

Implication of Genetic Polymorphisms in CYP2C9 and CYP2C19 on Drug Metabolism in Isolated Cryopreserved Human Hepatocytes

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Abstract

The cytochrome P450 (CYP) enzymes 2C9 and 2C19 in the IIC cluster on chromosome 10 are responsible for the metabolism of a number of clinically relevant drugs. Single Nucleotide Polymorphisms (SNPs) have been delineated in both genes which affect the enzyme activity or expression. The importance of certain polymorphisms is highlighted by the FDA recommendation for utilizing warfarin based on CYP2C9 genotype information and clopidogrel labeling based on the genotype of CYP2C19. Cryopreserved human hepatocytes have gained importance as a model for *in vitro* testing of drug metabolism and interactions in the modern drug discovery process. The ability of researchers to have access to reagents which are representative of the population at large is of critical importance. Here we explore the population representation and the correlation to activity of SNPs in 2C9 and 2C19 in our cryopreserved hepatocyte repository. Genomic DNA was isolated from cryopreserved human hepatocyte samples taken from 91 individual donors. Genotypes were generated utilizing Taqman PCR SNP assays for 2C9*2, *3 and 2C19*2, *3, *4, *5, *17. A metabolism assay was performed with tolbutamide and mephenytoin for these individuals generating a clearance rate based on formation of 4'-methylhydroxytolbutamide and 4'-hydroxymephenytoin respectively. The activity data, genotype, and race data were compiled and analyzed for basic statistical components and significance. Polymorphism frequency for 2C9*2, *3 and 2C19*2, *3, *4, *5, *17, closely match the frequency data from the HapMap survey. The mean rate of tolbutamide metabolism for CYP2C9*1/*2, *1/*3 and *2/*3, was significantly lower than that of *1/*1. 2C19*3, *4 and *5 were not present in our sample which correlates with their low population frequency. 2C19 *1/*2 heterozygous, wild type *1/*1 and heterozygous 2C19 *1/*17 showed a rank order relationship in mean rate of metabolite formation with mephenytoin corresponding with their Poor Metabolizer, Extensive Metabolizer, and Ultra Rapid Metabolizer phenotypes. The differences between these groups were not statistically significant and there was overlap in the range of values. These results confirm the utility of isolated cryopreserved human hepatocytes in *in vitro* drug development studies. The presence and proper phenotype of polymorphic alleles in isolated hepatocyte repositories allows for researchers to perform studies which require donors with specific genetic backgrounds earlier in development.

Introduction

The rising importance of pharmacogenetics in the clinical setting¹, highlighted by the identification of safety and efficacy of certain drugs in particular genetic backgrounds^{2,3}, has led to its increased inclusion in drug development programs. Testing for the impact of polymorphic CYP450 on a drug candidate is done with *in vitro* assays prior to clinical testing. In order to obtain accurate *in vitro* metabolism data and predict the impact in the clinical setting of specific polymorphisms, it becomes important to obtain well characterized *in vitro* systems.

In this study, we have characterized 91 cryopreserved human hepatocyte donors for genetic polymorphisms in CYP2C9 and CYP2C19. Polymorphisms were selected for high prevalence and physiologic relevance^{4,5,6}. Metabolic activity of these cells against prototypic substrates, tolbutamide for CYP2C9 and mephenytoin for 2C19, was also tested. The polymorphic frequency was stratified by race and compared to comparable HapMap data sets. The impact of the genotypes on the activity was analyzed for each CYP. Significant differences can be found in the rates of metabolism even in heterozygous backgrounds for some polymorphisms. This study highlights the utility of cryopreserved human hepatocytes in investigating the impact of genetic variation. With large donor libraries, rare variants can be found to generate more accurate predictions of clinical outcomes.

Material and Methods

Cryopreserved human hepatocytes and media were obtained from BioreclamationIVT(Baltimore, MD). Lots were thawed as outlined in the protocol from BioreclamationIVT. Briefly, vials were taken from liquid nitrogen and thawed immediately at 37°C into 49mls of InVitroGRO™ HT Media to wash out cryoprotectants, spun and resuspended in InVitroGRO™ KHB. Genomic DNA was isolated from cell pellets using the Qiagen DNA Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions.

Genotypes were determined using the isolated genomic DNA in TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA) (Table 1). Assays were run and analyzed with an Applied Biosystems 7500 Fast Real-Time PCR System running SDS Software V1.3. Allelic frequency was calculated as the number in each group divided by the total tested for each stratified race.

Activity of CYP2C9 and CYP2C19 in the cryopreserved human hepatocytes was determined using tolbutamide and mephenytoin, respectively. Cells were thawed as described above and incubated in InVitroGRO™ KHB buffer with 150uM tolbutamide and 20uM mephenytoin for 1hr. Reactions were stopped with an equal volume of methanol. The metabolite formation was determined by LC/MS/MS.

Cytochrome P450	Allele	Major Polymorphism	Type	SNP reference	AB Assay
2C9	*2	430C>T	Reduced function PM	rs1799853	C_25625805_10
2C9	*3	1075A>C	Reduced function PM	rs1057910	C_27104892_10
2C19	*2	681G>A	Truncation PM	rs4244285	C_25986767_70
2C19	*3	636G>A	Truncation PM	rs4986893	C_27861809_10
2C19	*4	1A>G	Loss of function PM	rs28399504	C_30634136_10
2C19	*5	1297C>T	Missense PM	rs56337013	C_27861810_10
2C19	*17	-806C>T	Increased transcription URM	rs12248560	C_469857_10

Table 1. Listed are the analyzed polymorphisms for CYP2C9 and CYP2C19, their effect on the gene product, NCBI database and PCR assay information. Included is the allele nomenclature defined by the Human Cytochrome P450 (CYP) Allele Nomenclature Committee.⁷ Type indicates the effect and phenotype of the mutant allele (PM, Poor Metabolizer; URM, Ultra Rapid Metabolizer). The SNP reference refers to the reference SNP archived by NCBI (<http://www.ncbi.nlm.nih.gov/>). The Taqman primer and probe set used are indicated by the Applied Biosystems assay number.

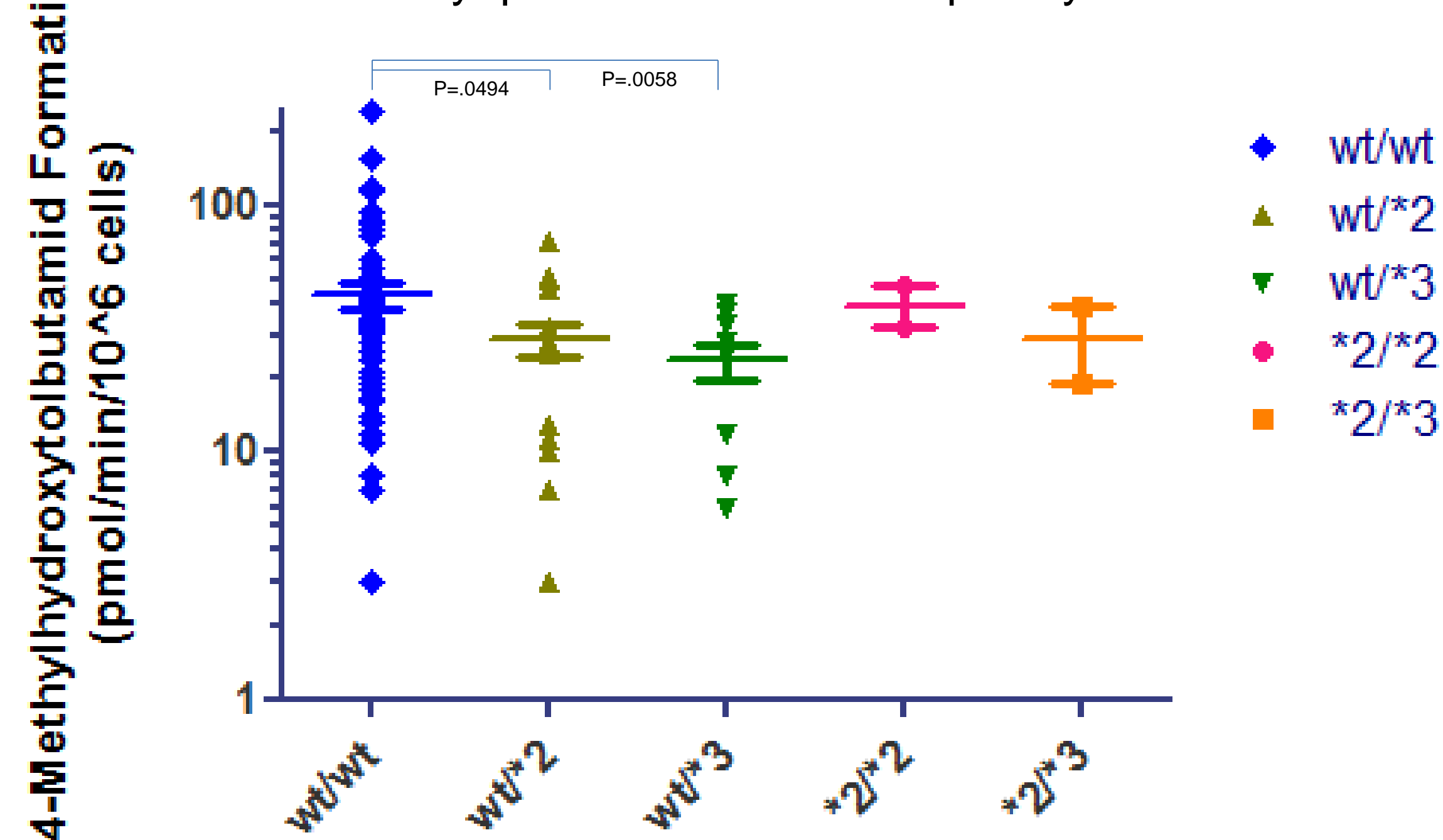
Results

	n=	Celsis IVT Allelic Frequencies			HapMap Allelic Frequencies		
		wt	*2	*2/*2	wt	*2	*2/*2
2C9*2							
Asian	1	1.000	0.000	0.000	1.000	0.000	0.000
African American	9	1.000	0.000	0.000	1.000	0.000	0.000
Caucasian	69	0.725	0.246	0.029	0.792	0.208	0.000
Hispanic	8	0.875	0.125	0.000			
Polynesian	1	1.000	0.000	0.000			
2C9*3							
Asian	1	1.000	0.000	0.000	0.911	0.089	0.000
African American	9	0.889	0.111	0.000	1.000	0.000	0.000
Caucasian	69	0.841	0.159	0.000	0.883	0.117	0.000
Hispanic	7	1.000	0.000	0.000			
Polynesian	1	1.000	0.000	0.000			
2C19*2							
Asian	1	1.000	0.000	0.000	0.489	0.511	0.000
African American	9	1.000	0.000	0.000	0.746	0.220	0.034
Caucasian	69	0.725	0.275	0.000	0.741	0.207	0.052
Hispanic	7	1.000	0.000	0.000			
Polynesian	1	0.000	1.000	0.000			
2C19*17							
Asian	1	0.000	1.000	0.000	0.956	0.044	0.000
African American	9	0.778	0.111	0.111	0.533	0.383	0.083
Caucasian	69	0.594	0.362	0.043	0.583	0.400	0.017
Hispanic	8	0.625	0.125	0.250			
Polynesian	1	1.000	0.000	0.000			

Table 2. Allelic frequency in cryopreserved human hepatocytes and that reported by the HapMap survey. For the Asian group, the CHB (Han Chinese in Beijing, China) group was used, for the African American, the YRI (Yoruba in Idaban, Nigeria), for Caucasian, the CEU (CEPH (Utah residents with ancestry from northern and western Europe)).

The genotypes of 88 tested cryopreserved human hepatocytes were compiled and stratified by both race and genotype (Table 2). The frequency data was compared to representative populations sequenced by the HapMap project. The allelic frequency data matched well with that seen in our African American and Caucasian populations for whom we had significant representation. Most groups were represented at low frequencies, preventing proper analysis. CYP2C19*3, *4 and *5 polymorphisms were tested but not detected in any of our donors, which follows their low population frequency, and this data was not reported.

Tolbutamide Metabolism in CYP2C9 Genotyped Cryopreserved Human Hepatocytes



Genotype	wt/wt	wt/*2	wt/*3	*2/*2	*2/*3
Mean Activity (pmol/min/10 ⁶ cells)	43.1	28.7	23.4	39.5	29.0
% of wt/wt	100%	67%	54%	92%	67%

Figure 1. The CYP2C9 activity of the individual cryopreserved human hepatocyte donors measured by the rate of formation of 4-methylhydroxytolbutamide is graphed versus the CYP2C9 genotype for these donors. The mean with SEM is marked for each group. The panel below the graph shows the mean and the relative activity to the wildtype genotype for each genotype.

Tolbutamide metabolite, 4-methylhydroxytolbutamide, formation in 91 cryopreserved human hepatocytes was analyzed with their 2C9 genotype (Figure 1). The wild type genotype displayed a large range of values spanning nearly two logs. The *2 and *3 heterozygous mutants also showed a wide range of values though with lower mean activities. These differences were shown to be significant in a paired t-test, with p-values displayed. The *2/*2 homozygous and *2/*3 genotypes contained only two individuals and showed no statistical significance. Values were higher than the single mutant donors though not out of line with some reports showing increased metabolism over heterozygotes.^{8,9}

Mephenytoin Metabolism in CYP2C19 Genotyped Cryopreserved Human Hepatocytes

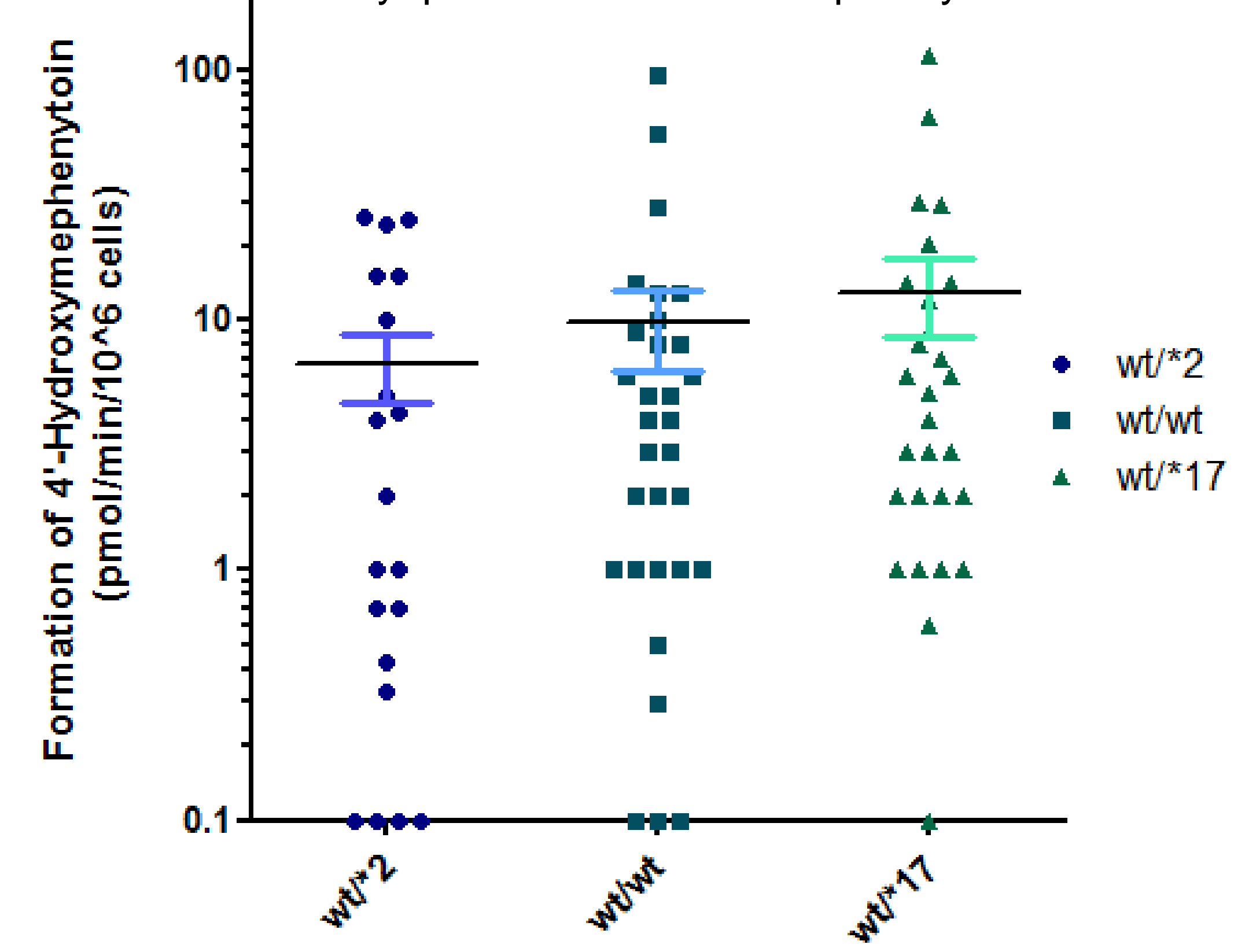


Figure 2. The CYP2C19 activity of the individual cryopreserved human hepatocyte donors measured by the rate of formation of 4-hydroxymephenytoin graphed versus the CYP2C19 genotype for these donors. The mean with SEM is marked for each group. The panel below the graph shows the mean and the relative activity to the wildtype genotype for each genotype. The data points marked on the x-axis is below quantifiable levels.

Mephenytoin metabolite, 4-hydroxymephenytoin, formation in 91 cryopreserved human hepatocytes was analyzed in relation to their 2C19 genotype (Figure 2). All genotypes displayed a large range of metabolism values spanning over two logs. The data means and range was lower for the wt/*2 poor metabolizer genotype when compared to the wildtype. The heterozygous wt/*17 genotype samples showed an increase in the mean rate of metabolism as expected. The differences between the genotypes were not statistically significant.

Conclusion

- Allelic frequencies seen in liver donor populations closely match that seen the larger HapMap survey of genetic variation.
- CYP2C9 variants show reduced mean metabolism of tolbutamide; however, a wide range of variation is present in all groups.
- The CYP2C9 *2 and *3 heterozygous wildtype samples are significantly different from the wildtype in formation of 4-methylhydroxytolbutamide.
- CYP2C19 variants show a difference in the mean rate of metabolism of mephenytoin corresponding to their PM or URM phenotypes.
- A large range of metabolic activities is seen in our human cryopreserved hepatocyte library independent of the tested phenotypes, which may be due to other polymorphic sites, donor physiological differences, or exposure to CYP inducing or inhibiting agents.
- Cryopreserved human hepatocytes provide a useful reagent for exploring the effect of genetic background on drug metabolism *in vitro* and may allow for researchers to identify idiosyncratic responses to candidate compounds in rare genetic backgrounds prior to clinical trials.

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