DMD Fast Forward. Published on December 13, 2012 as doi: 10.1124/dmd.112.048108

The Influence of Sex, Ethnicity, and *CYP2B6* Genotype on Bupropion Metabolism as an Index of Hepatic CYP2B6 Activity in Humans

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Running Title: Influences on CYP2B6 Activity using Bupropion

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The number of text pages: 27
The number of tables: 4
The number of figures: 0
The number of references: 31
The number of words in the *Abstract*: 250
The number of words in the *Introduction*: 625
The number of words in the *Discussion*: 1,366

ABBREVIATIONS: CYP2B6, Cytochrome 2B6; HIV, Human immunodeficiency virus; AAF, African-American Female; HAF, Hispanic-American Female; CAF, Caucasian American Female; CAM, Caucasian American Male; CA, Caucasian; BU, Bupropion; HB, Hydroxybupropion; BMI, Body Mass Index; AUC, area under the curve; PCR, polymerase chain reaction; MR, Metabolic Ratio; CNV, copy number variation; SNP, single nucleotide polymorphisms; VAF-Variant Allelic Frequency; h-hour.

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ABSTRACT:

The effects of sex, ethnicity, and genetic polymorphism on hepatic CYP2B6 expression and activity were previosuly demonstrated *in vitro*. Race/Ethnic differences in CYP2B6 genotype and phenotype were observed only in women. To identify important covariates associated with inter-individual variation in CYP2B6 activity *in vivo*, we evaluated these effects in healthy volunteers using bupropion as a CYP2B6 probe substrate. A fixed 150 mg oral sustained-release dose of bupropion was administered to 100 healthy volunteers comprising four sex/ethnicity cohorts (n=25 each): Caucasian males Caucasian, African American and Hispanic females. Blood samples were obtained at 0 and 6 hours post-dose for the measurement of serum bupropion (BU) and hydroxybupropion (HB) concentrations. Whole blood was obtained at baseline for *CYP2B6* genotyping. To characterize the relationship between CYP2B6 activity and ethnicity, sex, and genotype when accounting for serum BU concentrations (dose-adjusted $\log_{10}$-transformed), ANCOVA model was fitted in which the dependent variable was CYP2B6 activity represented as the $\log_{10}$-transformed, metabolic ratio of HB to BU concentrations. Several *CYP2B6* polymorphisms were associated with CYP2B6 activity. Evidence of dependence of CYP2B6 activity on ethnicity, or genotype-by-ethnicity interactions was not detected in females. These results suggest that C*YP2B6* genotype is the most important patient variable for predicting the level of CYP2B6 activity in females, when measured by the metabolism of bupropion. The bupropion metabolic ratio appears to detect known differences in CYP2B6 activity associated with genetic polymorphism, across different ethnic groups. Prospective studies will be needed to validate the use of bupropion as a probe substrate for clinical use.

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Introduction

Cytochrome 2B6 (CYP2B6) represents approximately 5% of the total liver microsomal CYP content (Gervot et al., 1999; Stresser, Kupfer 1999). Its expression and enzyme activity has been shown to vary approximately 100 fold in human liver (Faucette et al., 2000). CYP2B6 is also expressed in extrahepatic tissues such as brain, kidney, intestine (Gervot et al., 1999), uterine endometrium (Hukkanen et al., 1998), skin (Jammohamed et al., 2001), lung (Ding and Kaminsky, 2003), and heart (Thum and Borlak, 2000). CYP2B6 can partially or completely metabolize approximately 70 substrates (Ekins et al., 1998; Ekins and Wrighton, 1999). It has been shown that CYP2B6 contributes to the metabolism of the anti-cancer pro-drugs cyclophosphamide (Chang et al., 1993) and ifosphamide (Roy et al., 1999) and to the non-nucleosidic reverse transcriptase inhibitors (NNRTIs) such as efavirenz (Ward et al., 2003) and nevirapine (Erikson et al., 1999).

CYP2B6 is one of the most polymorphic CYP genes in humans and currently has 30 defined alleles with over 100 described polymorphisms (http://www.imm.ki.se/CYPalleles/cyp2b6.htm). *CYP 2B6* gene polimorphism includes copy number variants (CNV), missense mutations, insertions and deletions, and mutations affecting gene expression and activity. The polymorphic nature of the cytochrome P450 (*CYP450*) genes affects individual drug response and adverse reactions to a great extent (Ingelman-Sundberg et al, 2007). Studies of human liver tissues have shown that females tend to express higher levels of CYP2B6 in liver than males (Lamba et al., 2003). Race/ethnic differences in CYP2B6 genotype and phenotype were observed in women, but not in men. The CYP2B6 activity was 3.6- and 5.0-fold higher, respectively, in Hispanic females than in Caucasian ($p < 0.022$) and African-American ($p < 0.038$) females. The level of CYP2B6

expression has also been linked to genetic polymorphism. Single nucleotide polymorphisms (SNPs) in intron-3 break point 15582C>T (which is frequently in combination with the exon-4 516G>T and influences CYP2B6 alternative splicing), and the exon-9 1459C>T SNP were reported to result in low CYP2B6 activity in women but not men (Lamba et al., 2003).

Several probe substrates have become extremely useful *in vitro* and *in vivo* for evaluating cytochrome P450 activity (Tucker et al., 1998; Streetman et al., 2000). However, only two CYP2B6 substrates have emerged as potential probe substrate for quantifying the level of CYP2B6 activity in humans. *In vitro* investigations using human liver microsomes and recombinant CYPs suggest that CYP2B6 is the primary CYP enzyme catalyzing the metabolism of bupropion (Faucette et al., 2000; Hesse et al., 2000) and efavirenz (Ward et al., 2003). Stereoselective bupropion hydroxylation has been used as *in vivo* phenotypic probe for cytochrome P4502B6 (CYP2B6) activity (Kharasch et al, 2008) and to investigate potential CYP2B6 drug interactions *in vivo* pharmacokinetic studies (Hogeland et al., 2007). It has been shown that *CYP2B6* polymorphisms influence the pharmacokinetics of bupropion (Kirchheiner, 2003). However reports of the effects of variant alleles on bupropion's pharmacokinetics and clinical outcomes are conflicting and the relationship between its pharmacokinetics and CYP2B6 activity has not been firmly established *in vivo*. In contrast, *CYP2B6* polymorphisms could influence metabolism of efavirenz and consequently be associated with differences in clinical outcomes (Gouden et al., 2010). Thus efavirenz might be a useful CYP2B6 probe substrate *in vivo*. However, evidence of important drug-drug interactions between efavirenz and other CYP2B6 substrates is limited, and practical use of efavirenz as a probe substrate is restricted by concerns regarding human immunodeficiency virus (HIV) resistance in individuals for whom HIV status is not known.

In this cohort-comparison study, we investigated the relationship between bupropion metabolism and sex, ethnicity, and *CYP2B6* genetic polymorphism. Four cohorts of normal healthy adult volunteers were studied: Caucasian American males (CAM), Caucasian American females (CAF), Hispanic American females (HAF), and African American females (AAF). The polymorphisms investigated were defined by single nucleotide polymorphisms known to alter CYP2B6 function.

Material and Methods

Study Design. All subjects in the four cohorts received a fixed open-label dose of bupropion (BU) as a probe substrate for the determination of CYP2B6 activity. Activity was measured as the $\log_{10}$-transformed- metabolic ratio of hydroxybupropion (HB) to BU concentrations in blood drawn 6 hours post-dose. The single time point concentration ratio method was supported by an analysis of pharmacokinetic data from a previous bupropion study with 12 healthy volunteers (Hogeland et al, 2007) in which serial blood concentrations suggested that at time points between 6 and 48 hours, $\log_{10}$(HB) and $\log_{10}$(HB/BU) concentrations were correlated with $\log_{10}$(HB $AUC_{0-\infty}$) and $\log_{10}$[(HB $AUC_{0-\infty}$) / (BU $AUC_{0-\infty}$)], respectively. Upon analysis the six hour time point was chosen because of its high correlation (correlation coefficient 0.91, R^2= 0.76) between the concentration ratio and AUC ratio (unpublished data). Each of the four cohorts comprised 25 healthy adult volunteers of ages 18-50 years. All subject groups studied lived in the same area. Each subject had reported that their four grandparents shared a common race / ethnicity. Eligibility did not depend on height, weight or body mass index (BMI). Screening for eligibility was based on self-reported ethnicity, medical history, screening physical examination and interview, vital signs, and clinical laboratory measurements (sodium,

potassium, chloride, bicarbonate, the blood urea nitrogen - BUN test, creatinine, glucose, calcium, magnesium, phosphorous, total bilirubin, total protein, albumin, aspartate aminotransferase - AST, alanine aminotransferase - ALT, alkaline phosphatase, gamma-glutamyl transpeptidase - GGT, and lactate dehydrogenase - LDH, coagulation panel [prothrombin time - PT/ partial thromboplastin time - PTT], and urinalysis). Exclusion criteria included the following: ongoing medical illness; allergy to bupropion; history of seizures or gastrointestinal / hepatobiliary surgery; reported history of alcohol abuse, smoking, illicit drug use; use of prescription medication within the past month or grapefruit juice and over-the-counter medication during the last two weeks; use of oral contraceptives, or lactating or pregnant. Any lab value clinically significantly outside the reference ranges previously established by the McLendon Laboratories of the University of North Carolina Hospitals was also grounds for exclusion.

The study was conducted at the Verne S. Caviness General Clinical Research Center at the University of North Carolina (UNC) at Chapel Hill from July 1, 2006 to November 6, 2007. Subjects arrived after an overnight fast. Women of child bearing potential had a urine pregnancy test (Bayer Status pregnancy test) prior to dosing performed at McClendon UNC Hospital Lab. Each subject received a fixed sustained-release oral dose of 150 mg bupropion (Wellbutrin SR® GlaxoSmithKline, Research Triangle Park, North Carolina, USA Lot# 6ZP2500), along with 240 mL of water. In this case subjects received a fixed amount (150 mg) rather than the same dosage of bupropion. This necessitated a weight-adjusted dose normalization of plasma concentrations before statistical analysis, because subjects received different bupropion dosage calculated on a per-kg-body weight basis. All subjects were asked not to eat and drink alcoholic

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or caffeine-containing beverages until the 6 hour blood sample was drawn. However, drinking water was allowed.

The study protocol and subject-informed consent were approved by The University of North Carolina Institutional Review Board (IRB), and Research Involving Human Subjects Committee (RIHSC) of the Food and Drug Administration (FDA). The study has been carried out in accordance with The Declaration of Helsinki. Written consent was obtained from each participant.

Blood Sample Collection. For BU and HB quantification, blood was taken prior to BU dosing and 6 hours post-dose. A 7 mL sample of blood was drawn from a forearm vein into sterile sodium Ethylenediaminetetraacetic Acid (EDTA) tubes. Upon collection, tubes were placed on ice for 30 minutes; plasma was separated by centrifugation at 3000 rpm for 15 minutes at -4°C. Plasma was then aliquoted into cryovials and stored at -80°C until analysis. For genotyping of *CYP2B6*, blood was collected prior to BU administration.

CYP2B6 Genotyping. Genomic deoxyribonucleic acid (DNA) was extracted from the peripheral whole blood of each subject by use of a DNA extraction kit (Qiagen). *CYP2B6* SNPs at intron 3 branchpoint (15582C>T), exon 4 (516G>T; Q172H, rs3745274), exon 5 (785A>G; K262R, rs22794343), exon 7 (983T>C, I328T, rs2399499) and exon 9 (1459 C>T, R487C, rs3211371) were genotyped by resequencing. Respective exons were amplified using intronic primers and the polymerase chain reaction (PCR) conditions described earlier [18]. Prior to sequencing, unincorporated nucleotides and primers were removed by incubation with Shrimp Alkaline Phosphatase (USB; Cleveland, OH) and Exonuclease I (USB) for 30 min at 37°C,

followed by enzyme inactivation at 80°C for 15 min. Sequencing was carried out with an ABI Prism 3700 Automated Sequencer using the PCR primers or internal primers (sequence available on request). Sequences were assembled using the Phred-Phrap-Consed package (University of Washington; Seattle, WA; http://droog.mbt.washington.edu/PolyPhred.html), which automatically detects the presence of heterozygous single nucleotide substitutions by fluorescence-based sequencing of PCR products (Nickerson et al., 1997).

Bupropion and Hydroxybupropion Assays. Bupropion and hydroxybupropion plasma concentrations were quantified using an Agilent 1100 Series LC/MSD with positive electrospray ionization. Blood plasma samples obtained from subjects 6 hours after bupropion administration were thawed and 10 µl of 4 µg/ml tripolidine (internal standard) was added to 250 µl of the sample. Chromatographic separation was carried out on a Phenomenex C8 (3.2x100 mm, 3.0 µm) column with gradient elution (4% to 50% acetonitrile in 5mM ammonium acetate, pH 4.6) at a flow rate of 0.3 ml/min. All analytes were converted to free bases by addition of 100 µl of 0.1N potassium hydroxide. Liquid-liquid phase extraction was performed with 1.5 ml hexane: isoamyl alcohol (96:4). The organic layer was collected and 50 µl of 4N hydrochloric acid was added to each sample. The eluted solutions were taken to dryness in a Turbo Vap LV evaporator (Zymark corporation, Hopkinton, MA), then the residues were reconstituted in 150 µl of the mobile phase and 25 µl of sample solution was injected into Agilent 1100 Series LC/MSD spectrometer. Liquid chromatography - Mass spectrometry (LC/MS) analyses were carried out with the use of positive electrospray ionization. Chromatographic separation was performed on a Phenomenex C-8 (3.2 x 100mm, 3.0µm) column with the use of gradient elution with a flow rate of 0.3 ml/min. Mobile phase A consisted of 4% acetonitrile and 96% water containing 5 mM

ammonium acetate at pH 4.6. Mobile phase B consisted of 50% acetonitrile and 50% water containing 5mM ammonium acetate at pH 4.6. The lower limit of quantification was 1 ng/mL for both analytes. Inter-and intraday coefficients of variation were ≤10%. The analytical precision of each series was controlled by including two standard solutions spiked with 20 and 100 ng/ml of bupropion and 50 and 200 ng/ml hydroxibupropion, respectively. Buproprion was purchased from Sigma, (St Louis, MO, USA) and hydroxybuproprion was purchased from Gentest, BD Bioscience (Woburn, MA, USA).

Data Analysis Strategy. Descriptive statistical methods were used to compare the four cohorts in terms of age (years), height (cm), weight (kg), and body mass index (kg/m^2). At the time of the study, bupropion was only available in a fixed, 150 mg sustained-release dosage form. Therefore, subjects could not receive a dose of bupropion adjusted for differences in body weight. Without normalizing bupropion plasma concentrations for differences in the actual dose per kg body weight each subject received, bupropion plasma concentrations would vary inversely to body weight and volume of distribution. Accordingly, prior to analysis, all 6-hour BU and HB concentrations were adjusted to correspond to a standard dose for a 70 kg weight individual. These dose-adjusted (normalized) bupropion and hydroxybupropion concentrations were then transformed to $\log_{10}$ scale because previous studies had suggested that these concentrations arise from underlying population distributions that are approximately log-normal (Helms, 1998; Julious and Debarnot, 2000). The dose-adjusted and transformed variables, $\log_{10}$ (BU) concentrations and $\log_{10}$ (HB) concentrations, are hereafter referred to as logBU and logHB, respectively. Log_{10} (HB/BU) or $\log_{10}$ Metabolic Ratio is hereafter referred to as logMR.

The primary aims of the study were achieved by model-based analysis of mean logMR relative to logBU concentrations and subject characteristics. The assumed linear statistical model for the logarithm of the metabolic ratio represented mean logMR as a function of logBU concentrations and other covariates representing cohort and genotype, and other selected subject characteristics. This analysis of covariance (ANCOVA) model for logMR is linearly equivalent (Helms, 1988) to an ANCOVA model for logHB in which the regression equation includes the very same covariates: logBU concentrations, cohort, genotype, and other characteristics. By virtue of this linear equivalence, all results of the ANCOVA analysis are invariant regardless of whether the dependent variable is logHB or logMR. We chose to focus on logMR as the primary outcome variable for analysis. The analysis results presented would not be altered by using logHB as the primary outcome variable.

To compare the three ethnic populations (AAF, CA, HAF) with respect to prevalence of variant genotypes, ethnicity-specific genotype frequencies were tabulated along with corresponding 95% confidence intervals. For each of five *CYP2B6* polymorphisms (in intron 3, exon 4, exon 5, exon 7, exon 9), a Fisher's exact test procedure of size $\alpha = 0.0125$ was used to test the null hypothesis "no differences among the three ethnic populations." The size of the test, $\alpha = 0.0125 = 0.05/4$ instead of 0.05/5 (i.e., a Bonferroni adjustment for multiple comparisons) was used to account for the well described linkage disequilibrium between exon 4 and exon 5 and the almost perfect redundancy between these two alleles noted in Table 3. If at least one of the five p-values was smaller than $\alpha = 0.0125$ then the over-arching null hypothesis (H_o) "no differences among the three ethnic populations with respect to genotype prevalence rates for these five genotypes" was to be rejected.

To characterize the relationship between logMR and ethnicity, sex, and genotype, an analysis of covariance (ANCOVA) model was fitted in which the dependent variable was log MR, and the independent variables included the covariate logBU concentrations centered at its mean (1.83 ng/mL), terms representing membership in the four cohorts, and terms representing genotype with respect to intron 3, exon 5, and exon 9. Due to differences in *CYP2B6* haplotypes in different ethnic groups, the effects of *CYP2B6* genetic variation may vary in these populations. Therefore we assumed that the effects of any variant genotype may not be the same from one ethnic group to another. Accordingly, the model also included interaction terms representing differential effects of intron 3, exon 5, and exon 9 across cohorts. It should be noted that the data did not support additional inclusion of exon 4, exon 7 and exon 9 in the model: the values for exon 4 were highly redundant of those for exon 5 due to linkage disequilibrium (note: these two polymorphisms are commonly designated as the *CYP2B6*6* allele), and the values for exon 7 and exon 9 varied little across subjects.

Fitting the primary ANCOVA model to the data provided statistical estimates of the parameters (i.e., regression coefficients). These statistical estimates were used to compute tests of hypotheses and to compute estimates of mean log MR at given levels of cohort, genotype (intron 3, exon 5, exon 9), and logBU. Mean differences defining direct effects of cohort membership, and mean differences defining direct effects of genotype, as well as differences between mean differences that define genotype-by-cohort interaction effects also were estimated.

A second primary test was performed regarding genotype: to establish that intron 3, exon 5, and exon 9 have predictive value even when cohort membership is taken into account, an F-test procedure of size $\alpha = 0.05$ was used to test the null hypothesis (H_o) "intron 3, exon 5 and exon 9 have no predictive value". If, as expected, H_o was rejected, then further tests of

component sub-hypothesis were to be performed; e.g., $H_{o\ (1)}$ "intron 3 has no predictive value," etc. Similarly, a secondary hypothesis test was performed, designed to detect the existence of any association between logMR and cohort membership that remained after having accounted for genotype. If this overall H_o was rejected, then further tests of component sub-hypotheses were to be performed (HAF vs. AAF, etc.). This hierarchical hypothesis testing strategy was designed to control the overall rates of type I error while optimizing statistical power (Muller and Stewart 2006).

In support of the primary analyses, we performed conventional diagnostic computations (e.g., analysis of residuals) with graphical and tabular summaries of the results. We also performed a number of auxiliary analyses to evaluate the robustness of the primary results to perturbations of the assumptions and methods. The auxiliary analyses included fitting the primary model with the following: exon4 replacing exon 5 in the regression equation; omitting the genotype-by-subpopulation interactions; and several other analyses.

Several secondary and exploratory analyses also were performed as part of the comprehensive examination of the data. As an aid to interpretation of primary results, the genotype variables were excluded from the primary model and a secondary hypothesis (H_0), "the sex/ethnicity subpopulation differences are zero", was tested to demonstrate the existence of sex/ethnicity differences when genotype was not taken into account. The predictive value of age and BMI or weight was explored by modifying the primary model to include additional covariates in the model. Additional covariates considered were centered at their sample means ((age-27), (BMI-26), (weight-72), (height-167), (timing of blood draw - 6)) and terms representing them were included in the regression equation. Finally, as an aid to the interpretation of the primary results, an auxiliary analysis was performed to examine the

relationship between logBU concentrations and ethnicity, sex, and genotype. For this ANCOVA model the dependent variable was logBU concentrations and the independent variables were the same as in the primary analyses.

All statistical computations were performed using SAS System software (version 9.2, SAS Institute Inc., Cary, NC). The fitting of the linear regression models reported relied on algorithms provided by PROC GLM and an F-test procedure of size $\alpha = 0.05$ was used to test hypotheses. Confidence intervals for variance parameter estimators were obtained via PROC MIXED. Influence diagnostics (e.g., Cook's Distance, the PRESS statistic, etc.) were computed using PROC REG. In sensitivity analyses, auxiliary robust regression methods were implemented using PROC NLMIXED.

Results

Descriptive Tabulations. The four cohorts were similar in age. The males were taller, on average, than the females. Average weight was greatest for the AAF and CAM cohorts. Weights across the four cohorts ranged from 44 to 130 kg. The CAF cohort had the lowest average BMI, while the AAF cohort had the highest average BMI (Table 1).

Blood samples used were withdrawn 6 hour postdose. At 6-hour time point mean sampling times and standard deviations were: 6.01h ± 0.41h; 6.03h ± 0.94h; 6.03h ± 0.64h and 6.03h ± 0.12h for CAM, CAF, AAF and HF, respectively. Geometric means with corresponding 95% confidence intervals are shown in Table 2 for dose-adjusted BU and HB concentrations at 6 hour postdose, and for the calculated MR. While the mean BU concentration for Hispanic females was lower compared to the other three cohorts, mean HB concentrations were similar which resulted in an approximately 50% higher MR for Hispanic females.

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Association between CYP2B6 Genotype Frequency and Race/Ethnicity. Table 3 depicts the distribution of the genotypes of the major functional CYP2B6 polymorphisms for each of the four cohorts. Associations between ethnicity and frequency for any of the five studied genotypes were not detected. Although there appeared to be differences in variant allelic frequency among ethnic groups for Intron 3, we were unable to conclude these differences are real based on a pre-specified adjustment for multiple genotype comparisons.

Dependence of CYP2B6 Activity on Race/Ethnicity, Sex, and CYP2B6 Polymorphisms. The primary analysis used an ANCOVA model for logMR to evaluate the predictive value of logBU concentrations, sex, ethnicity, genotypes (intron 3, exon 5, exon 9) and genotype-by-sex/ethnicity interactions. Since exon 4 and exon 5 polymorphisms are in almost complete linkage disequilibrium, only exon 5 was included in the first analysis in order to save degrees of freedom and preserve power. Within the range of log-BU concentrations experienced by the subjects, the fitted model indicated that logHB concentration increases by 0.13 units for every one-unit increase in log BU concentration ($p < 0.0001$). Association of logMR with sex and ethnicity was not detected (p=0.7325). In contrast, the null hypothesis that the five genotypes have no predictive value was rejected (p=0.0033). Occurrence of variant intron 3 (p=0.0013) or exon 5 (p=0.0017) was associated with lower activity compared to subjects with wild type intron 3 and exon 5. Association with exon 9 was not detected (p=0.7415). The null hypothesis that the magnitudes of the genotype effects are the same for all three ethnic populations was not rejected; i.e., the genotype-by-ethnicity interaction terms in the model were not statistically significant (p=0.3275).

Table 4 illustrate estimates of mean logMR for the four sex/ethnicity cohorts for four genotype profiles: wild type for all three *CYP2B6* genotypes, variant only in intron 3, variant only in exon 5, or variant only in exon 9. HB/BU metabolic ratio was much greater in Hispanic females, as 16 out of 26 HAFs had a variant intron 3, and this variant is associated with lower metabolism ($p < 0.0148$).

When exon 4 was included in the regression equation in place of exon 5 the results were identical using either exon 5 or exon 4, which is expected since the two polymorphisms are in almost complete linkage disequilibrium. Two subjects had extremely low log MR values. Neither a deletion of these two subjects nor the use of robust regression methods provided a better fit to the data or led to a different interpretation of the results. Finally, inclusion of age and BMI in the primary model produced results that were fully consistent with those obtained via the primary model.

Relationship between Bupropion Concentrations and Subject Characteristics. A supportive analysis with logBU concentrations as the dependent variable was performed. The null hypothesis (H_0) that cohort effects and genotype-by-cohort interactions are all zero was rejected (p=0.0243). Hierarchical testing of component sub-hypotheses detected ethnicity differences, with Hispanic Americans having a lower mean logBU concentration than African Americans and Caucasian Americans. No differences between African Americans and Caucasian Americans were detected. An additional test designed to detect the existence of genotype differences not accounted for by sex/ethnicity cohort membership also was not statistically significant.

Discussion

Bupropion is an anti-depressant and in humans is metabolized to three major metabolites: erythrohydrobupropion, threohydrobupropion, and hydroxybupropion. The hydroxylation of bupropion to hydroxybupropion has been validated *in vitro* (Faucette et al., 2000) as a specific measure of CYP2B6 activity in liver microsomes. However, as opposed to *in vitro* incubations with microsomal preparations, clinical investigations are potentially complicated by competing metabolic pathways, as well as by renal and/or biliary elimination (Welch et al., 1987). Such alternative pathways of elimination may confound the relationship between *CYP2B6* polymorphisms and CYP2B6 activity in the liver as measured by the *in vivo* clearance of bupropion. The extent to which carbonyl reduction of bupropion to erythrohydrobupropion and threohydrobupropion competes with CYP2B6-mediated hydroxylation of bupropion in the liver is not known.

The purpose of this study was to identify important covariates that are associated with inter-individual variation in CYP2B6 activity using the metabolism of bupropion to hydroxybupropion as a surrogate measure. Knowledge of such covariates would further inform investigations intended to validate bupropion as a useful clinical probe. We hypothesized that specified *CYP2B6* polymorphisms would account for any sex or ethnic differences in bupropion that might be observed. In this study, there was no association of sex or ethnicity with CYP2B6 activity as measured by the hydroxybupropion/bupropion MR. However, an association was seen between CYP2B6 activity and polymorphisms in exon 5 and intron 3. In addition, the results also suggest that the effect of the variant alleles on CYP2B6 activity was similar for both genders and all ethnic groups examined.

The current study is the first to use bupropion metabolic ratio at a single time-point to develop a model for evaluating CYP2B6 activity. A traditional dense concentration-time method of pharmacokinetic data analysis for this study would be preferable, but would require upwards of 10 to12 concentration-time samples per patient and would be clinically prohibitive. Therefore, a single 6-hour time-point was selected for calculation of MR, guided in part by data from a previous study conducted by our research group. Using this approach, we found that the exon 5 allelic variant of *CYP2B6*, as well as the intron 3 variant, was independently associated with lower metabolic ratio compared to subjects with wild type exon 5. The association of the exon 5 variant with lower metabolic ratio, and our approach, are supported by the recent report by Chung et al. (Chung et al., 2011) where a metabolic ratio, calculated as the ratio of hydroxybupropion $AUC_{0\text{-}36h}$ to Bupropion BU $AUC_{0\text{-}36h}$, was lower in *CYP2B6*6* (this nomenclature denotes the linked exon 4 and exon 5 variants) carriers than in *CYP2B6*6* noncarriers among 35 healthy volunteers administered the same bupropion product at the same dose as used in our study. However, the small sample size per group, the absence of male group for each ethnicity, the reliance on the limited pharmacokinetic data based on 6 hr sampling time, the influence of alternative routes of elimination, and the extent of secondary metabolism of hydroxybupropion that has been described (Chung et al., 2011), should be considered as factors that potentially limit the findings of this study.

Female livers have been reported to have higher amounts of CYP2B6 messenger ribonucleic acid (mRNA), protein, and activity than male livers and a *CYP2B6* genotype-phenotype association was observed in livers obtained from females (Lamba et al., 2003). However, these previous investigations used human liver microsomes and the biotransformation of S-mephenytoin to nirvanol as a measure of CYP2B6 activity. We found no sex differences in

bupropion metabolic ratio in our log-scale model-based analysis, although our findings are limited to Caucasian males and females. Our data suggest that there may not be a clinically significant sex differences in human CYP2B6 activity, or our methods were not sensitive enough to detect a small difference. Ethnic differences in CYP2B6 activity have also been observed when a human liver microsome assay was used (Lamba et al., 2003; Parkinson et al, 2004). Samples obtained from Hispanic females had 3.6- and 5.0- fold higher CYP2B6 activity compared to liver microsomes taken from Caucasian females and African-American females, respectively (Lamba et al., 2003). In this *in vivo* study, ethnic differences were observed in bupropion concentrations, with Hispanic females having lower concentrations even after dose-adjustment compared to Caucasian and African-American females. However, there were no statistically significant differences in the bupropion metabolic ratio, using our primary model. As mentioned above, the reduction of bupropion by hepatic carbonyl reductase represents a competing pathway of metabolism and may account for our observations in Hispanic females. For example, higher rates of carbonyl reduction in Hispanic females might result in lower plasma bupropion concentrations without observing an effect on CYP2B6-mediated hydroxylation if Michaelis constant (K_m's) for bupropion reduction are much greater than the K_m for bupropion hydroxylation. Such effects would increase variability in the bupropion MR perhaps confounding our ability to detect differences. Similarly, differences in the disposition and retention of bupropion and hydroxybupropion in various tissue compartments (e.g. related to their partitioning in low BMI or high BMI subjects), reflecting differences in their physiochemical properties, and in the polymorphic drug transport processes involved in their uptake and elimination, will also increase variability in the MR. In addition, slow release of bupropion in the gastrointestinal tract, associated with the sustained release bupropion

formulation used in our study, would have a more rate-limiting effect on bupropion metabolism in individuals with high CYP2B6 activity compared to those with low activity. Similarly, the hydroxylation of bupropion is also catalyzed to a lesser extent by other highly polymorphic CYP450s which vary in concentration in human liver and these would have a greater contribution in individuals with low CYP2B6 activity. These other factors would tend to reduce the range in the MR observed in our study.

Association between the *CYP2B6* genotypes and sex/ethnicity cohorts was not detected in this study. It had been previously conjectured that a possible reason for ethnic differences in CYP2B6 activity could be differences in genotypic frequency. It was thought that differences in allelic frequency could, in part, explain ethnic group differences observed in CYP2B6 liver microsomal activity. In previous studies, intron 3 was found to be associated with lower CYP2B6 expression in females, exon 9 was associated with the lowest level of CYP2B6 activity in female livers, and exon 4 516G>T was shown to disrupt an exonic splice enhancer (Lamba et al., 2003; Thorn et al., 2010). The exon 5 785A>G is frequently present in combination with exon 4 516G>T in the *CYP2B6*6* haplotype. This haplotype is associated with lower CYP2B6 protein expression and bupropion hydroxylation in liver microsomes (Thorn et al., 2010). In our study, we detected an association between these functional *CYP2B6* genotypes and CYP2B6 activity. Subjects having at least one variant allele tended to have a lower bupropion metabolic ratio than those carrying wild type alleles, however, our results suggest the association of these variant alleles with lower metabolism is similar across all ethnic groups examined.

Variability in CYP2B6 activity and expression may depend on the combination of polymorphisms present in an individual. Also different genotypic combinations (haplotypes) may have different effects in different ethnic groups depending on other factors such as the

transcription factor, constitutive androstane receptor CAR, which reflects environmental effects such as other drugs. However, because no interactions were detected between genotype and ethnicity, we have no direct evidence to conclude that there are differences in genotypic effects across different ethnic groups.

By using hydroxybupropion/bupropion metabolic ratio as a measure of CYP2B6 activity we were able to confirm the influence of genetic variants that may be responsible for altered CYP2B6 function. Alterations in CYP2B6 activity may lead to differences in systemic exposure of drugs metabolized by CYP2B6 and therefore result in variation in therapeutic and toxic responses to these drugs in certain populations. Our results from this preliminary study suggest that only *CYP2B6* genotype has an association with CYP2B6 activity. These results are encouraging and may allow for bupropion to be used as a probe substrate across genders and ethnicities as a measure of CYP2B6 activity. However, a formal prospective study that includes both males and females among all study groups and using a more optimized blood sampling time is needed to develop a robust predictive model for future clinical use.

Acknowledgements

We would like to thank Dr. Colleen R.M. Lemmon for her help with the preparation of this manuscript, and Drs. Tedi A. Soule and Christine Walko for their help in executing the study as well as the staff of the GCRC and McClendon UNC Hospital Lab's for their assistance. We would also like to thank all of the participants in this study. We thank Dr. Jatinder Lamba and Jeremy Hunt for their expert technical assistance with *CYP2B6* genotyping.

Authorship Contributions

Participated in research design: Lindley, Schuetz, Ilic, and Chen

Collected data /Conducted experiments: Ilic and Thirumaran

Performed data analysis: Hull, Ilic, Kashuba, and Stewart

Wrote or contributed to the writing of the manuscript: Ilic, Hawke, Hull, Stewart, Schuetz and Chen

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Footnotes

This work was supported by the Office of Women's Health, U.S. Food and Drug Administration [Grant FRP#: FDA-SOL-05-00050]. K. Ilic was a Drug research and development fellow (2006-2008) supported by GlaxoSmithKline. K. Ilic's work is supported by the Ministry of Education and Science, Republic of Serbia, Project No. 175064, 2011-2014.

Part of the data in this paper was presented at the ASCPT Annual Meeting, Orlando, FL, April 2-5, 2008. Clinical Pharmacology and Therapeutics 2008; 83(1):81 and published in an abstract form in Clinical Pharmacology and Therapeutics 2008; 83(1):81

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DMD Fast Forward. Published on December 13, 2012 as DOI: 10.1124/dmd.112.048108
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TABLE 1

Descriptive tabulations for age and body size (N=100)

	CAM (n=25)	**CAF** (n=25)	**AAF** (n=25)	**HAF** (n=25)
Age (years)	25.7 ± 7.5	27.6 ± 7.4	27.6 ± 7.6	26.2 ± 8.2
Range	18 - 46	18 - 41	18 - 49	19 - 44
Height (cm)	178.7 ± 11.0	164.5 ± 5.8	164.4 ± 4.9	161.4 ± 5.6
Range	159.5 - 211.0	152.0 - 176.4	156.0 - 173.0	149.3 - 172.1
Weight (kg)	80.3 ± 8.3	60.6 ± 9.1	83.1 ± 22.4	63.2 ± 8.9
Range	65.0 - 99.3	44.4 - 80.5	52.8 - 129.7	44.5 - 81.6
BMI (kg/cm^2)	25.4 ± 4.0	22.3 ± 2.7	30.9 ± 8.5	24.3 ± 3.4
Range	17.5 - 39.0	18.1 - 29.9	20.1 – 51.4	19.1 – 30.9

CAM, Caucasian American Males; CAF, Caucasian American Females; AAF, African American Females; HAF, Hispanic American Females. BMI, Body Mass Index.
Only descriptive statistics were performed on these data. Values are expressed as mean ± standard deviation. The potential influence of these demographic variables in the analysis was addressed by considering them as covariates.

DMD Fast Forward. Published on December 13, 2012 as DOI: 10.1124/dmd.112.048108
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TABLE 2

Bupropion and hydroxybupropion 6-hour concentrations (ng/mL) and metabolic ratio across sex / ethnicity cohorts (N=100)

	Unadjusted		Dose-Adjusted[a]		
	BU	**HB**	**BU**	**HB**	**HB/BU**
CAM	66.67 (57.99-76.65)	260.95 (206.40-329.92)	76.10 (66.19-87.50)	297.88 (235.44-376.89)	3.92 (3.08, 5.00)
CAF	83.62 (73.14-95.60)	354.77 (317.59-396.30)	71.56 (63.82-80.25)	303.61 (271.80-339.14)	4.24 (3.62, 4.97)
AAF	66.13 (53.67-81.49)	258.55 (191.14-349.73)	75.92 (61.43-93.84)	296.81 (224.46-392.49)	3.97 (2.80, 5.64)
HAF	55.60 (43.89-70.44)	330.90 (282.86-387.10)	49.74 (38.48-64.31)	296.05 (256.49-341.71)	6.01 (4.60, 7.85)

CAM, Caucasian American Males; CAF, Caucasian American Females; AAF, African American Females; HAF, Hispanic American Females. Each group had 25 patients.

Values are expressed as geometric means (± 95% confidence intervals)[b].

[a] Prior to computation of the geometric mean, plasma concentrations were adjusted to correspond to a standard dose for a 70 kg weight individual.

[b] As statistical analysis is more appropriately performed using ANCOVA on log transformed data, these tabulations are provided only for descriptive purposes.

TABLE 3

DMD Fast Forward. Published on December 13, 2012 as DOI: 10.1124/dmd.112.048108
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Genotype frequencies by sex / ethnicity cohorts

Genotypes		**CAM** (n=25)	**CAF** (n=25)	**AAF** (n=25)	**HAF** (n=25)	**Total** (N = 100)	***p*-value (Test of Association)[a]**
Intron 3	**Variant**	11	13	6	16	46	*0.0148
	Wild-Type	14	12	19	9	54	
	VAF	22%	34%	12%	44%	28%	
Exon 4	**Variant**	10	12	16	12	50	0.4029
	Wild-Type	15	13	9	13	50	
	VAF	24%	28%	34%	28%	28.5%	
Exon 5	**Variant**	11	13	16	12	52	0.1163
	Wild-Type	14	12	9	13	48	
	VAF	26%	30%	36%	30%	30.5%	
Exon 7	**Variant**	0	0	1	0	1	0.5000
	Wild-Type	25	25	24	25	99	
	VAF	0%	0%	2%	0%	0.05%	
Exon 9	**Variant**	5	5	2	3	15	0.4380
	Wild-Type	20	20	23	22	85	
	VAF	10%	10%	4%	6%	7.5%	

CAM, Caucasian American Males; CAF, Caucasian American Females; African American Females, AAF; HAF, Hispanic American Females. VAF, Variant Allelic Frequency.

[a] Fisher's Exact Test of the null hypothesis of no association between genotype and ethnic group (AAF, HAF, CA). CAM and CAF pooled for this test. *A p-value less than $\alpha = 0.0125$ was considered statistically significant.

The variant allelic number represents heterozygous and homozygous subjects for the polymorphism.

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TABLE 4

Effect of genotype variants across sex / ethnicity cohorts (N=100)

Genotype	CAM (n=25)	CAF (n=25)	AAF (n=25)	HAF (n=25)
Wild type (only)	0.774 (0.648-0.901)	0.812 (0.672-0.952)	0.845 (0.678-1.013)	0.726 (0.460-0.992)
Intron 3 (only)	0.638 (0.530-0.746)	0.676 (0.567-0.784)	0.500 (0.314-0.686)	0.680 (0.540-0.819)
Exon 5 (only)	0.587 (0.480-0.693)	0.624 (0.518-0.731)	0.614 (0.496-0.731)	0.643 (0.494-0.792)
Exon 9 (only)	0.809 (0.659-0.960)	0.847 (0.689-1.004)	0.900 (0.579-1.221)	0.600 (0.324-0.847)

CAM, Caucasian American Males; CAF, Caucasian American Females; AAF, African American Females; HAF, Hispanic American Females.

Values are expressed as mean estimates of Log10 (MR) ± (95% Confidence Intervals).