The Influence of Two CYP2C9 Promoter SNPs on CYP2C9 Expression and Caffeine’s Impact on CYP1A2

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THESIS
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Chicago, Illinois

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This thesis is dedicated to my parents and grandparents without whom none of this would be possible.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>3-MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>AhR</td>
<td>Aromatic Hydrocarbon Receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CLpo</td>
<td>Oral Clearance</td>
</tr>
<tr>
<td>Cp</td>
<td>Plasma Clearance</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>D'</td>
<td>Deviation of the observed frequency of a haplotype from the expected</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>DRE</td>
<td>Dioxin-responsive Element</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate Dehydrogenase</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human Embryonic Kidney 293 Cell</td>
</tr>
<tr>
<td>HEPG2</td>
<td>Human Liver Carcinoma Cell Line</td>
</tr>
<tr>
<td>INR</td>
<td>International Normalized Ratio</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage Disequilibrium</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NS</td>
<td>Statistically Not Significant</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>R²</td>
<td>Correlation</td>
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<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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LIST OF ABBREVIATIONS (CONTINUED)

TCDD 2,3,7,8-Tetrachlorodibenzo-p-dioxin
SUMMARY

1. The Influence of Two CYP2C9 Promoter SNPs on CYP2C9 Expression

We have previously shown that one of the polymorphic forms of CYP2C9, CYP2C9*8, has been observed to cause a decrease in the dose of warfarin necessary for therapy. This is in part explained by decreased CYP2C9 enzyme activity upon an R150H change of CYP2C9*8. The goal behind this project was to determine the role two SNPs, that are in linkage disequilibrium with CYP2C9*8, play in the decreased warfarin dose requirement in CYP2C9*8 carriers.

A linkage disequilibrium study had revealed that CYP2C9*8 is linked with two promoter SNPs: -1766T>C and -1188T>C. As expected from the previous study where warfarin dose requirement was lower in CYP2C9* carriers, the warfarin dose was decreased in those African American patients with the SNP -1766 T>C. However, SNP -1188 T>C, which occurs with the SNP -1766 did not show a similar change in dose when present by itself. In fact, the SNPs are found in conjunction with each other but with a caveat: if -1766 is present, then it is found that -1188 is also present. However, if it is found that -1188 is present, it does not always occur that -1766 is present as well. It has also been shown that -1188 is associated with the presence of other CYP2C9 SNPs such as CYP2C9*2, CYP2C9*3, etc.

After discovery of the correlation between the promoter SNPs and warfarin dose requirement, our observation led us to determine if the result of the presence of the SNPs yields a decrease in CYP2C9 mRNA levels. However, we found that the presence of the SNP did not affect the mRNA levels of CYP2C9. Our ongoing research into the causation of reduced dosing, revealing no change in mRNA levels using standard qRT-PCR, led us to undertake mRNA comparisons using a method called allelic expression imbalance (AEI). This method allows scientists to compare mRNA values based on the SNP’s presence in the mRNA. The results from this test did in fact reveal that the presence of the SNP at -1766 showed there was
SUMMARY (CONTINUED)

a significant decrease in the mRNA yielded from the presence of this SNP. To determine the true cause of the decrease in CYP2C9 mRNA, we expressed a plasmid containing the CYP2C9 sequences without the promoter in HEK293T cells to determine if the promoter region was responsible for the decrease or if a comparison between CYP2C9*8 and CYP2C9 wild type would reveal a difference. Our results once again showed that there was no difference between the presence of the CYP2C9*8 versus the CYP2C9 sequence. This comparison allowed us to conclude that the true causation of the decreased gene expression was the presence of the promoter SNPs -1766T<C and -1188T<C in CYP2C9*8 African American patients. What remains to be determined is how the SNPs -1766 and -1188 are interacting, if at all, to affect the dosing of those patients with the presence of the SNPs.

2. Caffeine’s impact on CYP1A2 expression

In rats, caffeine was shown to induce the CYP1A2 enzyme. This leaves open the possibility that the induction of CYP1A2 in humans could be partially responsible for the tolerance of caffeine observed in humans. The goal for the caffeine project became to determine if caffeine was in fact an inducer of CYP1A2 in humans as seen in rats. Previously, it had been shown that caffeine tolerance is most likely due to a pharmacodynamic interaction of caffeine and its receptors. Effectively, the adenosine receptors respond to a daily interaction with caffeine by producing more receptors for the caffeine present. More receptors equate to the necessity of more caffeine to have the same effect; thus, tolerance is achieved. However, primary literature research had shown that in animals caffeine was shown to induce CYP1A2. That left open the possibility that caffeine tolerance was not only a pharmacodynamic result but also could occur because of a pharmacokinetic reason like the induction of CYP1A2.
SUMMARY (CONTINUED)

Consequently, we decided to express the plasmid, pGudLuc1.1 where luciferase gene expression is driven by the AhR response elements, in HepG2 cells and treated with increased doses of caffeine. The results showed that while caffeine did not increase luciferase activity at ‘normal’ doses of caffeine, it left open the possibility that caffeine could increase activity at higher doses, approximately eight times the normal dose.

Because CYP1A2 can be increased through other means besides the traditional binding to the XRE regions of the promoter region, we decided to obtain human hepatocytes and determine their response to caffeine dosing. We found once again that human hepatocytes do not respond in a similar fashion as rat hepatocytes. The data showed that at regular doses CYP1A2 is not induced by the presence of caffeine but only at higher non-normal doses, once again approximately eight times the normal dose. The data showed that caffeine was not an inducer at normal doses of caffeine. It does leave open the possibility that it can occur at higher doses of caffeine. The most likely reason for the difference between humans and rats is the interspecies difference in the CYP1A2 promoter region.
I. INTRODUCTION

A. INFLUENCE OF CYP2C9 PROMOTER SNPs ON CYP2C9*8

1. Current warfarin use and risks

Warfarin consistently ranks among the top ten drug-related causes of serious adverse effects, including death and hospitalization,\(^1\) prompting a black box warning on warfarin labeling. Further complicating warfarin therapy is the substantial intra-patient variability in the dose necessary to produce optimal anticoagulation, with dose requirements varying as much as 20-fold among patients.\(^2\) Failure to achieve optimal anticoagulation significantly increases the risks for bleeding and thrombosis.\(^3,4\)

2. Potential side effects of warfarin

Warfarin is a racemic mixture, with the S-enantiomer possessing a 3 to 5 times more potent anticoagulant effect than R-warfarin.\(^5,6\) S-warfarin is metabolized exclusively by cytochrome P450 (CYP) 2C9 to inactive metabolites, whereas R-warfarin is metabolized by CYP1A2, 3A4, and 2C19. Reductions in CYP2C9 activity secondary to genetic variation can lead to toxic plasma concentrations of warfarin and other CYP2C9 substrates.\(^7\) For example, bleeding with warfarin, neurotoxicity with phenytoin, gastrointestinal bleeding with non-steroidal anti-inflammatory drugs, and severe hypoglycemia with oral sulfonylureas have been reported with reduced function CYP2C9 alleles.\(^8-11\)

3. Influence of genetics on CYP2C9 expression

Over 30 alleles have been identified in CYP2C9 (http://www.cypalleles.ki.se). The CYP2C9*2 (R144C) and *3 (I359L) alleles, the most extensively studied, lead to significant reductions in enzyme activity.\(^12,13\) Accordingly, the CYP2C9*2 and *3 alleles are associated with reduced warfarin dose requirements and an increased risk for bleeding with warfarin.\(^2-8,14-19\)

While these alleles occur in over 30% of individuals of European ancestry, they occur in only about 5% of African Americans.\(^14,20,21\) The CYP2C9*5 (D360E), *6 (10601delA), and *11 (R335W) alleles occur almost exclusively in African Americans and lead to reductions in
enzyme activity; however, each has a frequency <3%.\textsuperscript{22-25} The CYP2C9*8 (R150H) allele is one of the most common variants in persons of African descent (allele frequency of approximately 6%).\textsuperscript{26, 27} Of interest, we recently found that the R150H variant, comprising the CYP2C9*8 allele, is associated with significantly lower warfarin dose requirements,\textsuperscript{26} and this could be explained in part by decreased enzyme activity of CYP2C9 R150H against S-warfarin.\textsuperscript{28} Previous haplotype analysis revealed that two SNPs in the upstream regulatory region of CYP2C9, i.e., c.-1766T>C and c.-1188T>C, are in linkage disequilibrium (LD) with R150H.\textsuperscript{24} Studies have reported no association between the -1188T>C allele and either S-warfarin clearance or warfarin dose requirement in those of European descent; however, it has not been examined in African Americans, in whom the associated CYP2C9*8 variant occurs almost exclusively.\textsuperscript{29, 30} On the other hand, the effects of the -1766T>C variant, alone or together with -1188T>C allele, on CYP2C9 expression, warfarin dose requirements, and S-warfarin clearance have not been reported.

4. Association of SNPs -1766 and -1188 with warfarin dose and their influence on CYP2C9 expression

In this study, we examined the association of the -1766T>C and -1188T>C SNPs with warfarin dose and S-warfarin clearance in an African American patient cohort. Also, we examined whether the CYP2C9 -1766T>C allele (along with -1188T>C) influences CYP expression using human liver tissues from African Americans. The effects of the promoter region variants on CYP2C9 promoter activity were further studied by using luciferase reporter assays.

B. INFLUENCE OF CAFFEINE ON CYP1A2 EXPRESSION

1. Caffeine use and tolerance

Caffeine is a psychoactive compound found in many daily-consumed drinks and food such as tea, coffee, energy drinks, and chocolate.\textsuperscript{31} Caffeine exhibits various pharmacological effects, including increased heart rate, diuresis, motor activity, and mental alertness. Repeated
administration of caffeine (e.g., daily intake of coffee) can produce a tolerance to the pharmacological effects of caffeine.\textsuperscript{32} Tolerance to the motor-activating effect of caffeine is in part explained by decreased sensitivity to adenosine receptors.\textsuperscript{33} However, whether altered caffeine elimination potentially plays a role in development of the tolerance remains unknown.

2. CYP1A2 induction mechanism

Caffeine is eliminated mainly through hepatic metabolism by the CYP1A2 enzyme. CYP1A2 expression is regulated by a ligand-activated transcription factor, aromatic hydrocarbon receptor (AhR) (reviewed in \textsuperscript{34,35}). Upon binding of a ligand such as 3-methylchoanthrane (3-MC), AhR translocates into the nucleus and forms a heterodimer complex with a nuclear protein, aromatic hydrocarbon receptor nuclear translator protein. The complex binds to a DNA sequence, called dioxin-responsive element (DRE), in the upstream regulatory region of target genes (including CYP1A1 and CYP1A2) and enhances transcription.

3. Animal Studies relating to CYP1A2 and caffeine

Previous studies in rats have shown that caffeine induces CYP1A2 expression. In Fischer 344 rats, oral administration of caffeinated green tea and black tea led to an approximately 5-fold induction in hepatic CYP1A2 enzyme activity as compared to the vehicle-treated animals,\textsuperscript{36} and administration of caffeine alone led to an 11-fold increase in CYP1A2 activity in Wistar rats.\textsuperscript{37} These results suggest that in rats, caffeine induces CYP1A2 expression. This CYP1A2 induction by caffeine may be in part responsible for development of caffeine tolerance. In humans, it remains unknown whether caffeine is capable of activating AhR and/or enhancing CYP1A2 expression.

C. SIGNIFICANCE OF THE PROJECT
Warfarin is a drug that has an extensive history associated with differential dosing of patients related to diet, race, and pharmacogenomics. The study of pharmacogenomics relates how the human genome imposes its effect on the drugs that consumers use. The pharmacogenomics of warfarin has been an area of specific interest for a long period of time due to its extensive profile in the realm of pharmacogenomics. This drug has become a stepping stone to how we will continue to study other drugs and their relationship to the human genome. Further investigation of warfarin pharmacogenomics will enable us to use warfarin as the best example of how to study pharmacogenomics of other drugs in the future. It is important that we as scientists try to discover as much about warfarin to aid us in the future of the proper development of drugs. This study’s impact will help us better understand how genetic changes related to warfarin will have clinical impacts. Also, the distinctiveness of our finding will help scientists to unlock the endless combinations of genetic changes and how they relate to their own studies.

Caffeine is one of the most widely consumed drugs in the world. It can be found in tea, coffee, energy drinks, and chocolate. Its impacts on the body are well known and established: increased heart rate, diuresis, motor activity, and mental alertness. What is also widely accepted as fast is that caffeine tolerance develops in perpetual use of caffeine primarily through consumption of coffee. A simple reason for caffeine tolerance could be the pharmacokinetic impact of CYP1A2. Because of its widespread use and impact on everyday lives, it would be of importance to determine if there are pharmacokinetic contributions to caffeine tolerance. It would have the potential to impact the co-administration of other drugs with caffeine.

D. HYPOTHESIS

Caffeine tolerance as related to CYP1A2:
We hypothesized that caffeine induces CYP1A2 via AhR activation in humans, which could be in part responsible for caffeine tolerance developed. Our investigation focused on the capability of AhR activation by caffeine.

Impact of CYP2C9 promoter SNPs on CYP2C9 expression:

We hypothesized that there is an association between the SNPs -1766T>C and -1188T>C with warfarin dose and S-warfarin clearance. This association led us to investigate whether these alleles influenced the expression of CYP2C9 in human liver tissues from African Americans.
II. Two CYP2C9 Promoter Region SNPs Linked to the *8 Allele in African Americans are Functional

A. MATERIALS AND METHODS

1. CELL CULTURE

HepG2 and HEK293T cells were cultured in complete DMEM supplemented with fetal bovine serum (10%; Gemini, Woodland, CA), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and MEM nonessential amino acids (1%).

2. METHODS

Clinical Dose Association and Pharmacokinetic Studies

Genetic samples were collected from 227 African Americans on a stable dose of warfarin, defined as the same dose for 3 consecutive clinic visits with an average international normalized ratio (INR) within target range, as previously described. In a subset of 40 patients, blood was collected 12 to 16 hours after the warfarin dose for determination of S- and R-warfarin plasma concentrations, which were used to calculate enantiomer concentration, as previously described. Patients taking moderate to potent inducers or inhibitors of CYP2C9-mediated warfarin metabolism (e.g. phenytoin, carbamazepine, rifampin, amiodarone, metronidazole, sulfonamides) or with a history of hepatic disease or nonadherence to warfarin were excluded from the pharmacokinetic analysis. The study protocol was approved by the institutional review board and conducted according to the Declaration of Helsinki.

CYP2C9 Genotyping

Genomic DNA was isolated from buccal cells or whole blood using a Puregene kit (Qiagen, Valencia, CA). The CYP2C9*2, *3, *5, *6, and *11 alleles were determined by PCR
and pyrosequencing, and CYP2C9*8 was determined by PCR and capillary sequencing, as previously described. The -1766T>C and -1188T>C variants were determined by PCR and capillary sequencing using forward and sequencing primer 5'-ATTGCTTTTCTTTGCCCTGT-3' and reverse primer 5'-CTCCAGACATGGCTGCTTTC-3'. The PCR reaction consisted of 25 µl of HotStarTaq Master Mix (Qiagen), primers (25 pmol), 15 µl of H2O, and 20-100 ng of DNA. Thermocycling consisted of denaturation for 15 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds; annealing for 30 seconds at 60°C, and extension at 72°C for 45 seconds, with a final extension of 72°C for 10 min. Both variants could be detected from the same PCR and sequencing reaction.

**Linkage Disequilibrium**

Pairwise linkage disequilibrium between the CYP2C9 variants was determined using Haploview software (Haploview 4.2, Cambridge, MA). LD was calculated as pairwise r² values and LD blocks were estimated by previously published methods.

**RNA Isolation and quantitative real time-PCR (qRT-PCR)**

RNA was isolated from human liver tissues (Life Technologies, Carlsbad, CA) by using Trizol (Life Technologies), and then cDNA was synthesized using High Capacity cDNA archive kit (Life Technologies). Using the cDNA as template, qRT-PCR was performed using StepOnePlus Real-Time PCR System and the following TaqMan® Gene expression assays (Life Technologies): CYP2C9 (Hs00426397_m1) and GAPDH (Hs99999905_m1). The fold change in mRNA levels of CYP upon drug treatment was determined after normalizing the gene expression levels by those of GAPDH (2⁻∆∆Ct method).

**Quantitative Analysis of Allelic Ratio in genomic DNA and mRNA using SNaPshot**
A previously published method was used with modifications. Coding region SNP rs7900194 (CYP2C9*8) was used as marker to measure allelic RNA expression of CYP2C9 in African American livers. A fragment of DNA or RNA (after conversion to cDNA) surrounding the marker SNP was PCR amplified, followed by a primer extension assay (SNaPshot) that targets the polymorphic site. Then the primer extension products were analyzed on an ABI 3730 capillary electrophoresis DNA instrument, using Gene Mapper 3.0 software (Life Technologies). Allelic gDNA ratios, normalized to 1, served as internal control. The allelic RNA ratios were normalized by the average of DNA ratios. To measure the allelic RNA ratio in CYP2C9*1 and CYP2C9*8 co-transfected cells, we used plasmid DNA isolated from transfected cells as internal control. The following is sequences of primers: gDNA PCR, forward 5′-TCCCTCCTAGTTTCGTTTCTCTTC-3′, reverse 5′-AAGGTCAGTGATATGGAGTAGGGTT-3′; cDNA PCR, forward 5′-GAGAGGAGTTTTCTGGAAGAGCATT-3′, reverse 5′-GGATCCAGGGCTGCTCAA-3′; SNaPshot assay primer: 5′-CCGTGTTCAAGAGAAGCCC-3′.

Cell Transfection and RNA Preparation

The day before transfection, HEK293T cells were plated into 6-well plates. Cells were co-transfected and total RNA prepared with Trizol (Life Technologies). The RNA samples were treated with DNase I and further purified using RNeasy kit (Qiagen) to eliminate plasmid DNA contamination. A portion of untreated total RNA was saved as source of plasmid DNA, serving as internal control for allelic RNA expression experiment.

Luciferase Reporter Assay
HepG2 cells were seeded in 12-well plates at a density of $6.0 \times 10^5$ cells/ml, and on the next day transfected with luciferase construct (0.3 µg), pcDNA3 plasmid (0.3 µg), and β-galactosidase expression plasmid (0.1 µg) using Fugene HD transfection reagent (Roche Applied Sciences) following the manufacturer’s protocol. After 48 hr incubation, cells were harvested, and activities of luciferase and β-galactosidase were determined using assay kits from Promega (Madison, WI). The luciferase activity was normalized to β-galactosidase activity to control for differences in transfection efficiency.

**Plasmids**

To generate luciferase vectors, -1828/+25 (from transcription start site) of CYP2C9 was PCR-amplified using p2C9-5K-Luc plasmid obtained from Dr. Dexi Liu (University of Pittsburgh School of Pharmacy) and cloned into pGL3-basic vector (Promega, WI) upstream of the luciferase gene using KpnI and XhoI cloning sites (named pCYP2C9). The -1766T>C and -1188T>C mutations were introduced using the QuikChange II XL site-directed mutagenesis kit (Stratagene). At the end, the following 4 luciferase constructs were obtained: pCYP2C9 [-1188T/-1766T], pCYP2C9 [-1188C/-1766T], pCYP2C9 [-1188T/-1766C], and pCYP2C9 [-1188C/-1766C]. CYP2C9 expression vector, named pcDNA3-CYP2C9, was constructed by subcloning CYP2C9 from BacFast1-CYP2C9 (kindly provided by Dr. Allan Rettie, University of Washington at Seattle) into pcDNA3. All insert sequences were confirmed by capillary sequencing. β-Galactosidase expression vector has been previously described.

3. **STATISTICAL ANALYSIS**

For the clinical data, warfarin doses and pharmacokinetic parameters were compared between genotype groups by the two-sided Student's unpaired $t$-test or one-way ANOVA, as
appropriate. S-warfarin clearance was not normally distributed and was log transformed prior to analysis.
B. Results

1. Linkage Disequilibrium among CYP2C9 Variants and Association with dose requirements

   Among 227 African Americans, the frequencies of the -1766C, -1188C, and 150H variants were 0.08, 0.37, and 0.07. The LD analysis of CYP2C9 in 227 African Americans revealed that the -1766T>C variant was in strong LD with the CYP2C9 R150H polymorphism ($r^2=0.90$) (Figure 1a). Interestingly, all patients with the -1766T>C or R150H variant also had the -1188T>C variant (D’=1), as shown in Figure 1b, indicating existence of a haplotype that contains all three SNPs. The frequency of the -1766C/-1188C/150H haplotype in our population was 0.07. The -1188C variant commonly occurred without the -1766C and 150H polymorphisms, thus accounting for the low $r^2$. However, interestingly, the -1188T>C variant was always present in all patients with a CYP2C9*2, *3, *5, or *6 (but not with the CYP2C9*11) allele (data not shown).
Figure 1a and 1b. Linkage disequilibrium plots for CYP2C9 variant alleles. The LD plots are created using Haploview and the color code on each plot follows the standard color scheme for Haploview. A. White ($r^2 = 0$), grey ($0 < r^2 < 1$) and black ($r^2 = 1$). B. White=$(|D'|<1, LOD<2)$, pink/red=$(|D'|<1, LOD\geq2)$, blue=$(|D'|=1, LOD<2)$, bright red=$(|D'|=1, LOD\geq2)$. The numbers in the squares on these LD plot are the D’ or $r^2$ values. D’ of 1 are not listed (shown as a colored square with no number). The LD blocks (shown as black triangles) were defined by the confidence interval of D’ $^{39}$ (data kindly provided by Dr. Larisa Cavallari).
The dose association study revealed that the -1766T>C variant is associated with lower warfarin doses, to the same extent as the R150H (*8) variant (Figure 2), as expected from the strong LD between -1766T>C and R150H. Similarly, the -1188T>C variant is associated with lower warfarin doses in the overall cohort. Given that all individuals with a CYP2C9 *2, *3, *5, *6, or *8 allele also carried -1188C, and the presence of these reduced function alleles may contribute to lower dose requirements with the -1188C allele, we examined the association between the -1188T>C polymorphisms and warfarin dose in CYP2C9*1 homozygotes. When the analysis was limited to individuals without a CYP2C9 -1766C, *2, *3, *5, *6, *8, or *11 allele, there was no longer an association between the -1188T>C variant and warfarin dose (Figure 2), suggesting that -1188T>C alone is not a key factor leading to decreased warfarin dose requirement and that other CYP2C9 SNPs co-existing with the -1188C allele (e.g., CYP2C9 -1766C, *2, *3, *5, or *8) are likely responsible for the lower warfarin dose requirement in -1188C allele carriers.
Figure 2. Association of -1766T>C and -1188T>C variants with warfarin dose requirements in 227 African Americans. Data with the -1188T>C variant are shown for all patients and when limited to patients with the CYP2C9*1/*1 genotype (absence of a CYP2C9*2, *3, *5, *6, *8, or *11 allele). The number of patients in each group is shown in parentheses below the genotype. (data kindly provided by Dr. Larisa Cavallari)
2. Effect of Promoter Region Variants on Warfarin Clearance

The -1766T>C SNP was in complete LD with CYP2C9*8 among the 40 patients included in the pharmacokinetic study. Thus, as we previously reported for CYP2C9*8, the -1766C allele was associated with lower oral clearance of unbound S-warfarin [CLpo,u (S)] and the ratio of R- and S-warfarin plasma concentration (Cp R/S) compared to the -1766TT genotype (Table 1). Oral clearance of R-warfarin, which is not metabolized by CYP2C9, was similar between genotype groups, as expected. The -1188T>C variant was also associated with S-warfarin clearance and Cp R/S, with lowest values with the variant CC genotype. The difference in pharmacokinetic parameters between -1188T>C genotype groups was no longer evident when limiting our analysis to those without a variant CYP2C9*2, *3, *5, *6, *8, or *11 allele (i.e., limited to CYP2C9 wild-types). Specifically, pharmacokinetic parameters were similar among the 3 genotype groups and between -1188T allele homozygotes and -1188C allele carriers when limited to CYP2C9*1 homozygotes.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>CLpo (R) (ml/min/m²)</th>
<th>CLpo,u (S) (ml/min/m²)</th>
<th>Cp R/S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>-1766T&gt;C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC or TC (n=12)</td>
<td>0.90 ± 0.22</td>
<td>85 ± 27*</td>
<td>1.28 ± 0.42*</td>
</tr>
<tr>
<td>TT (n=28)</td>
<td>0.94 ± 0.26</td>
<td>121 ± 63</td>
<td>1.73 ± 0.66</td>
</tr>
<tr>
<td><strong>-1188T&gt;C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC (n=8)</td>
<td>0.83 ± 0.22</td>
<td>70 ± 18*</td>
<td>1.12 ± 0.26*</td>
</tr>
<tr>
<td>TC (n=17)</td>
<td>0.93 ± 0.22</td>
<td>105 ± 38</td>
<td>1.54 ± 0.37</td>
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<tr>
<td>TT (n=15)</td>
<td>0.97 ± 0.28</td>
<td>138 ± 74</td>
<td>1.91 ± 0.82</td>
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<tr>
<td><strong>-1188 T&gt;C in wild-types†</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC (n=2)</td>
<td>0.79 ± 0.02</td>
<td>74 ± 21</td>
<td>1.26 ± 0.43</td>
</tr>
<tr>
<td>CT (n=10)</td>
<td>0.93 ± 0.26</td>
<td>108 ± 47</td>
<td>1.60 ± 0.31</td>
</tr>
<tr>
<td>TT (n=14)</td>
<td>0.95 ± 0.29</td>
<td>143 ± 74</td>
<td>1.98 ± 0.81</td>
</tr>
</tbody>
</table>

(data kindly provided by Dr. Larisa Cavallari)

*p < 0.05 for comparison between genotype groups

†Wild-types were patients without a CYP2C9*2, *3, *5, *6, *8, or *11 allele

Table 1. Pharmacokinetic parameters in 40 African Americans by CYP2C9 promoter region variants
3. Effects of promoter region variants on CYP2C9 Expression

To determine whether the CYP2C9 -1766C/-1188C/150H haplotype affects CYP2C9 expression, we examined CYP2C9 mRNA expression in 32 African American liver tissue samples by using qRT-PCR. Twenty-six liver samples were found to be homozygous as CYP2C9*1/*1, and six liver samples heterozygous as CYP2C9*1/*8. The six *1/*8 heterozygous liver tissues were absent for CYP2C9*2, *3, *5, *6, and *11 (data not shown). We set the CYP2C9 expression level in one liver tissue (liver number 495) as an arbitrary unit of 1 and compared CYP2C9 expression among different liver tissues. As shown in Figure 3, CYP2C9 expression in 32 liver tissues exhibited marked interindividual variation, and CYP2C9 mRNA expression levels did not differ between the livers carrying the CYP2C9*1 and CYP2C9*8 alleles.
Figure 3. CYP2C9 mRNA levels in liver tissues from CYP2C9\*8 carriers. mRNA expression levels of CYP2C9 in 32 liver tissues from African Americans were determined by qRT-PCR in CYP2C9\*1/\*1 (n=26) and CYP2C9\*1/\*8 carriers (n=6). CYP2C9 expression in liver number 495 was arbitrarily set as one (mean ± S.D.). NS, statistically not significant.
To control for potential environmental and genetic (other than CYP2C9*8) influences on CYP2C9 expression that may contribute to interindividual variability, allelic expression of CYP2C9*1 versus CYP2C9*8 was determined in the 6 human livers carrying CYP2C9*1/*8 genotype. RNA and genomic DNA were extracted from all 6 samples, and then mRNA and genomic DNA expression levels of the CYP2C9*8 allele (i.e., cDNA 449G>A) were measured and normalized by that of CYP2C9*1 allele. As expected from *1/*8 heterozygosity, the 449A/G allele ratio for genomic DNA was close to 1 (Figure 4a). Interestingly, however, mRNA of CYP2C9*1 and CYP2C9*8 revealed allelic expression imbalance; mRNA expression from the CYP2C9*8 was significantly lower than that from the CYP2C9*1, the A/G allele ratios ranging from 0.36 to 0.77 (Figure 4a). Of these 6 samples, 5 had the -1766C/-1188C genotype, and one sample (liver number 1057) had the -1766T/-1188C genotype. Of note, liver number 1057 exhibited the least allelic expression imbalance (A/G ratio 0.77).
Figure 4. Allelic expression of CYP2C9*8 in comparison with CYP2C9*1. A. RNA from six liver samples carrying CYP2C9*1/*8 were obtained. Allelic mRNA expression of CYP2C9 was obtained for each allele. Results represent ratio between CYP2C9*8 (A allele) and CYP2C9*1 (G allele) for mRNA and genomic DNA (mean±S.D. from triplicate or quadruplicate measurement of each liver sample). B. Allelic mRNA expression of CYP2C9 was obtained in HEK293T cells cotransfected with CYP2C9*1 and CYP2C9*8.
To rule out potential contribution of the coding SNP in -1766C/-1188C/150H haplotype to the allelic expression imbalance shown in *1/*8 livers, we examined allelic expression of CYP2C9*1 and CYP2C9*8 after transient transfection of CYP2C9*1 and CYP2C9*8 expression vectors into human cells. To this end, equal amount of two CYP2C9 expression vectors each carrying cDNA of CYP2C9*1 and CYP2C9*8 were co-transfected into HEK293T cells, and allelic expression of CYP2C9*1 and CYP2C9*8 was determined. The results showed that allelic expression levels of CYP2C9*1 and CYP2C9*8 are similar to each other (Figure 4b), suggesting that it is likely the associated promoter region SNPs that are responsible for decreased allelic expression of -1766C/-1188C/150H haplotype.

4. Effects of -1766T>C and -1188T>C on CYP2C9 Promoter Activity

To determine whether -1766T>C and -1188T>C SNPs decrease CYP2C9 expression by affecting promoter activity, we performed a luciferase assay. HepG2 cells were transfected with one of the four luciferase vectors (each carrying -1828/+25 of CYP2C9 harboring wild-type or variant allele at -1766 and/or -1188) and luciferase activities were measured. The results (Figure 5) showed that the T>C mutation at -1766 or -1188 led to enhanced luciferase activities (2.6- and 1.7-fold induction, respectively) whereas mutations at both -1766 and -1188 (i.e., the variants associated with CYP2C9*8) led to 5-fold reduction in luciferase activity. These results indicate that -1766T>C and -1188T>C SNPs that occur clinically in CYP2C9*8 carriers lead to decreased CYP2C9 promoter activity.
Figure 5. Effects of Promoter SNPs -1766 and -1188 on promoter binding of CYP2C9. HepG2 cells were transfected with a luciferase construct harboring the promoter SNPs of CYP2C9 (i.e., -1766T/-1188T, -1766C/-1188T, -1766T/-1188C, or -1766C/-1188C), and β-galactosidase expression plasmid. The transfected HepG2 cells were transfected and after 48 hours luciferase assay was performed. Results represent relative luciferase activity in comparison with the wild-type sequence of -1766T/-1188T (mean ± S.D.; n = 3). *, p < 0.05; **, p < 0.01 compared to wild-type.
C. Discussion

African Americans comprise a significant proportion of the population requiring warfarin and other CYP2C9 substrates. However, African Americans are largely underrepresented in pharmacogenetic studies, and much less is known about the metabolic effects of CYP2C9 variants on warfarin pharmacokinetics and dosing in African Americans compared to those of European descent. Accordingly, existing pharmacogenomics dosing algorithms derived from populations of mostly European descent are significantly less accurate in African Americans.\(^44,45\)

In this regard, we have previously characterized the effects of a CYP2C9 variant commonly occurring in African Americans, CYP2C9*8 (R150H), on warfarin therapy. We showed that the CYP2C9*8 variant is associated with reduced warfarin dose requirement,\(^26\) and this is in part explained by decreased warfarin-metabolizing activity of CYP2C9 by R150H substitution.\(^28\)

The CYP2C9*8 allele occurred in a haplotype containing two promoter variants (-1766C and -1188C) in our African American population, as evidenced the high D’ value between R150H, -1766T>C, and -1188T>C. Expectedly, we found that -1766T>C was associated with decreased warfarin dose requirement and S-warfarin clearance, similar to CYP2C9*8 (these variants are in high LD; \(r^2 = 0.9\)), whereas the -1188C variant alone, without 150H, -1766C, or the *2, *3, *5, or *6 alleles, did not impact warfarin maintenance dose or S-warfarin clearance. Similarly, previous studies in European descent populations found no association between -1188T>C and warfarin dose requirements.\(^29\)

To examine whether the promoter SNPs linked with the R150H polymorphism potentially lead to altered CYP2C9 expression, and thus contribute to the effects of the CYP2C9*8 allele, we measured mRNA levels of CYP2C9 in liver tissues obtained from African Americans. CYP2C9 expression showed up to 500-fold differences among individuals, probably due to effects of various environmental factors (e.g., diet, concomitant medications, alcohol use, etc) on CYP2C9 expression. Likely because of this large interindividual variability, there was no statistically significant difference found in total CYP2C9 mRNA expression between
CYP2C9*1/*1 and CYP2C9*1/*8 carriers. To overcome the large interindividual variation in total CYP2C9 expression, we examined potential allelic expression imbalance between CYP2C9*1 and CYP2C9*8, in liver tissues from CYP2C9*1/*8 carriers. Since allelic RNA expression measures the relative amount of RNA derived from each of the two alleles in the same cells from the same individuals, the influence from trans-acting factors (e.g., environmental factors) can be cancelled out. Therefore, allelic RNA expression is a more accurate and sensitive measure than total RNA expression for detecting the effects of cis-acting regulatory polymorphisms on RNA expression. The results revealed significant allelic expression imbalance, with CYP2C9*8 being associated with 1.3- to 2.8-fold lower mRNA expression as compared to CYP2C9*1. Interestingly, the liver that showed the least amount of allelic expression imbalance had the homozygous -1766T/T (wild-type) rather than -1766T/C genotype present in the other livers containing the 150H and -1188C variants. This suggests that -1766T>C, potentially along with -1188T>C variant, leads to decreased CYP2C9 expression. Consistent with this idea, results from our promoter reporter assays showed that the -1766T>C and -1188T>C variants together led to a decreased CYP2C9 promoter activity.

*In silico* analysis of CYP2C9 upstream regulatory region using MatInspector (Munich, Germany) predicted that transcription factors in homeodomain protein family bind to the -1766 and -1188 regardless of presence of the variant alleles (data not shown). Homeodomain transcription factors behave as activators or repressors of target gene expression and are involved in regulation of developmental processes in eukaryotes. Different members of homeodomain transcription factors are known to display similar DNA-binding specificity, yet regulate a distinct set of target genes by selective interaction with coregulators. Our *in silico* promoter analysis indicate that mutations at -1766 and -1188 do not create binding sites for new transcription factors (other than homeodomain transcription factors), suggesting that it is likely subtle changes in recruitment of homeodomain transcription factors and coregulators that are responsible for the decreased CYP2C9 promoter activity from -1766T>C and -1188T>C
variants. Similar subtle changes in actions of homeodomain transcription factors may be responsible for the increased CYP2C9 promoter activity upon mutation at -1766 or -1188 alone. Whether the presence of polymorphisms at -1766 and -1188 indeed lead to recruitment of different homeodomain transcription factors and coregulators remains to be determined.

Taken together, our results indicate that the combination of the -1188T>C and -1766T>C SNPs is associated with decreased mRNA expression, likely due to reduced CYP2C9 promoter activity. The decreased CYP2C9 expression from promoter region SNPs, in addition to the decreased warfarin-metabolizing activity of the R150H variant provides mechanistic explanation for the association between CYP2C9*8 and decreased warfarin dose requirement in African Americans. Further, our results suggest that the CYP2C9*8 allele is comprised not just of the R150H polymorphism, but also of the -1766T>C and -1188T>C variants, and that these latter variants are important in predicting the overall effects of the CYP2C9*8 allele. Indeed, while only one of the carriers of the 150H allele in our study had the wild-type -1766TT genotype, liver tissue from this individual showed the least amount of allelic expression imbalance. These findings suggest that at a minimum, both the R150H and -1766T>C allele should be considered when determining presence of the CYP2C9*8 allele.
III. CAFFEINE INDUCES CYP1A2 EXPRESSION IN RAT HEPATOCYTES BUT NOT IN HUMAN HEPATOCYTES

A. MATERIALS AND METHODS

1. MATERIALS

DMSO, caffeine, omeprazole, and 3-MC were purchased from Sigma (St. Louis, MO).

2. CELL CULTURE

Hepatocytes: Primary human and rat hepatocytes were obtained from Liver Tissue Cell Distribution System (Pittsburgh, PA) and Invitrogen (Durham, NC), respectively. Upon receipt, media were replaced with serum-free Williams’ E media as previously described. On the next day, the cells were used for drug treatment.

HepG2: Cells were cultured in complete DMEM supplemented with fetal bovine serum (10%; Gemini, Woodland, CA), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and MEM nonessential amino acids (1%).

3. METHODS

Plasmids

pGudLuc1.1 is a gift from Dr. Michael Denison (UC Davis). β-Galactosidase expression vector has been previously described.

Luciferase Reporter Assay

HepG2 cells were seeded in 12-well plates at a density of 6.0 x 10^5 cells/ml, and on the next day transfected with 0.6 µg of luciferase construct and 0.1 µg of β-galactosidase expression plasmid using Fugene 6 transfection reagent (Roche Applied Sciences) following the
manufacturer’s protocol. The transfected cells were grown for 24 hr and treated with vehicle or drugs. After 24 hr incubation, cells were harvested, and activities of luciferase and β-galactosidase were determined using assay kits from Promega (Madison, WI). The luciferase activity was normalized to β-galactosidase activity to control for differences in transfection efficiency.

RNA Isolation and Quantitative Real time-PCR

Total RNAs were isolated from human hepatocytes using Trizol (Invitrogen, Carlsbad, CA) and used as a template for cDNA synthesis using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Using the cDNA as template, qRT-PCR was performed using StepOnePlus Real-Time PCR System and TaqMan® Gene expression assays (Applied Biosystems). The following TaqMan probes (Applied Biosystems and Integrated DNA Technologies) were used: human CYP1A2 (Hs01070369_m1), human GAPDH (Hs99999905_m1), rat CYP1A1 (Rn.PT.49.7201670), rat CYP1A2 (Rn00561082_m1), and rat β-actin (Rn0066789_m1). The fold change in mRNA levels of CYP upon drug treatment was determined after normalizing the gene expression levels by those of β-actin or GAPDH (2^{ΔΔCt} method).40

4. STATISTICAL ANALYSIS

Analysis was performed by using Student’s t-test.
B. RESULTS

1. Caffeine Activation of Human AhR

To determine whether caffeine activates human AhR, we performed luciferase reporter assays in human HepG2 cells using the pGudLuc1.1 vector, which harbors a luciferase gene driven by a DRE. HepG2 cells were transfected with pGudLuc1.1 and β-galactosidase (for normalization of transfection efficiency). The transfected cells were treated with vehicle (DMSO), caffeine (50-400 µM), or 3-MC (a known AhR ligand) for 24 hours, and luciferase activities were measured. The 50 µM concentration of caffeine corresponds to the maximum plasma concentration obtained after administration of 12-oz. coffee. The results showed that caffeine, at a concentration as high as 400 µM, showed minimal effects on the DRE-driven luciferase activity, whereas 3-MC caused a significant (41-fold) induction when compared to vehicle-treatment as expected (Fig. 1). These results indicate that caffeine, at concentrations attained from ordinary coffee drinking, is not an AhR activator in HepG2 cells.
Figure 6. Effects of caffeine on AhR transactivation. HepG2 cells were transfected with a luciferase construct (pGudLuc1.1) and β-galactosidase expression plasmid. The transfected HepG2 cells were treated with vehicle (DMSO), caffeine, or 3-MC (0.5 µM) for 24 hr, and luciferase assay was performed. Results represent fold changes in luciferase activity by drug treatment relative to vehicle treatment (mean ± S.D.; n = 3). *, p < 0.05 compared with vehicle-treated group.
2. Effects of caffeine on CYP1A2 expression

A regulatory mechanism distinct from AhR action is known to mediate induction of CYP1A2 expression by chemicals, such as omeprazole. To determine whether caffeine is capable of inducing human CYP1A2 expression (despite not being an AhR activator), we examined the effects of caffeine on CYP1A2 expression in primary human hepatocytes. Primary hepatocytes were treated with DMSO (vehicle), caffeine (50-400 µM), omperazole, or 3-MC for 72 hours, and mRNA levels of CYP1A2 were measured by using qRT-PCR. The results showed that 3-MC and omeprazole significantly induced CYP1A2 expression (by 48- and 130-fold, respectively) although caffeine up to 200 µM had a minimal effect on CYP1A2 expression (Fig. 2A). Interestingly, caffeine at 400 µM enhanced CYP1A2 expression by 2.3-fold in human hepatocytes (p < 0.05) although the magnitude of this change was small as compared to that by 3-MC or omeprazole. The lack of CYP1A2 induction at 50 µM caffeine concentration was shown in 3 additional different batches of human hepatocytes (data not shown). On the other hand, in rat hepatocytes, 3-MC and caffeine (50 µM) both led to significant induction in CYP1A2 expression (281-fold and 9-fold, respectively) (Fig. 2B), consistent with the previously reported induction of CYP1A2 expression upon caffeine administration in rats. Similar result was obtained in additional batch of rat hepatocytes. Taken together, our results suggest that caffeine, at concentrations attained from ordinary coffee drinking, enhances CYP1A2 expression in rat hepatocytes but not in human hepatocytes.
Figure 7. Effects of caffeine on mRNA expression of CYP1A2. Primary human hepatocytes (A) or rat hepatocytes (B) were treated with vehicle (DMSO), caffeine (50-400 µM for human hepatocytes; 50 µM for rat hepatocytes), omeprazole (100 µM), or 3-MC (0.5 µM) for 72 hr. mRNA expression levels of CYP1A2 were determined by qRT-PCR. The data shown are representative of results obtained from 4 and 2 different batches of human and rat hepatocytes, respectively (mean ± S.D.; n = 3 wells/drug treatment). *, p < 0.05; ***, p < 0.001 compared with vehicle-treated group.
3. DISCUSSION

Our results from luciferase reporter assays and human hepatocytes revealed that caffeine, at concentrations attained from average coffee drinking (50 µM), is neither an activator of AhR nor an inducer of CYP1A2 expression in human cells. Interestingly however, at higher concentrations (≥ 400 µM), caffeine significantly induced CYP1A2 expression in human hepatocytes (Fig 7A) while not activating AhR in HepG2 cells (Fig 6). Possibly, mechanisms not involving direct AhR activation by caffeine may be responsible for the CYP1A2 induction in hepatocytes at 400 µM caffeine concentration. For example, metabolites of caffeine produced from human hepatocytes (that express drug-metabolizing enzymes at much higher levels than in HepG2 cells) may activate AhR and induce CYP1A2 expression. Regardless of the underlying mechanisms, the high concentration (400 µM) of caffeine is likely unattainable from average coffee drinking; thus its physiological significance appears minimal.

Studies have reported that consumption of caffeine-containing food or beverages modulates CYP1A2 activity in humans. Djordjevic et al reported that heavy coffee drinking (i.e., regular daily intake of ≥ 3 cups of coffee) is associated with increased CYP1A2 activity index (i.e., plasma paraxanthine/1,3,7-trimethylxanthine ratio) in Serbian and Swedish. Also, another study in healthy Caucasians reported that caffeine increases CYP1A2 activity by 45% for every liter of coffee consumed per day. Considering our data showing that caffeine at concentration attained by average coffee drinking does not activate AhR, the induced CYP1A2 activity seen in previous clinical studies may be attributable to other substances present in roasted coffee beans (e.g., polycyclic aromatic hydrocarbons).

Unlike in human hepatocytes, caffeine is a weak inducer of CYP1A2 expression in rat hepatocytes at 50 µM (Fig. 7B and ). Whether this is due to AhR activation by caffeine (or its metabolites) is unknown. It was previously shown that caffeine was unable to displace
TCDD from its binding to AhR.\textsuperscript{37} As the experiment was performed using high concentrations of TCDD, which limits detection of low-affinity ligands to AhR,\textsuperscript{37} the possibility of AhR activation by caffeine (or its metabolites) still remains. In our study, caffeine treatment enhanced expression of CYP1A1, another AhR target gene, by 6-fold in rat hepatocytes (as compared to 53-fold induction by 3-MC; data not shown), suggesting potential AhR activation by caffeine. Furthermore, the chemical structure of caffeine resembles atypical AhR ligands that have been recently discovered.\textsuperscript{56} The investigation on whether caffeine or its metabolites bind to and activate AhR is beyond the scope of this exploratory study. Together, the involvement of AhR in the CYP1A2 induction by caffeine remains to be further verified.

In conclusion, our results show that caffeine has minimal effects on AhR activity or CYP1A2 expression in human hepatocytes. This suggests that in humans mechanisms other than CYP1A2 induction by caffeine mediate development of caffeine tolerance. Whether other components of coffee or tea affect CYP1A2 expression in humans remain to be determined.
V. CONCLUSIONS

In summary, we found that the decrease in warfarin dosing in CYP2C9*8 patients is in part due to the presence of the linked SNPs -1766T<C and -1188T<C. Previously, studies had shown that -1188T<C was linked to other CYP2C9 SNPs like CYP2C9*2, CYP2C9*3 etc., but the presence of -1766T<C is linked to -1188T<C. A comparison of CYP2C9*8 carriers to wild-type CYP2C9 carriers was unable to show a difference in the expression of mRNA; however, AEI analysis was able to show that there is in fact a difference in the mRNA expression between CYP2C9*8 and the wild-type CYP2C9. As a result, we determined that the -1766/-1188 SNPs have a profound effect on CYP2C9 expression, and the genotype at these two promoter SNPs is an important consideration to understand the effects on warfarin dose. Our data were able to show the distinct difference in gene expression was due to the presence of the promoter SNPs and not the exonic SNP, CYP2C9*8.

In summary, we found that there is no induction of CYP1A2 at normal doses, but it is possible to induce CYP1A2 at higher, non-normal doses of caffeine. The luciferase data was able to show no difference in luciferase activity at normal doses of caffeine. Also, the mRNA changes in human hepatocytes showed similar changes leading us to the conclusion that caffeine is not an inducer of CYP1A2 except at extremely high doses of caffeine. While not shown, it is reasonable to conclude that this difference between rats and humans is due most likely to an interspecies difference in XRE binding domains or additional chemicals that were not studied for the changes of CYP1A2 induction. Conclusively, we were able to determine there is no difference in CYP1A2 induction with caffeine treatment.
VI. FUTURE DIRECTIONS

In our study, we have shown that CYP2C9 expression is reduced in CYP2C9*8 carriers because of the presence of the promoter SNPs -1766T<C and -1188T<C working in concert in the promoter to have its effect. However, much remains unknown about how these two SNPs are working in concert together to reduce warfarin dosing in those carriers. Future experiments would include the determination of the different regulators—promoters or silencers—binding to the -1766 and -1188 regions with and without the presence of the SNPs. This would help us to determine that possibly with the presence of the two SNPs that there is an interaction between regulators, which essentially leads to a hampering of the ability to properly promote mRNA expression of CYP2C9. It would be important to determine this interaction to aid in our understanding of how SNPs work to affect the dosing of such drugs like warfarin. Often the research is done to determine that the presence of the SNP changes dosing but further investigation is not done behind why that SNP affects the dosing. Our research done so far is just a small step in the right direction to build up to further pharmacogenomic studies.

In our caffeine study, we were able to show that caffeine does not induce CYP1A2 at normal doses; however the possibility exists that caffeine increase CYP1A2 at higher doses. The next step would be to determine if another constituent in coffee is responsible for CYP1A2 induction or the true causation is the interspecies difference between the two promoter regions. This determination would be important because it remains possible that other substituents, like polyphenols, present in teas could lead to induction of CYP1A2. We would have to undertake similar tests to determine if these substances are responsible for induction of CYP1A2. Other experiments could also include the difference in caffeine binding to different XRE binding regions of the CYP1A2 promoters of rats versus humans.
VII. CITED LITERATURE


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David Vaynshtein and Hyunyoung Jeong, “Caffeine induces CYP1A2 expression in rat hepatocytes but not in human hepatocytes”, Drug Metabolism Letters 2012 (in print)  
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HONORS: Dean’s List Fall 2006-Spring 2010