2004

Characterization of CYP2D protein from human brain cerebellum

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Characterization of CYP2D Protein from Human Brain Cerebellum

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Thesis Submitted to the
School of Pharmacy
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Basic Pharmaceutical Sciences

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Morgantown, West Virginia
2004

Key words: 2-D blots, brain, CYP, CYP2D6, CYP2D7, immunoblot, immunoprecipitation, liver, metabolism, microsomes, nanospray LC-MS/MS, pharmacogenetics, SDS-PAGE

Abstract

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To date, knowledge in characterization of CYP proteins has been limited to immunoblotting, RT-PCR, immunohistochemistry and so forth; therefore, it is the intention of this study to investigate:

1) What kind of CYP2D protein is present in brain with modern proteomic tools;
2) Whether, CYP2D6 in brain is same as the liver CYP2D6; and
3) If CYP2D7 is present, can CYP2D6 polymorphism be found in the brain?

To answer these questions, this study used a one-step method of isolation of protein by immunoprecipitation followed by its identification using 2D-blots, nanospray LCMS, immunoblots and immunohistochemistry. Probing of immunoprecipitated proteins with polyclonal CYP2D6 antibody revealed two major CYP2D6 immunoreactive bands. Similar banding pattern resulted from matched human liver microsomes suggesting there are more than one CYP2D isoform(s) exist in these tissues.

Dedication

This thesis would be incomplete without the mention of the support given me by my family and friends, to whom this thesis is dedicated. Without them this thesis seemed interminable, I doubt it should ever have been completed.

Acknowledgments

I would like to express my gratitude and appreciation to Dr. Robert. L. Haining for his guidance, suggestions and patience, which encouraged me all the time of my research and writing of this thesis.

Acknowledgements are also made to my committee members, Drs. Grazyna Szklarz and Fei Chen for their support and acceptance to serve on my committee.

I deeply appreciate the help of my colleagues at the school of pharmacy, which not only helped me morally but with their valuable suggestions.

Special thanks go to the members of the tissue bank and the proteomics facility at West Virginia University who helped me with the procedures of immunohistochemistry and nanospray LC-MS/MS.

Finally, I would like to appreciate my family's unconditional love and their support throughout my life that has helped me reach this milestone.

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Abbreviations

2-D	2 -dimensional
APAP	*N*-acetyl-*p*-aminophenol
bp	Base pair
BHT	Butylated hydroxytoluene
CO	Carbon monoxide
Cerb	Cerebellum
CYP	Cytochrome P450
EDTA	Ethylenediaminetetraacetic acid
EM	Extensive metabolizer
Hippo	Hippocampus
HT	Histidine tagged
IHC	Immunohistochemistry

IM	Intermediate metabolizer
MAO	Monoamine oxidase
MPTP	1-methyl-4-phenyl-1, 2, 3, 6 tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium ion
NAPSQI	*N*-acetyl-*p*-benzosemiquinoneimine
NAPQI	*N*-acetyl-*p*-benzoquinoneimine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PM	Poor metabolizer
PMSF	Phenyl methyl sulfonyl fluoride
PTP	4-phenyl-1,2,3,6-tetrahydropyridine
SDS	Sodium dodecyl sulfate
URM	Ultra rapid metabolizer

Introduction

The CYP2D protein in the human brain is expressed as CYP2D6 in liver and is known to metabolize variety of drugs such as psychoactive, antihypertensives, and environmental toxins[1-3].

Metabolism refers to the manner in which the body detoxifies drugs, breaking some down in the liver so they can more easily circulate or be excreted. Every person has a unique rate of metabolism, and a variety of factors such as body weight, absorption, distribution of the drug, and rate of excretion may serve to influence drug levels in the blood. The CYP2D6 protein is also known to be highly polymorphic in nature; however, about 5-10% Caucasians are poor metabolizers of drugs that are metabolized by CYP2D6 because of the numerous polymorphs found in this gene[4,5].

Problem Statement. The significance of the CYP2D6 protein is that it is associated to variety of disease states such as Parkinson's disease, personality syndrome, lung cancer, skin cancer, systemic lupus erythematosus, Balkon nephropathy and ankolysing spondilitis [6-11]. To the extent that such enzymes can be fine-tuned to meet the unique needs of each individual will likely be the extent to which such treatments will be efficacious.

Hypothesis. It is hypothesized that the expression of CYP2D6 protein in specific regions of brain will locally affect the metabolism of many exogenous and endogenous substrates.

Rationale. It is possible that this enzyme can undergo splice mechanism to form many other proteins or closely related protein such as CYP2D7; for instance, CYP2D7 was recently found to undergo splicing and form an active protein that can metabolize codeine to morphine in the brain which was earlier known only to be metabolized by CYP2D6 [12]. Likewise, CYP1A1 and CYP1A2 (though not CYP2D) proteins have shown similar function to CYP2D, as they are also drug-metabolizing enzymes. The 1A1/2 has been shown to have targeting sequence to mitochondria as well as endoplasmic reticulum. The mitochondrial CYP1A1/2 is different from liver CYP1A1/2 since mitochondrial CYP1A1/2 is 30 amino acids shorter than liver 1A1/2. This 30 amino acid sequence had brought great change in the substrate specificity of CYP1A1/2 in the brain in a sense; it is similar to CYP3A4 drugs now [13]. According to sequence analysis of CYP2D protein, it is recognized that the CYP2D6 protein also has series of positively charged residues in its sequence that can act as mitochondrial target.

To date, knowledge in characterization of CYP proteins have been limited to immunoblotting, RT-PCR, immunohistochemistry and so forth. However, toxicogenomics is a new scientific field in which researchers' study how the genome responds to environmental stressors or toxicants[14]. This new field combines studies of genetics, genomic-scale mRNA expression (transcriptomics), cell and tissue wide protein expression (proteomics), metabolite profiling (metabonomics), and bioinformatics with conventional toxicology in an attempt to better understand the role of gene-environment interactions in disease processes. New molecular technologies such as DNA microarray analysis and protein chips can now measure the expression of hundreds to thousands of genes and proteins simultaneously, thereby providing researchers with the potential to

accelerate discovery of toxicant pathways and specific chemical and drug targets. "The power and potential of these new toxicogenomics methods are capable of revolutionizing the field of toxicology"[15].

It is the intention of this study, therefore, to investigate:

1. What kind of CYP2D protein is present in brain with modern proteomic tools;
2. Whether, CYP2D6 in brain is same as the liver CYP2D6; and
3. If CYP2D7 is present, can CYP2D6 polymorphism be found in the brain?

To answer these questions, this investigation used a one-step method of isolation of protein by immunoprecipitation followed by its identification using 2D-blots, nanospray LCMS, immunoblots and immunohistochemistry.

Literature Review

This literature review will examine the current scholarly work in the field of drug metabolism that will help to understand the putative role played by CYP proteins in the metabolism of endogenous and exogenous substrates, and how this information will be relevant to clinical pharmacokinetics.

Background and Significance: According to Damaris Christensen (2002), the differences in the way different people respond to drugs are in large part genetically determined. That diversity provides the basis for one of the most touted potential benefits of genetic knowledge: By teasing out the connections between a person's genes and his or her drug responses, it may be possible to customize medicine. The science behind this personalized medicine is called pharmacogenetics. "As you look at developing new therapies, new interventions, and even at the role of nutrition in health, being able to segment populations to see who is benefiting or who is at risk is very important," says Steven Lehrer, head of DNA Sciences in Fremont, Calif. "Who you are when you're being treated is the last thing we think of, but it should be the first thing"[16].

These researchers report that genes play an important role in drug response because they control how each person's body breaks down, or metabolizes, medicines. In addition, a number of drugs tend to target specific receptors, which are gene-specified proteins that sit on the surfaces of cells. These receptors are unique markers that allow substances, including drugs, to bind to cells and in some instances to penetrate them; furthermore, individual variations in genes affecting metabolism or cell-surface binding can influence responses to drugs[16].

In 2001, a study published in the Journal of the American Medical Association (JAMA) investigated whether various drugs are metabolized by one or more enzymes that have genetic variants that result in unusually slow breakdown. This study found that almost 60 percent of the drugs most commonly cited as triggering adverse reactions fit that description. By contrast, such enzymes only break down 22 percent of drugs within a random sample of those sold in the United States according to Kathryn A. Phillips of the University of California, San Francisco. Phillips said that, "These results suggest that genetic variability in drug-metabolizing enzymes is likely to be an important contributor to the incidence of adverse drug reactions"[16]. Individuals who metabolize drugs slowly may suffer problems for two fundamental reasons:

1) In some cases, the drug will only become active only after it is metabolized. In the event this happens more slowly than usual, or not at all, the patient may experience no benefit.

2) In other situations, where a drug is not metabolized as rapidly as expected, the effective doses may be required to be much higher than intended[16].

One of the first widely used applications of pharmacogenetics is within the arena of cancer treatment. In part, this is because most cancer drugs are relatively toxic, so physicians have much incentive to reduce side effects. Consider the drugs thioguanine and mercaptopurine, which are prescribed for acute leukemia, as well as to prevent rejection of organ transplants. An enzyme called thiopurine methyltransferase, or TPMT, normally inactivates the drugs. About 1 in 300 people does not have an effective version of this enzyme, and about 1 in 10 has one, rather than two, functioning copies of the gene, according to William Evans of St. Jude's Children's Research Hospital in Memphis.

These segments of the population continue to be at high risk of side effects. Today, U.S. oncologists routinely test patients for TPMT activity before prescribing these drugs. In these cases, the clinicians give patients with ineffective TPMT only low doses of the drugs. "It's the first pharmacogenetic test to make it all the way into the real world, into the clinic"[16].

Other metabolizing agents that are being carefully examined are a large family of enzymes called cytochrome P450s. These enzymes were once believed to be mainly a hepatic drug detoxication system, but is now understood that these P450s are included in a myriad of enzymatic reactions implicated in important life processes.

Advances in molecular biology and genomics facilitated the biochemical characterization of individual P450 enzymes, which in turn revealed many surprises about these enzyme systems in the body.

First, the cytochromes P450 act on many endogenous substrates, introducing oxidative, peroxidative, and reductive changes into small molecules of widely different chemical structures[17,18]. Substrates identified to date include saturated and unsaturated fatty acids, eicosanoids, sterols and steroids, bile acids, vitamin D3 derivatives, retinoids, and uroporphyrinogens [19-21].

Second, many cytochrome P450 enzymes can metabolize various exogenous compounds including drugs, environmental chemicals and pollutants, and natural plant products[22-24] .

Third, metabolism of foreign chemicals frequently results in successful detoxication of the irritant; however, the actions of P450 enzymes can also generate toxic

metabolites that contribute to increased risks of cancer, birth defects, and other toxic effects [25,26].

Fourth, expression of many P450 enzymes is often induced by accumulation of a substrate, For example, hepatic concentrations. The case of CYP-mediated APAP metabolism is rather different, however, in that this pathway generates the highly toxic quinone imine, NAPQI [27].

In addition to the two-electron oxidation catalyzed by the CYP pathway, it has also been reported that various peroxidases are capable of generating the one-electron oxidation product, the benzosemiquinone radical NAPSQI [28,29].

At least two possible products of CYP-mediated APAP oxygenation are known, NAPQI and 3-OH-APAP, the latter compound being considered non-toxic. At least three CYP isozymes have been shown to metabolize APAP, namely 2E1, 2A6, and 1A2. It is not known whether the oxidation of APAP carried out by CYP generates a transient radical species, or if a concerted two-electron oxidation occurs. Interestingly, 2E1 and 2A6 differ significantly in the ratio of NAPQI to 3-OH-APAP [30].

Although cytochromes are most frequently studied in relation to their role in the metabolism of xenobiotics, their involvement in endogenous metabolism, particularly that of steroids (fig. 1), is also very important; in fact, this function in the organism was probably the primary one [31]. It has been assumed that, in prehistoric organisms, cytochrome P450 was responsible for hydroxylation of organic substrates subsequently used as sources of energy. This function has been preserved up till now in some microorganisms and attempts have been made to develop microbes capable of degrading industrial contaminants of the environment that is otherwise difficult to break down[32].

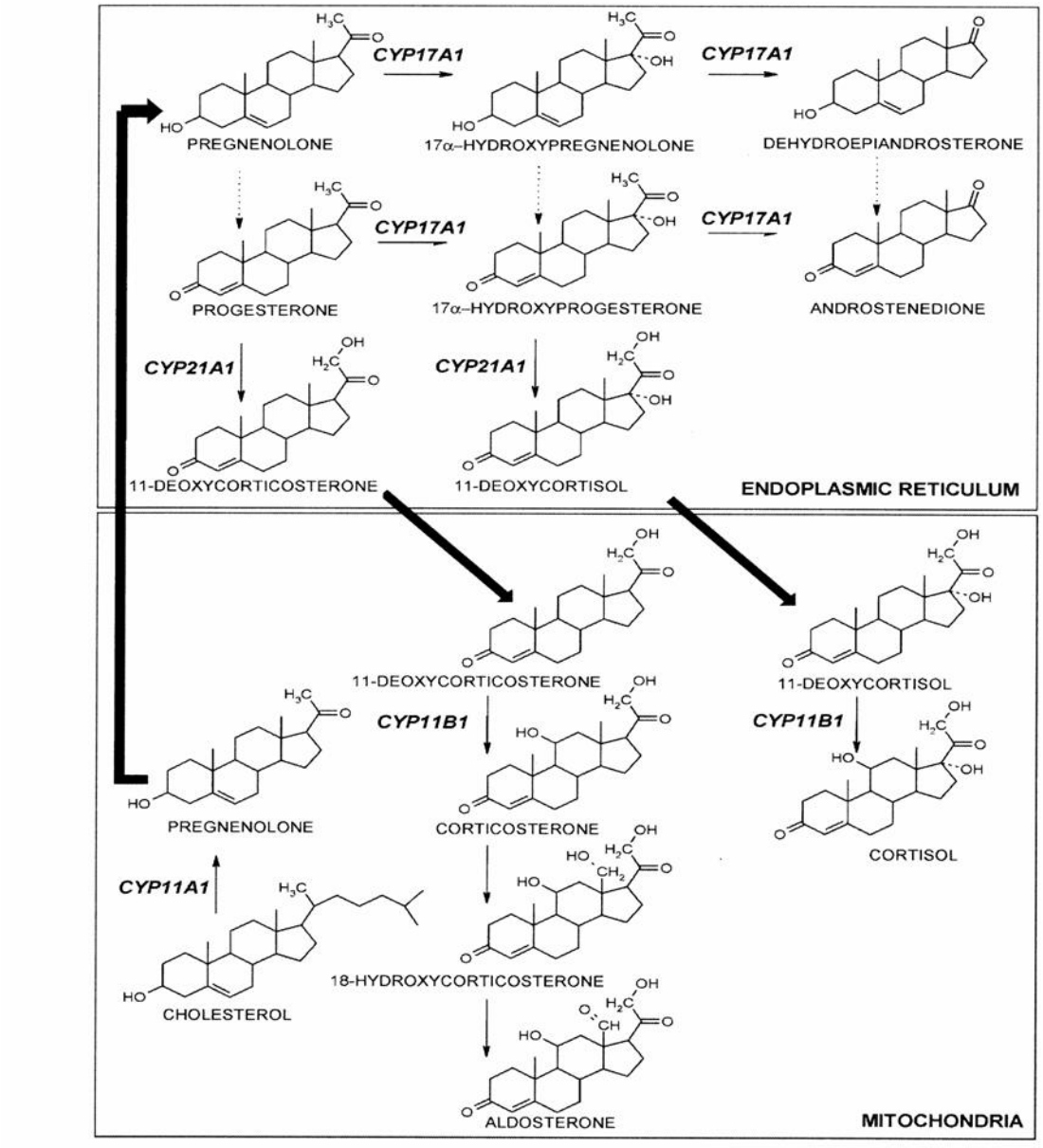


Figure 1 Cytochrome P450 mediated biosynthetic and degradation pathways of adrenal steroid hormones. Adapted from [33].

In plant also the production of many significant secondary metabolites, such as lignin, terpenoids, steroids, essential oils or opioid precursors, is based on cytochrome functions[34].

The CYP isoforms whose primary functions are to metabolize xenobiotics (families CYP 1, CYP 2 and CYP 3) are also known to be involved in endogenous metabolic processes in substrates such as melatonin and estradiol (CYP1A), testosterone (CYP3A), catecholamines (CYP2D), progesterone (CYP2C, CYP3A) and arachidonic acid (CYP2E) [34].

According to Miksys and Tyndale, the extrahepatic cytochrome P450 enzymes in the brain may also play a role in the activation or inactivation of centrally acting drugs, in the metabolism of endogenous compounds, and in the production of potentially harmful metabolites and/or oxygen stress[21]. Miksys and Tyndale report that, "CYPs are distributed unevenly among brain regions, and are found in neurons, glial cells, and at the blood-brain interface (table 1). They have been observed in mitochondrial membranes; in neuronal processes, and in the plasma membrane, as well as in endoplasmic reticulum". The highly localized nature of CYPs in brain strongly suggests a role of cerebral CYPs in local drug metabolism[21]. This contention is further supported by enzymatic assays performed in-vitro by brain microsomes using same probe substrates used to assess specific hepatic CYP activity. Therefore, modulation of brain CYPs could constitute a local regulatory mechanism of enzyme activity, thus influencing drug response; for tissues exhibiting low regenerative capacity, such as brain, such modulation would probably be of major toxicological significance[19].

Table 1 Regional brain distribution and forms detected for cytochrome P450 in different species. Adapted from reference[19].

CYP	Species	Form	Localisation
1A2	Human	mRNA	Basal ganglia, frontal and occipital lobules, pons, red nucleus, substantia nigra
	Rat	Protein	Cortex, cerebellum, multiple regions
	Rat	mRNA	Striatum, hypothalamus, olfactory bulbs
2C9	Human	mRNA	Brain tumour samples
2C	Rat	Protein	Substantia nigra, basal ganglia, multiple regions
	Rat	mRNA	Whole brain
	Mouse	mRNA	Whole brain
2D6	Human	mRNA, protein	Multiple regions, especially in hippocampus and cerebellum (Purkinje cells)
2D	Rat	mRNA, protein	Cerebellum, hippocampus, olfactory bulbs; individual subfamily members' locations vary across regions
3A	Human	mRNA	Primarily in basal ganglia, frontal cortex, cerebellum, pons and red nucleus
	Human	Protein	Cortex, brain tumour cell cultures and neurons in normal brain areas
	Rat	mRNA	Whole brain
	Rat	Protein	Brain microsomes
	Mouse	mRNA	Whole brain

Another interesting issue is to elucidate how this local modulation would take place. There are several indications suggesting that endogenous modulation of the CYPs present in brain is likely to occur. For example, in 2003, A. M. Yu and his colleagues at the Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, reported that screening for endogenous substrates revealed that CYP2D6 is a 5-methoxyindolethylamine O-demethylase. The objective of the investigation by Yu et al. was to screen for potential endogenous substrates for CYP2D6. By employing recombinant CYP2D6, together with hepatic microsomes from CYP2D6-transgenic mice, human liver microsomes, and a specific anti-CYP2D6 monoclonal antibody, it was determined that CYP2D6 did not significantly metabolize the endogenous phenylethylamines 2-phenylethylamine, octopamine, synephrine, 3-methoxy-p-tyramine, 4-methoxy-m-tyramine, metanephrine, and normetanephrine, nor the indolethylamines tryptamine, serotonin, 6-methoxytryptamine, and melatonin, nor the beta-carbolines

harman, norharman and tryptoline in the population investigated in this study; however, the indolethylamines 5-methoxy-N,N-dimethyltryptamine (5-MDMT) and pinoline (6-methoxy-1,2,3,4-tetrahydro-beta-carboline) did show relatively high affinity for CYP2D6 in a spectral binding assay (K(s) 28 +/- 5, and 0.5 +/- 0.3 microm (mean +/- SEM), respectively) and were O-demethylated only by CYP2D6 in a panel of 15 recombinant common human P450s. Pinoline and 5-MDMT O-demethylase activities were 35- and 11-fold greater in liver microsomes from CYP2D6-humanized mice, respectively, than those in liver microsomes from control mice. Furthermore, the increased activities were completely inhibited by an anti-CYP2D6 monoclonal antibody. Kinetic analysis with recombinant CYP2D6 resulted in K(m) and k(cat) values for 5-MDMT and pinoline O-demethylations of 12 +/- 1 microm and 65 +/- 1 min(-1) and 1.8 +/- 0.3 microm and 26 +/- 1 min(-1), respectively. Yu et al. note that these two substrates can therefore be added to 5-methoxytryptamine, which they recently reported to be an endogenous CYP2D6 substrate[35]. "CYP2D6 is therefore a relatively highly specific, high-affinity, high-capacity 5-methoxyindolethylamine O-demethylase. Polymorphic cytochrome CYP2D6 may therefore exert an influence on mood and behavior by the O-demethylation of these 5-methoxyindolethylamines found in the brain and pineal gland". In addition, these processes may have an effect on mental and neurological health. These findings may provide new avenues of investigation for the determination of CYP2D6 phenotype [36].

These broad spectrums of P450 reactions are due to multiple P-450 isozymes with differing but overlapping substrate specificities. Some of the xenobiotics that require cytochrome P450 for their metabolism are able to induce expression of this cytochrome

and thus increase its amount in the organism. The most important inducers of human CYP 1A, which is considered most relevant to pollution of the aquatic environment, include PAHs, nitrated polyaromatic hydrocarbons (NPAHs), PCBs, dioxins (TCDD) and some pesticides [37,38].

The members of the CYP1A subfamily are responsible for metabolic activation of the majority of known promutagens and carcinogens that, in a long term, may be involved in carcinogenesis, reproductive disorders, etc.[39].

The induction of CYP1A is mediated by the Ah receptor (AhR), a xenobiotic-binding protein present in the cytosol. The receptor-contaminant complex linked to a nuclear translocator is transported to the nucleus, where its linkage to DNA results in expression of the gene coding for this cytochrome [40,41]. Generally, the toxicity of a pollutant is related to the degree of its affinity to AhR. Pollutants with a high binding ability for AhR also have a high capacity to induce CYP1A, which has adverse consequences, as described by Billiard et al. [40].

Human CYP2D6 catalyzes the hydroxylation of debrisoquine and a variety of commonly used pharmaceuticals such as dextromethorphan[42,43]. The expression of CYP2D6 in human populations is polymorphic. Polymorphism in debrisoquine/sparteine oxidation is arguably the most highly studied pharmacogenetic trait[44]. The DNA sequence encoding the enzyme has been localized to the 4.3-kb, nine-exon cytochrome P450 2D6 (CYP2D6) gene found at chromosome 22q13.1[45]. To date more than 48 mutations and 53 alleles of CYP2D6 have been characterized in European populations[4]. The poor metabolizer (PM) phenotype follows an autosomal recessive pattern of inheritance. An allele duplication consisting of multiple functional copies of CYP2D6

confers the ultrarapid metabolizer (URM) phenotype. Individuals who demonstrate normal levels of CYP2D6 activity are referred to as extensive metabolizers. Intermediate metabolizers (IMs) typically produce lower than normal levels of functional enzyme[46,47].

The liver enzyme cytochrome P450 CYP2D6 (debrisoquine 4-hydroxylase) metabolizes numerous drugs, including many antidepressants, neuroleptics, antiarrhythmics, and antihypertensive agents[48]. Variability in the gene that encodes this enzyme is an important factor underlying variable drug treatment response. Huchinson et al reported the metabolism of hydromorphone from liver microsomes of six CYP2D6 extensive metabolizers (EM) and one CYP2D6 poor metabolizer (PM). They showed hydromorphone formation in liver microsomes from CYP2D6 EMs was dependent on a high affinity enzyme (K_m = 26 μM) contributing 95%, and to a lesser degree a low affinity enzyme (K_m = 3.4 mM). In contrast, only a low affinity enzyme (K_m = 8.5 mM) formed this metabolite in the liver from the CYP2D6 PM, with significantly decreased hydromorphone formation compared with the livers from the EMs. Norhydrocodone was formed by a single low affinity enzyme (K_m = 5.1 mM) in livers from both CYP2D6 EM and PM. Recombinant CYP2D6 and CYP3A4 formed only hydromorphone and only norhydrocodone, respectively. Hydromorphone formation was inhibited by quinidine (a selective inhibitor of CYP2D6 activity), and monoclonal antibodies specific to CYP2D6. Troleandomycin, ketoconazole (both CYP3A4 inhibitors) and monoclonal antibodies specific for CYP3A4 inhibited norhydrocodone formation. Extrapolation of in vitro to in vivo data resulted in a predicted total hepatic clearance of 227 ml h^{-1} kg^{-1} and 124 mlh^{-1} kg^{-1} for CYP2D6 EM and PM, respectively[49].

Nakamura et al in another study compared bufuralol 1'-hydroxylase activity among liver microsomes prepared from individuals whose CYP2D6 genotypes had been determined, they recognized that the activity tends to decrease depending on the number of the CYP2D6*10 allele. Pre-incubation of liver microsomes from individuals homozygous for the CYP2D6*10 allele resulted in a decrease in the enzyme activity more rapidly than those from individuals homozygous for the CYP2D6*1, suggesting that not only the catalytic activity but also the thermal stability of the enzyme appeared to be affected by the genetic polymorphism. To confirm this hypothesis, Nakamura and his colleagues compared the kinetic parameters of CYP2D6.1 and CYP2D6.10 for bufuralol 1'-hydroxylation and dextromethorphan O-demethylation using microsomes prepared from yeast transformed with plasmids carrying CYP2D6 cDNAs (*1A and *10B). Kinetic studies of these CYP2D6 forms indicated clear differences in the metabolic activities between the wild (CYP2D6.1) and the mutant enzymes (CYP2D6.10).

Furthermore, bufuralol 1'-hydroxylase activity in microsomes of yeast expressing CYP2D6.10 was rapidly decreased by heat treatment, supporting the idea that the thermal stability of the enzyme was reduced by amino acid replacement from Pro (CYP2D6.1) to Ser (CYP2D6.10). These data strongly suggest that the thermal instability together with the reduced intrinsic clearance of CYP2D6.10 is one of the causes responsible for the known fact that Orientals show lower metabolic activities than Caucasians for drugs metabolized mainly by CYP2D6, because of a high frequency of CYP2D6*10 in Orientals [50].

Defects in the CYP2D6 gene have been associated with a number of CNS diseases such as Parkinson's disease (PD), Alzheimer's disease, neuroleptic-induced

disorders such as tardive dyskinesia and certain types of CNS cancer[51], although not all studies agree.

Furthermore, genetic analyses has revealed the association of the CYP2D6 B mutation with PD[52,53]. The B mutation of the CYP2D6 gene is a G to A transition at the intron 3 - exon 4 junctions, which shifts the position of the 3' splice site, leading to a frameshift[45].

Using a sample of CYP2D6 duplication-negative ultrarapid metabolizer subjects and selected control subjects with extensive metabolism, Lovlie and his co-workers examined parts of the CYP2D7 pseudogene, and the promoter region and 5'-coding sequence of CYP2D6 for polymorphisms possibly associated with the ultrarapid metabolizer phenotype. In an initial screening of 17 subjects (13 ultrarapid metabolizers and four extensive metabolizers), they identified three DNA variants in the 5'-end of the CYP2D7 pseudogene and 29 variants in the 5'-end of the CYP2D6 gene. Five variants were selected thereof for examination in a larger sample of subjects having the ultrarapid metabolizer (n = 27) or extensive metabolizer phenotype (n = 77). Subsequent statistical analyses of allele, genotype and estimated haplotype distributions showed that the 31A allele of the 31G > A (Val (II) Met) polymorphism was significantly more frequent in ultrarapid metabolizer subjects than in extensive metabolizer subjects (P = 0.04). Also, estimation of haplotype frequencies suggested that one of the haplotypes with the 31A variant was significantly more frequent among the ultrarapid metabolizers compared with the extensive metabolizers (P = 0.03). The average metabolic ratio was significantly lower in subjects possessing the 31A allele compared with subjects homozygous for the 31G allele (P = 0.02)[54].

Huang et al. on the other hand reported variability in CYP2D mRNA from human breast tissue. Using gene-specific oligonucleotide probes, they were able to trace full length mRNA and six distinct variants that expressed from CYP2D7*P* pseudogene rather than CYP2D6. The full length mRNA was expressed in minor form whereas two variants b' and c were dominant[55]. Woo et al examined a similar pattern of splice variation from human brain. Out of 94 samples tested, they found that the majority of expressed transcript corresponded to the shortened clone as found by Huang et al from breast tissue[56]. More interestingly, Pai and co-workers reported a functional splice variant of CYP2D7 from brain tissue that can metabolize codeine to morphine more efficiently than CYP2D6. This brain variant contains a partial inclusion of intron 6 (57 bp) in the transcribed mRNA sequence of CYP2D*7* mRNA[12].

Various methods including catalytic, pharmacological, immunological, and molecular criteria have been used to identify cytochrome CYP2D in mammalian brain[1,57,58]. Niznik and co-workers made the initial observation of CYP2D in dog brain during screening of central and peripheral tissues with tritiated GBR-12935, which labels the dopamine transporter protein and the so-called piperazine acceptor site or mazindol-insensitive site in brain tissue[57]. High concentrations of the piperazine acceptor site were found in liver microsomes. The similarity between amphetamine derivatives that inhibited both GBR-12935 striatal binding and hepatic CYP2D6 activity prompted further studies that demonstrated correlations between the inhibitor profile at the piperazine acceptor site purified from dog striata and the inhibition constant (Ki) for human hepatic CYP2D6 ($r = 0.85$).

Subsequent studies by Tyndale (1991) focused on the catalytic activity of CYP2D in canine striata. One of the classic CYP2D6 substrates, sparteine, was used as the marker for CYP2D activity in dog striata. High ($r > 0.95$) correlations were observed between inhibition of sparteine oxidation (Ki values) in canine striata and in human hepatic microsomes, and in human CYP2D6 expressed in HepG2 cells ($r = 0.93$). (-)-Cocaine was found to have particularly high inhibitory potency (Ki= 74 nanomolars (nM) for canine striatal CYP2D), and a high degree of overlap was found between compounds binding to the dopamine transporter and striatal CYP2D. The distribution of CYP2D in dissected regions of human brain demonstrated a forty fold range in activity, with the highest level being found in supraorbital cortex and parietal cortex, and the lowest in the cerebellum[58].

Methods

Human Tissue Samples. Samples of normal human tissues including liver, cerebellum and cortex were collected from West Virginia University tissue bank, Department of Pathology (School of medicine; Morgantown, WV). The tissues obtained from tissue bank were obtained as frozen tissues that were either used directly or paraffinized before use depending upon the study.

Preparation of Brain Microsomes and Mitochondrial Fraction. Brain microsomes and mitochondrial fraction was prepared as suggested elsewhere[59,60]. Briefly, 4g of the brain were homogenized in 9 volumes of 0.1M Tris containing, 0.1mM dithiothreitol, 0.1mM phenylmethylsulfonylfluoride, 0.2mM EDTA, 1.15% potassium chloride and 10% glycerol at pH 7.4 (Buffer A). The homogenates were centrifuged at 17000 x g for 30 min. The crude mitochondrial fraction obtained was reconstituted in 15% percoll (10mls/g of tissue). The discontinuous density gradient was prepared by layering resuspended pellet onto preformed layers of percoll consisting of 23% and 40% percoll (3.5ml each 15, 23 and 40% percoll in centrifuge tube). The centrifuge tubes were spun at 30,000 x g for 10 min that formed three major bands. The material banding between lower two percoll layers was carefully aspirated and diluted 1:4 in buffer A and centrifuged again for 10 min at 15,000 x g. The pellet formed was reconstituted in small volume of buffer A and stored in -80°C for further investigation. The supernatant obtained previously at 17,000 x g was collected to which was added 8mM solid calcium

chloride and spun at 30,000 x g for 1h. The microsomal pellets formed were suspended in 3mL of buffer A and a reduced CO spectrum was recorded.

Preparation of Liver Microsomes. Four grams wet weight of human liver tissue was homogenized in four volumes of homogenization buffer (0.15M KCl, 0.25M potassium phosphate buffer at pH7.4) that was supplemented with fresh PMSF (1:1000). The homogenate was centrifuged for 5 min at 5000 rpm and for 15 min at 11,500 rpm at 4°C. The supernatant obtained above were combined and centrifuged for another 1h at 35000 rpm at 4°C. The pellets obtained in this step was resuspended in homogenization buffer containing PMSF (1:1000) (initially volume used) and centrifuged again for 1h at 35000 rpm. The pellets obtained were finally resuspended in small volume of storage buffer (100mM potassium phosphate buffer, 1mM EDTA, 20% glycerol, 1mM DTT and 20μM BHT) and stored in -80°C until further use.

Immunohistochemical Studies. The cerebellum tissue was obtained from West Virginia tissue bank. The sections were frozen in OCT embedding medium (Tissue-Tek; Torrance, CA), cut 10-μm thick on a cryostat and placed on 3 amino propyltriethoxysilane -coated slides. IHC staining was performed using Ventana/ View™ DAB kit (Ventana Medical Systems Inc; Tucson, AZ) on BenchMark IHC™ System (Ventana Medical Systems Inc; Tucson, AZ). Briefly, the sections were air dried and then immersed into acetone for 10 min each. The primary antibody specific to CYP2D6 (1:500; MAB 2D6; Gentest, Woburn, MA) was applied and incubated for 32 min at 42ºC. Then sections were washed with PBS, 5 min each and incubated with universal biotinylated secondary antibody (Ventana/ View™ DAB kit). Sections were then treated with streptavidin-HRP for 8 min at 42ºC, washed with PBS, and incubated with DAB

substrate for 4 min at 42ºC. Counterstaining was carried out with hematoxylin (Sigma–Aldrich; St Louis, MO). Controls were routinely included.

Immunoprecipitation. 10% w/v whole cell lysate was prepared from cerebellum tissue in Ripa Lysis Buffer (Santa Cruz Biotechnology Inc, Santa Cruz, CA). After protein measurement, the cell lysate was ultra-centrifuged at 48000 x g for 30 min to separate soluble proteins from insoluble proteins[61]. The pellets were suspended again in Ripa Lysis buffer. To the supernatant and to reconstituted pellets were added primary CYP2D6 polyclonal antibody and incubated for 1hr at 4°C. After 1 hr, protein G - Agarose beads (Santa Cruz Biotechnology Inc, Santa Cruz, CA) were added to the above suspension and incubated for additional 1hr at 4°C on a rocker platform. The immunoprecipitates were collected by centrifugation at 14000x g for 30 sec at 4°C. The supernatant from the soluble and the pelleted fractions were removed by aspiration and saved. The remaining precipitated beads were washed three times with Ripa Lysis Buffer followed by additional wash with PBS to remove the detergent. For controls the addition of primary antibody and protein G -Agarose beads were reversed.

Pre-made tissue lysates from brain regions including hippocampus, frontal cortex and cerebellum (Geno-Tech Inc., St. Louis, MO) were obtained and immunoprecipitated. They were analyzed for CYP2D6 as stated earlier.

SDS-PAGE and Immunoblotting. The immunocomplexes obtained above were resuspended in 100 µl of 5% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol, and 1.5 M Tris/HCl buffer, pH 6.8. Immunoprecipitated proteins were eluted from protein G-Agarose by heating at 95°C for 10 min, and 10 µl were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (7 x 8 cm; 10% separating

gel and 3.9% stacking gel). The gels were transferred on nitrocellulose paper at 200mA for 4h. Non-specific binding was prevented by blocking with 5% nonfat dry milk in TBST (25mM Tris (pH 7.5), 150mM NaCl) for 1h at room temperature. The antibodies used in this study are specific for CYP2D6 which were used in appropriate dilution (1:500 for MAB2D6 or polyclonal 2D6 1:9000 in 0.5% nonfat dry milk in TBST) to incubate the nitrocellulose for 1h and the immunocomplexes that were detected with 1:500 anti mouse IgG. Three washing with TBST for five minutes was followed by NBT/BCIP detection.

2-D Electrophoresis. For the first dimension, a 7 cm pH 3-10 linear IPG strip (Immobine™, Amersham Biosciences, Piscataway, NJ) was rehydrated with 125µl of 2-D solubilizing solution (8M urea, 4% CHAPS, 1% DTT, 0.5%v/v pharmalytes pH 3-10 and 0.002% bromophenol blue) containing about 1mg of protein. Isoelectric focusing was conducted at room temperature at a maximum for 2000V for 4h using Multiphor II (Pharmacia, Piscataway, NJ). For the second dimension, the IPG strip was equilibrated in equilibrium buffer (50mM Tris (pH 6.8), 6M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue) for 15 min containing 10mg/ml DTT and for another 15 min in equilibrium buffer containing 25mg/ml iodoacetamide. The IPG strip was subsequently placed on top of a 10% separating gel (7 x 8 cm). Gels were run on Mini Protean II vertical electrophoresis system (Bio-Rad, CA) at constant voltage of 200V followed by electroblotting on to nitrocellulose membrane at 200mA for 4h. The membrane was stained with ponceau S to detect the spot at the pI range of CYP2D6. After initial detection the spot was destained with TBST. Immunoblotting was carried out in a similar way as discussed previously under immunoblotting.

Pre-made 2-D gels (4-20% gradient) for cerebellum and liver samples were transferred onto PVDF membranes and supplied to us (Geno-Tech Inc., St. Louis, MO) which were immunoblotted for CYP2D6 as discussed under the section of immunoblotting.

In-Gel Tryptic Digest and Peptide Extraction. SDS-PAGE for immunocomplex was obtained as described under the section of SDS PAGE and immunoblotting. The molecular weight region on SDS-PAGE gel between 45 and 64 kDa was divided into three bands, and each band was carefully excised with scalpel. Bands were neutralized first with distill water and then completely destained with destaining solution (1:1 100mM ammonium bicarbonate/methanol). Bands were further macerated to $1mm^2$ pieces to increase the surface area then dried under vacuum for 5 minutes. Digestion of dried gel pieces were carried out using 50 µL of 2 µg/ml SDS (100 ng) trypsin covered with 25 mM ammonium bicarbonate solution for 12 h at 37°C. The samples were loaded onto ZipTip C18 (Millipore) which was previously equilibrated with 10 µl 10% ACN, 0.1% TFA. The peptides were eluted from the gel pieces with drawing samples up and down for 10 times into 4µl of 2% acetic acid and 50% ACN solution

LC-ESI-MS/MS. Nanoscale LC was performed using Thermo Finnigan LCQ deca XP Plus. Approximately, 2µl sample in 0.1% acetic acid was loaded onto column using helium pressure cell. The sample was washed for about 5min over C18 10 cm x 75 cm (5µm) column with 95% mobile phase A (0.1% acetic acid) and 5% mobile phase B (acetonitrile containing 0.1% acetic acid), at a flow rate of 300nl/min. After 5 min the flow of mobile phase B was increased linearly to 50% over 40 min. The column effluent

was continually detected into LCQ mass spectrometer fitted with nano-ESI source and spectra were recorded.

ESI was performed under the following conditions: positive ionization mode; spray voltage, 1.6kV; capillary voltage of 160°C; 35% MS/MS collision energy in ion trap and no sheath or auxiliary gas was used. Data was collected in a full scan mode and data dependent MS/MS mode. Three microscans were performed, with maximum ion injection time of 200ms. In full scan mode the ions were collected in *m/z* range of 400 to 2000.

Protein Identification. An MS/MS spectrum was searched using Sequest Browser software against a human database containing P450 proteins[62]. The Sequest finds the peptide sequence in database that best explains the fragment ion present in the spectrum. Candidate sequence is found in the database on the basis of intact peptide masses, and complete or partial spectra expected to result from the fragmentation pattern of peptide are generated and compared to experimental spectrum. The final score assigned to each candidate-fragmented peptide is called Xcorr, a measure of theoretical spectrum correlation to experimental spectrum. Minimum two peptides with high Xcorr values of $\geq$ 2.5 are considered significant for the identification of protein.

Results

Microsomal and mitochondrial P450: We investigated the expression of CYP2D protein from human cerebellum microsomes and mitochondrial fractions. The microsomes were prepared by calcium aggregation method[59]. Microsomes from liver were prepared according to the method of Anderson et al and were used as positive control[63]. The mitochondrial fractions were prepared along with microsomes by percoll discontinuous density gradient method[60]. The P450 peak measured by reduced carbon monoxide binding spectroscopy suggested low levels of P450 in cerebellum tissue compared to liver tissue (fig. 2 and 4). The microsomes prepared from cerebellum tissues contained further two fold less P450s compared to mitochondrial fraction (fig 2 and 3).

The microsomal and mitochondrial fraction was then analyzed for CYP2D protein by SDS-PAGE using specific CYP2D6 monoclonal antibodies as well as polyclonal antibodies. Our data was negative for the presence of CYP2D protein in mitochondria. The cerebellum microsomes when probed with CYP2D6 specific antibodies showed characteristic protein band.

Immunohistochemistry: We also investigated the presence of CYP2D protein in cerebellum tissues by immunohistochemistry. Immunohistochemistry was performed on both frozen as well as paraffin embedded tissues. The immunohistochemical localization of P4502D in human brain cerebellum depicted immunoreactive protein in the purkinje cells and in the granule cells in the cytoplasmic region (figure 5 A). The controls were also routinely included which did not show any immunostaining in the cerebellum (figure 5 B).

Immunoprecipitation and immunoblotting: We isolated CYP2D6 protein by immunoprecipitation method from cerebellum tissue using the specific antibody to CYP2D6. The antigen-antibody complex formed was then precipitated from solution by addition of an insoluble form of an antibody binding protein such as Protein G or second antibody. The precipitate formed was subjected to SDS/PAGE under denaturing conditions followed by western blot using CYP2D6 specific monoclonal antibody. Figure 6(A and B) shows CYP2D6 band at 50 kDa when cerebellum was immunoprecipitated with MAB 512-1-8[64]. No staining was observed when addition of Protein G- Agarose and CYP2D6 antibody was reversed for immunoprecipitation (lane 2, 3, 4). Immunoreactive proteins were also detected on three different brain regions namely, cerebellum, frontal cortex and hippocampus (Geno-Tech Inc., St. Louis, MO) with CYP2D6 specific antibody (fig. 6C). When CYP2D6 polyclonal antibodies were used, resulted in two immunoreactive bands on immunoprecipitation from brain cerebellum region (figure 6A). Similar pattern was also observed in matched liver, which was used as a control (figure 6D).

Furthermore, 2D- blot analysis for CYP2D protein from liver tissue (Geno-Tech Inc., St. Louis, MO), resulted in two spots about the pI range of 6-7, which suggests two CYP2D isoforms of CYP2D6 (figure 7B) existed in the liver tissue. The 2-D blots prepared from cerebellum tissue only detected one spot at the pI of 6.7 (figure 7A). This single spot confirmed the presence of CYP2D6 protein in the brain, the other spot corresponding to CYP2D isoform could not be ascertained due to background.

LC-MS/MS: Immunoblotting data from human brain cerebellum suggests two CYP2D isoforms. To further differentiate these P450 isoforms, mass spectrometric method was used. Our data generated by LC-MS/MS gave us three major peaks, which do not explain the P450 present in the brain. These three peaks represent the amount of alpha-tubulin protein only which large backgrounds (fig 8A and B).

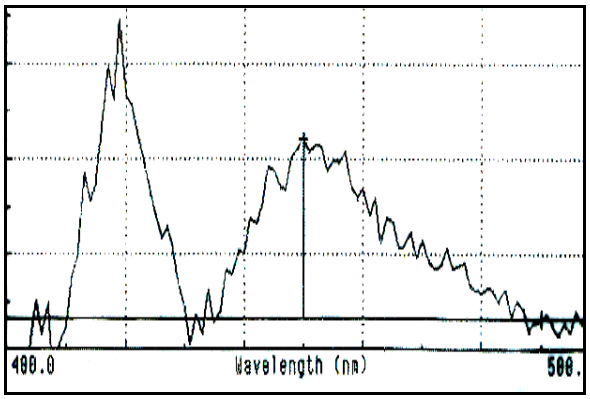


Figure 2 The reduced carbon monoxide spectra of human cerebellum microsomes prepared by calcium aggregation method [59]. The specific P450 content was estimated to be 0.3 pmoles/mg of tissue.

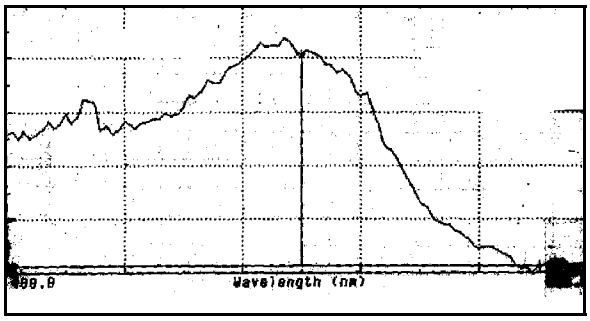


Figure 3 Dithionite reduced CO spectra obtained from mitochondrial fraction of cerebellum. The specific P450 content estimated was 0.65 pmoles/mg of tissue.

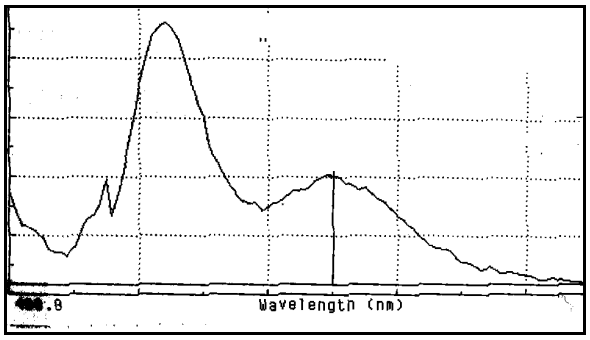
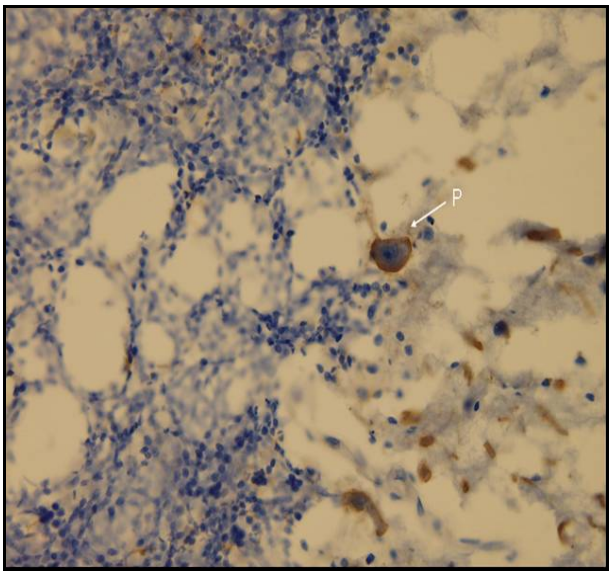


Figure 4 The CO difference spectroscopy from human liver microsomes. The estimated P450 content measured was 1nmoles/mg of tissue.

A



B

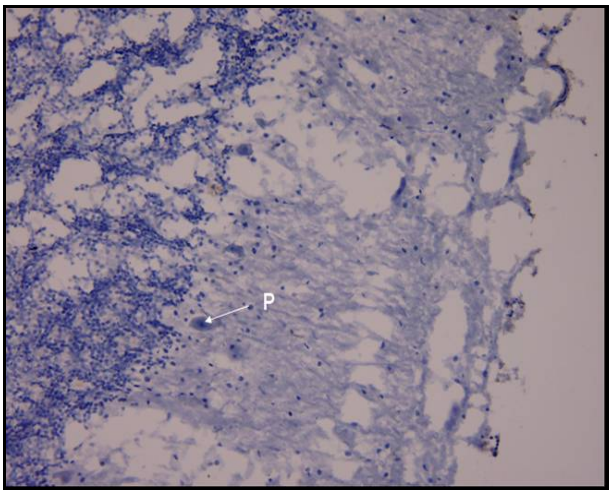


Figure 5 Immunohistochemical localization of CYP2D in human brain cerebellum. (A) Depicts the localization of CYP2D in granule cells and Purkinje fibers. (B) Depicts the control section with no staining in the plasma membrane.

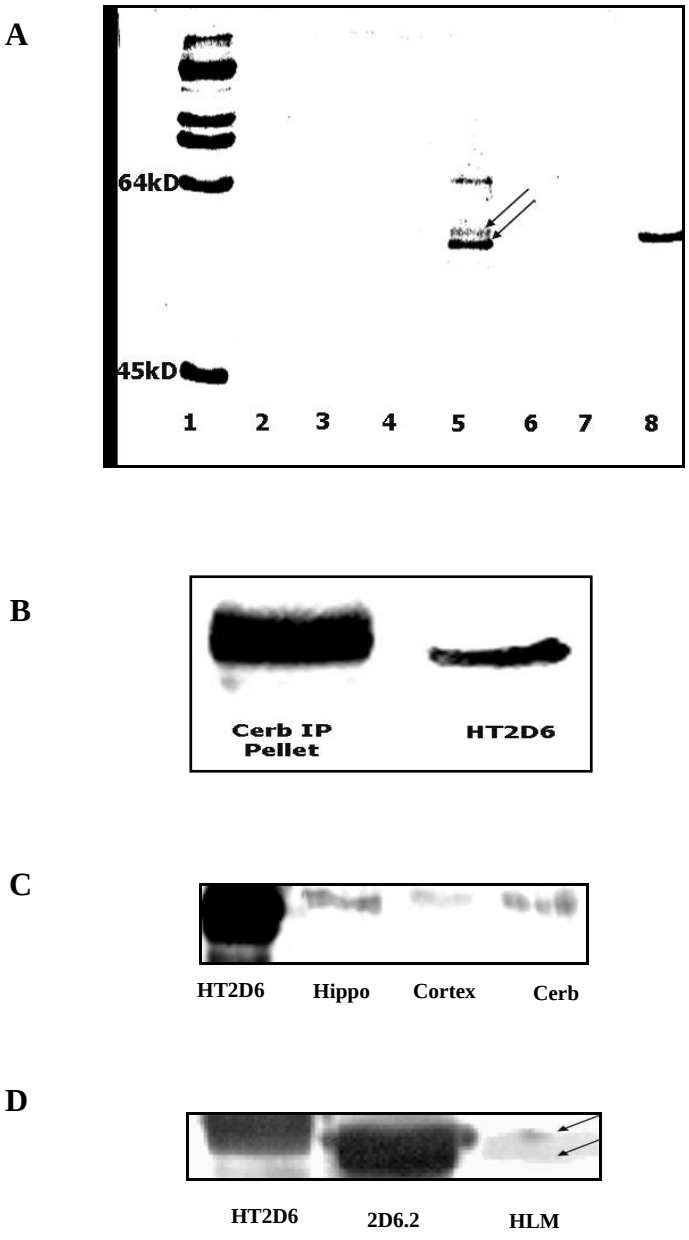


Figure 6. (A) Depicts the immunoprecipitation from human brain cerebellum lysate using polyclonal antibody to CYP2D6. Lanes 2,3,4 are lysate, supernatant and pellets prepared from the tissue respectively in, which the addition of antibodies was reversed. Lane 5 is a pellet fraction, which is immunoprecipitated with protein G-Agarose beads. Lanes 6 and 7 are the supernatant respectively treated similarly as lane 5. Lane 8 is the positive control containing recombinant CYP2D6 histidine tagged. (B) Lane 1 depicts the immunoblot analysis of immunoprecipitated CYP2D from the pellets. (C) Immunoprecipitation was carried out from pre-made lysates for cerebellum, hippocampus and cortex using monoclonal CYP2D6 antibodies. (D) Immunoblot analysis using polyclonal CYP2D6 antibodies from human liver microsomes depicts two immunoreactive bands.

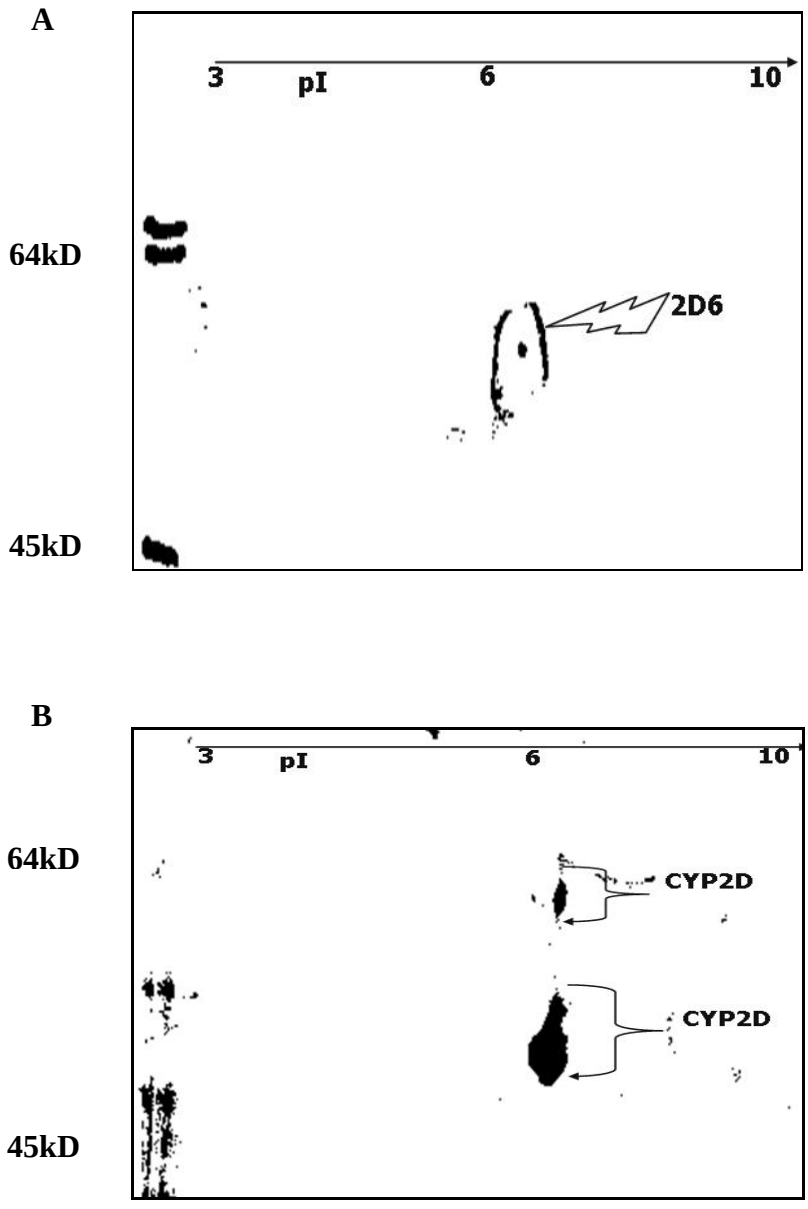
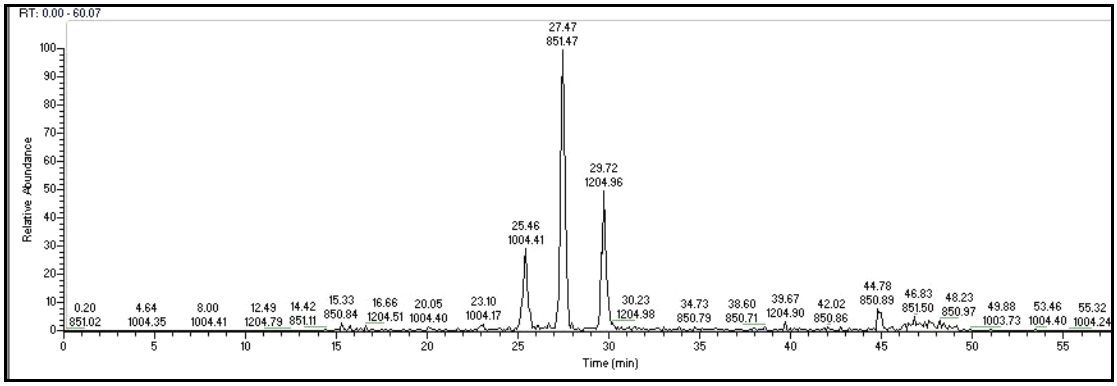


Figure 7 2-D western blots. (A) 2-D blot of human brain cerebellum when probed with monoclonal CYP2D6. The single spot was observed at the pI of 6.7 and a molecular weight region of 45-64 kD. (B) Pre-made 2-D western blot of human liver (Geno-Tech Inc.) showing two spots at the pI of 6.7 and molecular weight region of 45-64 kD when probed with polyclonal CYP2D6 antibodies.

A



B

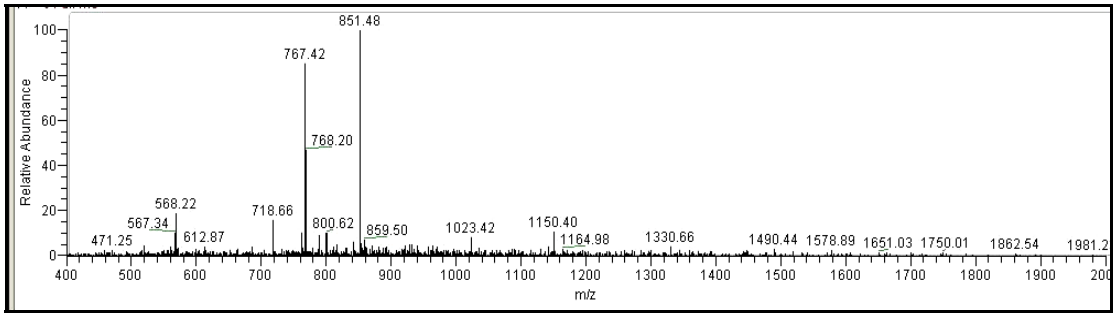


Figure 8 Nano spray LC-MS/MS performed on immunoprecipitated protein. (A) depicts the three most abundant peaks found on chromatography and (B) represents their molecular weights. The molecular weights corresponded to the sequence of alpha-tubulins.

Discussion

Cytochrome P450s are thought as liver enzymes associated with the endoplasmic reticulum. P450s therefore are accepted as the primary detoxification pathway for xenobiotics[18]. Typically there is a vast body of literature on P450s; still the information pertaining to human CYPs remains incomplete. For example, only CYP2D6 has been mapped throughout human brain[65]. This could be attributed to ethical issues with respect to human subjects. Therefore, most studies concerning distribution of P450s have been carried out only in animal models. The first P450 that was isolated and characterized was $P450_d$ from rat, which showed similar substrate specificity as human CYP1A2. This form was also shown to be induced by polyaromatic hydrocarbons and polychlorinated biphenyls [66]. Unfortunately, the rat is not a good model of human metabolism, which is dependent on two of the most important human P450 enzymes, CYP3A4 and CYP2D6. For example, the rat ortholog CYP3A1 is not induced by the typical CYP3A inducer rifampicin [67]. Moreover the CYP2D1 enzyme of rat; an orthologous of human CYP2D6, shows significant differences in mechanism of inhibition as illustrated by a lack of inhibition by quinidine [68]. Dexmethorphan, a marker substrate of CYP2D6, has been shown to be metabolized by CYP2D2 of rat and not CYP2D1 [69].

Rabbit CYP enzymes were the first mammalian P450s that were crystallized [70]. Though rabbit CYP P450 isoforms have close substrate specificity for human CYP2E1 and CYP1A2, they lacked a good partner for human CYP2D6 [71]. Many other animal models including beagle dogs, monkeys (Maccacus *rhesus, Cynomologus,* and *Marmoset)*, and minipig have been used, but all animals differ in between one or the

other human CYP counterparts [72,73]. In addition to these animal models, human CYPs have been characterized from different human tissues. Besides liver, CYP2D6 mRNA or/and protein has been demonstrated in lung, blood, skin, and brain [11,65,74].

Though human models would be ideal for most metabolism studies, many ethical issues prevent them. Moreover, studies from autopsies tissues though relate closely to the living human model, there is always scarcity and difficulty in acquiring these tissues. Also, post-mortem delays may have their own affect on the enzymatic activity. Therefore, recently Corchero et al described use of humanized mouse model. Here, FVB/N mouse stains were used to generate CYP2D6 transgenic animals[75]. Though these models were adequate to study CYP2D6 associated pharmacokinetics from liver, these model failed to express CYP2D6 protein in brain. One of the studies conducted in our lab suggested no significant protection from MPTP induced dopaminergic neurons atrophy (unpublished observation). Therefore, the best model that can be studied for CYP2D6 in entirety is from human autopsies samples.

Stobel's lab and many others have reported brain specific CYP2D6 to metabolize CYP2D6 specific drugs in vitro[23,24,76]. Such extra-hepatic metabolism of psychoactive drugs and regional specific distribution of CYP2D6 that possess the catalytic activity has generated immense interest in the possible contribution of this class of enzymes in neurophysiology. Furthermore, it is also possible that this enzyme can undergo splice mechanism to form many other proteins or closely related protein such as CYP2D7; for instance, CYP2D7 was recently found to undergo splicing and form an active protein that can metabolize codeine to morphine in the brain which was earlier known only to be metabolized by CYP2D6[12]. Likewise, CYP1A1 and CYP1A2 (though not CYP2D)

proteins have shown similar function to CYP2D, as they are also drug-metabolizing enzymes. The 1A1/2 has been shown to have targeting sequence to mitochondria as well as endoplasmic reticulum. The mitochondrial CYP1A1/2 is different from liver CYP1A1/2 since mitochondrial CYP1A1/2 is 30 amino acids shorter than liver 1A1/2. This 30 amino acid sequence had brought great change in the substrate specificity of CYP1A1/2 in the brain in a sense; it is similar to CYP3A4 drugs now[13]. According to sequence analysis of CYP2D protein, we found that the CYP2D6 protein also has series of positively charged residues in its sequence that can act as mitochondrial target. Therefore, we investigated the expression of CYP2D protein from the mitochondrial fraction prepared from human brain cerebellum. Mitochondrial fractions were prepared by percoll discontinuous density gradient according to Sims [60] and were studied for mitochondrial P450 CYP2D protein by probing it with CYP2D6 specific monoclonal as well as polyclonal antibodies. Our data does not suggest the presence of CYP2D protein in mitochondrial fraction (fig. 3 and data not shown), but it has to be appreciated here that this is possible. Most of the studies carried out for analysis of P450 in brain have been done using specific inducers of P450. Since CYP2D6 does not have any specific inducer, the complexity of isolation of small amount of protein from brain remained difficult. Moreover, localization of P450 in the inner membrane of mitochondrial membrane may cause difficulties for immunoglobulins to interact with the proteins. It has been suggested that freeze thawing the mitochondria couple of times may facilitate the antigen-antibody interaction[77].

To make sure the acquired tissue had enough CYP2D6 protein expression, we also investigated the presence of CYP2D protein in cerebellum tissues by

immunohistochemistry. Immunohistochemistry was performed on both frozen as well as paraffin embedded tissues. The immunohistochemical localization of P4502D in human brain cerebellum depicted immunoreactive protein in the purkinje cells and in the granule cells in the cytoplasmic region (fig 5). Interestingly, the frozen tissue gave better demonstration of protein localization compared to paraffin embedded slides. This could be because the frozen sections allow excellent antigen preservation compared to paraffin embedded tissues where antigen gets cross-linked to fixative reagents.

Furthermore, with the recent report of Pai et al that showed the presence of functional CYP2D7 splice variant in the brain[12], we carried our investigation further with the characterization of CYP2D protein from the human brain tissue. In our investigation, we used the novel method of one step immunoprecipitation to study the characterization of CYP2D protein. Immunoprecipitation method was used since it involves the complexation of protein with its specific antibody that can be precipitated from solution by addition of an insoluble form of an antibody binding protein such as Protein G or second antibody. This method not only helps in removal of many hindering protein but also concentrates the low levels of protein present in the tissue[78,79]. The precipitate formed was then subjected to SDS/PAGE under denatured conditions followed by western blot using CYP2D6 specific monoclonal antibody. Our results showed a characteristic CYP2D band at 50 kDa when cerebellum was immunoprecipitated with MAB 512-1-8 [64] (fig 6 A and B). No staining was observed when addition of Protein G-Agarose and CYP2D6 antibody was reversed for immunoprecipitation (fig 6A lanes 2, 3, and 4). Immunoreactive proteins were also detected on three different brain regions namely; cerebellum, frontal cortex and hippocampus (Geno-Tech Inc., St. Louis, MO),

which depicted expressed CYP2D6 (fig 6C). Interestingly, when CYP2D6 polyclonal antibody were used, resulted in two immunoreactive bands from brain cerebellum region (fig 6A). With recent report that CYP2D7 produced an active form of splice variant in brain, we assume that these two bands correspond to CYP2D6 and CYP2D7. Similar pattern was also observed in matched liver, which were used as a control (figure 6D).

Furthermore, on premade 2D- blot for liver (Geno-Tech Inc., St. Louis, MO), we saw two spots about pI 6.7 region which suggests two CYP2D isoforms of CYP2D6 (figure 7B) whereas the 2-D blots prepared from cerebellum tissue only detected one spot at the pI of 6.7 (figure 7A). This single spot detection though confirmed the presence of CYP2D6 protein in the brain, the other spot corresponding to CYP2D isoform could not be ascertained due to background. Also, since we are looking at the membrane bound proteins, it might be possible that the one of the protein did not migrate well.

In order to characterize the CYP2D isoform(s) obtained as a result of immunoprecipitation of cerebellum tissues, we studied these bands on nano-spray LC-MS/MS[80,81]. This is because it was hard to differentiate these corresponding bands with the specific antibodies due to their limited availability. Activity assays were also performed with the cerebellum microsomes using MPTP as a substrate, but the results were below detection as the expression of P450 in brain reported is about 1% that of liver. Therefore, an alternative method that can best be applied to characterize the P450 from brain homogenates was the use of mass spectrometric method as it may be able to analyze low levels of multiple proteins in single run. To date, the reports on analysis of P450 by mass spectrometry have been very few; the majority of which have relied on the analysis of the metabolite generated by P450-substrtae reaction. This method though

gives unique advantages over other conventional methods also have the limitation as the coverage of protein by this method is only about 40%. Our data generated by LC-MS/MS gave us three major peaks, which do not explain the P450 present in the brain. These three peaks represent the amount of alpha-tubulin protein only which large backgrounds (fig 8A and B). We did not detect cytochrome P450 isoforms by our LC-MS/MS analysis, but this does not exclude the presence of P450 from the sample. P450s might be present in very small amounts with the high levels of alpha – tubulin as an interfering protein.

Summary

Exposure to MPTP has been reported to cause symptoms of Parkinson's disease. It has been hypothesized that the expression of CYP2D6 in human brain may alleviate the symptoms of disease by metabolizing MPTP to non toxic metabolite PTP that may balance its activation by MAO-B to MPP^+ in brain. This hypothesis was further supported from epidemiological studies where Parkinson's disease has been reported to be prevalent in CYP2D6 poor metabolizers. Furthermore, CYP2D6 has been suggested to undergo splicing in brain, which may affect the activity of the substrates at the site of action. With this background knowledge and current report that CYP2D7 can also form an active protein by splicing mechanism in brain, prompted us to investigate the different form of CYP2D proteins that can be found in the brain. Therefore, we investigated the protein by novel method of immunoprecipitation as the amounts of these proteins were expected to be found in very low concentrations. Our immunoprecipitation data supports that there are two possible isoforms of CYP2D protein in brain that may affect the site dependent metabolism of many psychoactive drugs. The immunoblot analysis from the human liver microsomes prepared from the same subject also depicted the unusual two bands which inferred that more than one CYP2D isoforms may be expressed in human liver. This is a very interesting finding since the splicing of CYP2D7 is only recognized to be found in the brain. Further support to this finding was seen in pre-made 2-D blot of liver, which showed two distinct spots for CYP2D when probed with CYP2D6 polyclonal antibodies suggesting that the CYP2D isoforms may be available in other individuals. With all these finding we were still unable to confer with certainty the sequence analysis for these proteins by nano-spray LC-MS/MS. This was because the

amount of protein we are looking at is present in a very low concentration in human brain, the detection of which is also inhibited by lipids and the most abundant interfering proteins such as alpha and beta tubulins.

In future, more tedious methods have to be employed to immunoprecipitate and separate the proteins; as well as various denaturation buffers can be tried in order to separate the proteins from the membranes and their detection by LC-MS/MS.

Conclusion

The current study showed that the CYP2D protein have been conclusively identified by one-step immunoprecipitation method. This is a first report to best of our knowledge that has shown more than one CYP2D isoform(s) exist in the brain cerebellum tissue. Furthermore, our results are also consistent with the previously published studies describing expression profile of CYP2D protein in the human brain cerebellum. Clearly, there is a considerable advantage in using immunoprecipitation method in the characterization of P450s from complex tissues.

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Appendix

Brain microsome buffer	0.1M Tris containing, 0.1mM DTT, 0.1mM PMSF, 0.2mM EDTA, 1.15% KCl and 10% glycerol at pH 7.4
Storage buffer	100mM potassium phosphate buffer, 1mM EDTA, 20% glycerol, 1mM DTT and 20μM BHT
Liver microsome buffer	0.15M KCl, 0.25M potassium phosphate buffer at pH7.4
Sample buffer	5% SDS, 20% glycerol, 10% mercaptoethanol, and 1.5 M Tris/HCl buffer, pH 6.8
IP buffer	1X TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.004% sodium azide, 0.1% SDS, protease inhibitors, PMSF and sodium orthovandate
2-D buffer	8M urea, 4% CHAPS, 1% DTT, 0.5%v/v pharmalytes pH 3-10 with 0.002% bromophenol blue
Equilibration buffer	50mM Tris (pH 6.8), 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue