild to moderate liver diseases did not exhibit impaired CYP2D6, but reduced CYP2C19 activity[73]. The observed differences may be explained by the different types of hepatic diseases (moderate liver cirrhosis versus chronic hepatitis). The patients in our liver biopsy group had chronic hepatitis C viral infection with advanced liver damage (mainly stage II and III) and autoimmune liver diseases with chronic liver enzyme elevations which may contribute to the differences.

Overall, a comparison of the CYP2D6 enzyme activity and mRNA transcript reveals that livers with one functional allele express lower activity and decreased levels of mRNA compared to those with two functional alleles. The tissue concentration range of CYP2D6 mRNA transcripts reported in the current study was similar in magnitude to that observed by others[28]. When making a comparison of copy number across populations with two functional alleles, only the liver transplant group has lower levels of CYP2D6 mRNA transcripts than the control group. Given the limited sample size, it is difficult to draw a definitive conclusion regarding the potential effect of ischemia on mRNA degradation.

The ratios between CYP2D6 mRNA transcript levels and *in vitro* enzyme activity represent an indirect measure of translation of the transcripts into CYP2 D6 protein as reflected in enzyme activity (Table 5). If translation of CYP2D6 mRNA is closely regulated, the ratio of the two measures would be relatively constant across all subjects. Our results showed that *within* each test group (ignoring a few outliers: ID

1, 7 and 20 in liver biopsy and 24 in liver bank) mRNA transcripts-to-activity ratio remained relatively constant. The one outlier subject in the control group had a higher ratio compared to all the other subjects due to greater mRNA copy number relative to enzyme activity. The reason for this is unclear and remains to be evaluated.

When comparing the activity-to-mRNA transcript ratio across groups, the mean ratio is much higher for the control group relative to the others (Table 5). The higher enzyme activity content per copy of transcript may signify more efficient translation to CYP2D6 proteins and/or slower turnover of CYP2D6 protein. The cause for the ratio discordance may be multifactorial. It is possible that these liver biopsies are from healthier individuals without any of the pathology or collection damage associated with the other groups, including fatty infiltration, liver disease or ischemia, thus leading to CYP2D6 with higher catalytic efficiency or diminished degradation of protein. Unfortunately, we did not receive sufficient tissue from the biopsies to perform Western Blot analysis to analyze CYP2D6 protein.

However, the relatively constant activity-to-mRNA transcript ratio within the control population indicates that quantitation of the full-length mRNA transcript may be predictive of both *in vitro* and *in vivo* CYP2D6 function, particularly for those liver samples collected under conditions that preserve enzymatic and RNA integrity. It is noteworthy that the relationship holds true for livers with two functional alleles and is somewhat less clear for livers with only one functional allele.

We have presented the inter-relationships between CYP2D6 genotype, mRNA transcript, and *in vitro* liver enzyme activity. The detection and quantitation of the full-length CYP2D6 transcript is an improved technique to monitor mRNA transcripts as truncated message fragments are not translated into CYP2D6 protein. The data

from this study indicate that mRNA level is well-correlated to *in vitro* enzymatic activity for liver samples obtained from healthy individuals with two functional alleles.

5.4 Characterization of the human *MDR1* gene

P-glycoprotein (Pgp), an ATP-dependent efflux transporter that protects the body from environmental toxins and xenobiotics, is encoded by the human *MDR1* gene. Human *MDR1* is located on chromosomal region 7q21. Although several different single nucleotide polymorphisms (SNPs) were shown to influence Pgp expression and activity, the reported length of the *MDR1* gene in Genbank and other databases continues to evolve and varies between 6.3 kb and 210 kb.

Another goal of our investigations was to determine the MDR1 gene length by PCR and Southern Blot experiments using different human tissue samples and cell cultures. As the Genbank data are often based on the analysis of limited DNA specimens, we used several different human cell lines as well as lymphocyte and liver samples to investigate eventual differences between tissues and/or subjects regarding the *MDR1* gene locus. Both sets of experiments confirm the length of the *MDR1* gene being much longer than 6.3 kb, and it is most likely 209 kb as indicated in the database (accession no. NT007933). The results were consistent in the cases of different human liver tissue samples, several cell lines and lymphocytes, showing that there is very little, if any, interindividual variation with respect to the gene length (Figures 8 and 9). The recent research interest to characterize differences in gene expression related to variation in drug response among individuals, has begun to provide some clues in *MDR1* gene regulatory mechanisms. Discoveries of *MDR1* sequence variation in contig sequences have provided some clues. However, detailed

studies of *MDR1* gene regulation require a validated *MDR1* sequence. Toward this end, we have provided data validating the 209 kb *MDR1* sequence.

The results of our study confirming the size of the *MDR1* gene will be of great utility when extended to analyses of mechanisms of drug-induced resistance. In other words, knowledge of both intron-exon structure and overall gene length can be used to differentiate between aberrant mRNA splicing and gene deletions in multidrug resistance. In addition, there are a number of references in the literature regarding "mini-Pgp" [74, 75], but there have been no molecular analyses to explain this phenomenon or its cause; our reference for *MDR1* gene structure will aid in these investigations.

Overall, our data presented have clarified and established that *MDR1* gene length is likely to be 209 kb.

6. SUMMARY

1. We have developed an efficient and reliable assay to detect and quantitate full-length 1.5 kb CYP2D6 mRNA transcripts from any tissue or cell source. The CYP2D6 activity appeared to relate more closely to full-length CYP2D6 mRNA concentration than a short-sequence of CYP2D6 RNA estimated with a real-time quantitative RT-PCR assay. This assay could be adapted to detect other mRNA transcripts of interest.

2. The CYP2D6 activity in tissue homogenates of liver biopsy specimens collected from control subjects (with no apparent liver disease), liver biopsy subjects, liver transplant subjects, and liver bank specimens were assessed with a newly developed and validated a CYP2D6 probe substrate, R-568, that offers a 20-fold higher sensitivity than dextromethorphan. The improved assay sensitivity allowed evaluation of CYP2D6 enzyme activity in a few milligrams of tissue collected from biopsy.

3. The liver samples grouped in four populations were genotyped for the six most common CYP2D6 genetic variants (i.e., *3, *4, *5, *6, *7 and *8). The overall allelic frequency agreed well with data available in the literature.

4. A combination of genotyping and mRNA level determination could allow a quantitative estimation of functional CYP2D6 activity in healthy human livers with a reasonable degree of confidence.

5. Using PCR and Southern Blot techniques with DNA derived from different human cell lines and tissues, we have characterized the *MDR1* genomic sequence and established that the total gene length is likely to be 209 kb.

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