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***In Vivo* Functional Effects of *CYP2C9* *MIL*, a Novel and Common Variant in the Yup'ik Alaska Native Population**

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Abbreviations Used

AN, Alaska Native; CYP, cytochrome P450; HLM, human liver microsomes; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography mass spectrometry; YK, Yukon-Kuskokwim

Abstract

Alaska Native people are under-represented in genetic research, but have unique gene variation that may critically impact their response to pharmacotherapy. Full resequencing of *CYP2C9* in a cross-section of this population identified *CYP2C9MIL* (*MIL*), a novel, relatively common single nucleotide polymorphism hypothesized to confer *CYP2C9* poor metabolizer phenotype by disrupting the start codon. *MIL* is present at a minor allele frequency of 6.3% in Yup'ik Alaska Native people and, thus, can contribute to the risk of an adverse drug response from narrow therapeutic index *CYP2C9* substrates, such as (*S*)-warfarin. This study's objective was to characterize the catalytic efficiency of the *Leu1* variant enzyme *in vivo* by evaluating the pharmacokinetic behavior of naproxen, a probe substrate for *CYP2C9* activity, in genotyped Yup'ik participants. We first confirmed the selectivity of (*S*)-naproxen O-demethylation by *CYP2C9* using activity phenotyped human liver microsomes and selective CYP inhibitors, then developed and validated a novel LC/MS method for simultaneous quantification of (*S*)-naproxen, (*S*)-O-desmethyl naproxen, and naproxen acyl glucuronide in human urine. The average ratio of (*S*)-O-desmethyl naproxen to unchanged (*S*)-naproxen in urine was 18.0 ± 8.0 ($n = 11$) for the homozygous *CYP2C9 Met1* reference group and 10.3 ± 6.6 ($n = 11$) for the *Leu1* variant carrier group ($P = 0.011$). The effect of *MIL* variation on *CYP2C9* function and its potential to alter the pharmacokinetics of drugs metabolized by the enzyme has clinical implications and should be included in a variant screening panel when pharmacogenetic testing in the Alaska Native population is warranted.

Significance Statement

Our group recently identified the novel *CYP2C9 Met1Leu* variant in Alaska Native people. Here, we validated (*S*)-naproxen as a CYP2C9 probe substrate to characterize the *in vivo* functional activity of the *CYP2C9 Leu1* variant. The results of our pharmacogenetic-pharmacokinetic study suggest that the *CYP2C9 Leu1* variant exhibits loss of enzyme activity. This finding may be important to consider when administering narrow therapeutic index medications metabolized by CYP2C9 and also compels further investigation to characterize novel genetic variation in understudied populations.

Introduction

The cytochrome P450 family 2 subfamily C member 9 (CYP2C9) enzyme is responsible for the elimination of approximately 15% of all medications cleared through a P450-mediated biotransformation pathway (Zanger et al., 2008; Van Booven et al., 2010). CYP2C9 has a broad range of clinical substrates, including anticoagulants, anticonvulsants, angiotensin II blockers, hypoglycemic agents, and nonsteroidal anti-inflammatory drugs. The *CYP2C9* gene is highly polymorphic, with coding-region variation (*CYP2C9**2 and *3) that confers poor metabolizer phenotype, dramatically influencing the pharmacokinetics and drug response of commonly used narrow therapeutic index medications (e.g., (*S*)-warfarin, phenytoin) (Caudle et al., 2014; Flora et al., 2017; Johnson et al., 2017).

Recently, our group identified the novel *CYP2C9 Met1Leu* (*MIL*) variant in the Alaska Native (AN) population (Fohner et al., 2015). The substitution of leucine for methionine at the first amino acid position is predicted to markedly slow or stop RNA translation. Indeed, *in vitro*

studies with *MIL* gene transfected HepG2 cells demonstrated that the *CYP2C9 LeuI* variant protein does not accumulate in this liver-derived cell line (McDonald et al., 2020). In the Yup'ik AN population, the *MIL* variant is found at a higher minor allele frequency (6.3%) than the well-characterized *CYP2C9*2* (0.3%) and *CYP2C9*3* (2.1%) alleles (Fohner et al., 2015). The historical home of the Yup'ik people is southwestern Alaska, along the Bering Sea, including the relatively remote Yukon-Kuskokwim (YK) Delta. There are 58 communities in the YK Delta (total population ~23,000), a 75,000 square mile area, and all are accessed by air, water or other non-road system travel. Communities have health clinics staffed by community health aids and primary care is offered through five sub-regional health clinics or the regional hub hospital in Bethel, Alaska. This geographic isolation of communities away from primary care providers creates challenges to medical service that may not be experienced in urban areas. For example, pharmacotherapy with narrow therapeutic index drugs can be more difficult to manage because of geographical barriers to monitoring drug responses. With specific regard to *CYP2C9* substrates, such as warfarin, phenytoin, and tolbutamide, variation in the *CYP2C9* gene contributes to inter-individual differences in dose requirement (Becker et al., 2008; Caudle et al., 2014; Flora et al., 2017; Johnson et al., 2017). Genetic testing, as a form of precision medicine, has been adopted by many urban medical centers and may have enhanced clinical utility for managing these and other drug therapies in geographically isolated populations. To advance the goals of precision medicine for AN people, it is necessary to fully understand the frequency and function of variation in important pharmacogenes such as *CYP2C9*. Moreover, it is critical to investigate previously unknown variants, such as *MIL* and *N218I*, that are common in the AN population (Fohner et al., 2015) and are expected to impair *CYP2C9* activity.

Characterization of enzyme function *in vivo* is commonly accomplished with a pharmacokinetic study that involves administration of a probe drug selectively metabolized by the enzyme of interest. Established CYP2C9 probes include the narrow therapeutic index drugs warfarin, phenytoin and tolbutamide, as well as the non-steroidal anti-inflammatory drugs celecoxib and flurbiprofen. However, for a study in the Yup'ik population, selection of a commonly used drug known to be safe and recognizable to potential participants (over the counter) was considered just as important as selectivity for CYP2C9 activity. Thus, we elected to validate and use (*S*)-naproxen as the *in vivo* enzyme probe. (*S*)-naproxen undergoes O-dealkylation primarily by CYP2C9, with minor involvement from other P450 enzymes (Miners et al., 1996; Tracy et al., 1997). It is well absorbed (Runkel et al., 1972; Davies and Anderson, 1997), highly bound to albumin (Davies and Anderson, 1997), and almost completely eliminated in the urine as naproxen glucuronide (60% of the dose), unchanged naproxen (1%), and secondary glucuronide and sulfate metabolites of (*S*)-O-desmethyl naproxen (20%) (Sugawara et al., 1978; Kiang et al., 1989; Vree et al., 1993; Davies and Anderson, 1997). Although not the major pathway of (*S*)-naproxen elimination, a low total urinary (*S*)-O-desmethyl naproxen/(*S*)-naproxen concentration ratio is indicative of a low CYP2C9 intrinsic formation clearance. This study's objective was to verify the selectivity of the (*S*)-naproxen O-dealkylation reaction for CYP2C9 and then determine the catalytic efficiency of the novel *MIL* variant *in vivo* to inform on its potential to affect the drug disposition and pharmacological response of medications metabolized by CYP2C9.

Materials and Methods

Setting

Study recruitment was conducted in 10 communities found in the YK Delta of Alaska. Approximately two-thirds of the AN population in Alaska live in rural communities with populations of 50 – 1000 people, many only accessible by air or water (Norris et al., 2012). Dr. Bert Boyer and Ms. Scarlett Hopkins, formerly at the University of Alaska Fairbanks and now based at Oregon Health & Science University (OHSU), have ongoing genetic research partnerships with 11 of the 58 rural communities in the YK-Delta.

Study Participants

Study participants were selected from a cross-sectional population of Yup'ik men and women over 18 years old, for whom *CYP2C9* *MIL* genotype was previously determined, and who consented to be contacted for future research investigations. Participants were in good health and not taking non-steroidal anti-inflammatory agents or other drugs known or suspected of altering CYP2C9 function.

Study Design

The University of Alaska Fairbanks and OHSU Institutional Review Boards (IRB) and the Yukon-Kuskokwim Health Corporation (YKHC) Human Studies Committee and Executive Board approved this study. The University of Washington (UW) IRB approved the overall research project, as UW was the academic home of the grant funding this research (NIH P01 GM116691) and its principal investigators. The study is registered at ClinicalTrials.gov (NCT04449471).

Following written informed consent, participants were asked to fast for 12 hours prior to the start of the pharmacokinetic study, and then provided a baseline urine sample. A single 220 mg naproxen sodium caplet (200 mg (*S*)-naproxen), was administered with a glass of water. Urine was collected for the next 24 hours after the naproxen dose. Due to the instability of naproxen acyl glucuronides in alkaline media, urine pH was stabilized by adding 13.6 g monobasic potassium phosphate to each urine collection container before use. At the end of the collection interval, study participants returned the urine collection container to the study site, where the urine volume was measured and recorded. The urine was well mixed and two 5 mL aliquots were taken from the collection container and stored initially at -15°C in a portable freezer, and then at -80°C, until analysis.

Genotyping

To identify *Met1/Leu1* heterozygotes and *Leu1/Leu1* homozygotes from the Yup'ik population, the Fluidigm platform was used to perform genotype analysis of DNA extracted from white blood cells, targeting the *CYP2C9* exome, as previously described (Fohner et al., 2015). Based on prior gene sequencing work, the following *CYP2C9* variants (cDNA position and base change indicated for variants without rs number) were tested: *Met1Leu* (1A>T), *Asn218Ile* (653A>T), *2 (rs1799853), *3 (rs1057910), *8 (rs7900194), *11 (rs28371685), *13 (rs72558187), *14 (rs72558189), and *29 (rs182132442). A total of 1112 individuals from the Yup'ik population were genotyped.

Validation of (*S*)-Naproxen as a Selective *CYP2C9* Probe Substrate

Comprehensive *in vitro* studies were performed to validate the selectivity and sensitivity of naproxen as a probe for CYP2C9 activity. Unlabeled (*S*)-naproxen and racemic O-desmethyl naproxen- d_3 were purchased from Toronto Research Chemicals (Ontario, Canada). Unlabeled O-desmethyl naproxen, furafylline, sulfaphenazole, and NADPH were purchased from Sigma Aldrich (St. Louis, MO). Pooled HLMs were purchased from XenoTech (Kansas City, KS). Individual HLMs were isolated from the University of Washington School of Pharmacy Human Liver bank, as previously reported (Shirasaka et al., 2016). Individual recombinantly-expressed cytochrome P450 Supersome™ preparations were obtained from Corning Life Sciences (Woburn, MA). All other chemicals were analytical grade or better and obtained from various commercial vendors.

(*S*)-naproxen was incubated with pooled HLMs (0.5 mg/mL final concentration) in the presence of NADPH (1 mM final concentration) in a buffer consisting of 50 mM KH_2PO_4 with 1.27 mM EDTA, pH 7.4, at a total volume of 200 μ L. In experiments using selective CYP isoform inhibitors, sulfaphenazole (prepared in methanol, with final concentration below 0.2%) and furafylline (prepared in DMSO, with final concentration below 0.1%), the final inhibitor concentration was 10 μ M. Microsomal incubations with furafylline underwent a 20 minute preincubation with the CYP1A2 inhibitor prior to (*S*)-naproxen reaction initiation. Reactions ran for 20 minutes at 37°C, and were conducted over a (*S*)-naproxen concentration range of 5 – 1800 μ M. The microsomal reaction was quenched with the addition of 1 mL ice cold methanol containing 2% formic acid. To the quenched samples, 80 ng of O-desmethyl naproxen- d_3 , internal standard, were added. The samples were then centrifuged at 3000 *g* for 10 minutes, decanted into glass culture tubes, and dried with nitrogen gas, and resuspended in 50 μ L mobile phase. A volume of 20 μ L was injected onto the LC/MS.

A CYP Supersome™ screen was performed by evaluating CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9*1, CYP2C9*2, CYP2C9*3, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, and CYP3A7 metabolic activity towards (*S*)-naproxen. For this experiment, (*S*)-naproxen was incubated with 10 pmol of each Supersome™ preparation in 50 mM KH₂PO₄ with 1.27 mM EDTA buffer (except CYP2A6, for which 50 mM Tris buffer was used) in a total volume of 200 µl. Reactions were initiated with the addition of NADPH (1 mM final concentration) and incubated for 20 minutes at 37°C at the (*S*)-naproxen concentrations of 25 µM (below *K_m*) and 1000 µM (saturating concentration). The incubation reaction was quenched with 1 mL ice cold methanol containing 2% formic acid and 200 ng of O-desmethyl naproxen-d₃, internal standard, was added. The samples were centrifuged at 3000 *g* for 10 minutes, decanted into glass culture tubes, and dried with N₂ gas, and resuspended in 50 µl mobile phase. A volume of 5 µl was injected onto the LC/MS. For CYP enzymes that catalyzed (*S*)-O-desmethyl naproxen formation, additional reactions were carried out for 20 minutes at 37°C, over the (*S*)-naproxen concentration range of 5 – 1800 µM, to determine Michaelis-Menten kinetic parameters. The data was normalized for the absolute amount of recombinant P450 added to the incubations (i.e., pmol P450).

To evaluate the effect of varying CYP1A2 content on naproxen metabolism, single donor HLMS from the UW Human Liver Bank were selected based on CYP1A2 protein content (determined by LC-MS/MS analysis). Five high CYP1A2 expressors and five low CYP1A2 expressors, all with comparable CYP2C9 protein expression, were incubated with 20 µM (*S*)-naproxen, NADPH (1 mM final concentration), in the presence or absence of 10 µM sulfaphenazole and furafylline, in 50 mM KH₂PO₄ with 1.27 mM EDTA, pH 7.4, and in a total volume of 200 µl. Reactions were carried out as described above with pooled HLM experiments.

Calibration curves for (*S*)-naproxen metabolites were prepared by spiking variable amounts of unlabeled (*S*)-O-desmethyl naproxen, into 200 μ l of potassium phosphate buffer in order to generate standard mixtures with final concentrations of 0.2 – 10 μ M for HLM incubations, 0.1 – 5 μ M for HLM inhibition experiments, 0.1 – 30 μ M for Supersome™ experiments. Standard solutions, prepared in duplicate for each concentration, were immediately worked-up and analyzed in an identical fashion to that described for the incubation samples above. GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, CA) was used to estimate K_m and V_{max} parameters.

Urine Sample Preparation

For (*S*)-naproxen detection, urine samples (50 μ l) were prepared by adding 100 μ l of HPLC grade water and 100 μ l of 1 nmol racemic naproxen- d_3 (internal standard for (*S*)-naproxen). For naproxen acyl glucuronide detection, urine samples were diluted 1:20 in blank urine then 50 μ l of the diluted sample was combined with 100 μ l of HPLC grade water and 100 μ l of 1 nmol racemic flurbiprofen acyl glucuronide (internal standard for naproxen acyl glucuronide). For total (*S*)-O-desmethyl naproxen detection, urine was diluted 1:4 in blank urine, then the diluted urine sample (50 μ l) was combined with 80 μ l HPLC grade water, 20 μ l 6M HCL, 100 μ l of internal standard (1 nmol racemic O-desmethyl naproxen- d_3) followed by vortexing and incubating at 90°C for 60 minutes to facilitate glucuronide and sulfate cleavage via acid hydrolysis. This heated acid hydrolysis approach was adapted from a published protocol for a similarly structured acyl-glucuronide (Zgheib et al., 2007), since O-desmethyl naproxen glucuronide can hydrolyze back to O-desmethyl naproxen or isomerize to glucuronidase-resistant isoglucuronides, under alkaline conditions (Davies and Anderson, 1997). All samples were

vortexed, centrifuged at 14,000 *g* for 5 minutes, and then 50 μ l of sample supernatant was transferred to autosampler vials and 2 μ l were injected onto the LC/MS.

Urine Sample Analysis

To evaluate the effect of *MIL* variation on CYP2C9 function, the urinary (*S*)-*O*-desmethyl naproxen to unchanged naproxen metabolite to parent ratio was determined from the 24-hour urine collection. Naproxen and metabolite concentrations were accessed by LC/MS using an Agilent 1956B single quadrupole mass spectrometer coupled with an Agilent 1200 series (Santa Clara, CA) liquid chromatography system. Chromatographic separation was achieved on a Luna C18 (2 mm x 50 mm x 5 μ m) column (Torrence, CA) with a mobile phase flow rate of 0.3 mL/min. The mobile phase consisted of 10 mM ammonium formate (A, pH 3.5) and methanol (B), and linear gradients were applied with B% increasing from 40% to 80% between 3 and 8 minutes and decreasing to 40% at nine minutes. Quantitation was achieved by selected ion monitoring centered on *m/z* values of 248.1 for (*S*)-naproxen, 251.1 for racemic naproxen-*d*₃, 234.1 for (*S*)-*O*-desmethyl naproxen, 237.1 for racemic *O*-desmethyl naproxen-*d*₃, 424.1 for naproxen acyl glucuronide, and 438.1 for racemic flurbiprofen acyl glucuronide. Data acquisition and analysis were performed using the Agilent MassHunter software. Calibration curves were constructed by plotting the peak area ratio of each compound to the respective internal standard against a range of targeted analyte concentrations. We measured the urinary concentration of the major naproxen metabolite, naproxen acyl glucuronide, in order to ensure comparable dose recovery and urine collection compliance. The intra-day variation for quantitation of each analyte did not exceed 2% for the low concentration quality control (QC) for (*S*)-*O*-desmethyl naproxen and did not exceed 6% for the high concentration QC. The relative

errors of the two QC concentrations tested in three independent experiments were within 5% and 8% for the low and high concentration QCs, respectively.

Statistical Analysis

We proposed a regression analysis for the statistical analysis plan to test for an additive-gene dose effect (0, 1, or 2 functional alleles) of *Leu1*. From our power calculations, a sample size of 30, with 10 participants per genotype group, resulted in a power of 0.8 at a 0.05 significance level. Considering possible difficulties in recruiting 10 *Leu1/Leu1* homozygotes, we also proposed *a priori* an alternative statistical analysis plan where the homozygous variant *Leu1/Leu1* group would be combined with the heterozygous variant *Met1/Leu1* group. Under this plan, a sample consisting of 15 reference homozygotes and 15 *Leu1* carriers and homozygotes would achieve the same power at the same level of significance. Recruitment was halted by travel restrictions instituted by OHSU and the YKHC due to the COVID-19 pandemic, and our final sample size was 22, with 11 *Met1* homozygotes and 11 *Leu1* carriers and homozygotes. Travel is still restricted, as of time of submission of this manuscript. The regression analysis for the comparison between the *Met1/Met1* reference and combined *Met1/Leu1* heterozygotes and *Leu1/Leu1* homozygotes allowed for heteroscedasticity, and was completed using RStudio version 1.2.1335 (RStudio, Inc., Boston, MA). With regard to the analysis of the *in vitro* data, comparisons between recombinant enzymes and single donor HLMs were assessed using two-tailed unpaired t-tests, allowing for heteroscedasticity.

Results

Study Enrollment Based on *MIL* Genotype

A total of 1112 Yup'ik adults were genotyped for *CYP2C9* variants. After removing duplicate records from repeat visits ($n=193$) and genotypes with no calls ($n=6$), a total of 913 genomes were considered for the pharmacokinetic study (Figure 1). Individuals with one or more copies of *CYP2C9**2, *3, *8, *11, *13, *14, *29, or *N218I* alleles were excluded due to their confounding effects on CYP2C9 activity. A total of 8 *Leu1/Leu1* homozygotes were identified from 5 different communities, with an average age of 36 years. These individuals were all unrelated at the parent-child and sibling level. A total of 85 *Met1/Leu1* heterozygotes, with an average age of 37 years, and 629 in the *Met1/Met1* reference (wildtype), with an average age of 36 years, were also identified.

Selectivity and Sensitivity of (*S*)-Naproxen as a Probe for CYP2C9 Enzyme Activity

Comprehensive *in vitro* studies determined that CYP2C9 is the predominant enzyme metabolizing (*S*)-naproxen to (*S*)-O-desmethyl naproxen. A representative Michaelis-Menten plot of (*S*)-O-desmethyl naproxen formation in pooled human liver microsomes (HLMs) is shown in Figure 2, with a mean K_m of $420 \pm 2 \mu\text{M}$ and V_{max} of $0.92 \pm 0.06 \text{ nmol/min/mg}$ microsomal protein from three repeated experiments. The CYP Supersome™ screen showed that (*S*)-O-desmethyl naproxen was formed in incubations containing $25 \mu\text{M}$ naproxen by only three P450 enzymes – CYP2C9, CYP2C8, CYP1A2 (Figure 3). The activity of CYP2C9 was much higher than that of CYP1A2 and CYP2C8. Twenty-five μM is a more clinically relevant concentration, considering that (*S*)-naproxen exhibits extensive protein binding (>99%) and its C_{max} is $\sim 250 \mu\text{M}$ following a single 440 mg dose under fasting conditions (NDA, 2005). The dominant role of CYP2C9 is less apparent at higher (*S*)-naproxen concentrations, as

demonstrated in the incubation with 1000 μM of (*S*)-naproxen, where the contribution of CYP1A2 increased by over 30-fold and small amounts of product were detected in incubations with several other P450 SupersomesTM (Figure 3). In a separate experiment, we evaluated the activity of a vector control SupersomeTM preparation, compared to CYP2C9. There was no (*S*)-O-desmethyl naproxen product detected in incubations with the vector control, at both the 25 μM and 1000 μM substrate concentrations, compared to a robust product formation rate produced by CYP2C9.

Full kinetic experiments were conducted to assess the (*S*)-O-desmethyl naproxen intrinsic formation clearances of CYP2C9, CYP1A2, and CYP2C8 SupersomesTM (Figure 4). The mean V_{max} values for CYP2C9 and CYP1A2 were 31.7 and 41.7 pmol/min/pmol P450, respectively, while their K_m values were markedly different, 280 μM for CYP2C9 and 1000 μM for CYP1A2 ($P = 0.005$) (Table 1). The intrinsic clearance by CYP2C9 was significantly greater than for CYP1A2 ($P = 0.008$). Given liver abundances of 73, 52, and 24 pmol P450/mg protein (Rowland-Yeo et al., 2004) for CYP2C9, CYP1A2, and CYP2C8, respectively, the average contribution from each of these enzymes to (*S*)-O-desmethyl naproxen formation was predicted to be 78% for CYP2C9, 20% for CYP1A2, and 2% for CYP2C8.

We also estimated the fraction of (*S*)-naproxen metabolized to (*S*)-O-desmethyl naproxen in HLMs by CYP2C9 and CYP1A2 from selective enzyme inhibitor experiments conducted with 20 μM (*S*)-naproxen, a substrate concentration 5-fold below the K_m determined in pooled HLMs. (*S*)-O-desmethyl naproxen formation was reduced by $76.9 \pm 1.5\%$ with 10 μM sulfaphenazole, a selective CYP2C9 inhibitor, by $21.5 \pm 1.6\%$ with 10 μM furafylline, a selective CYP1A2 inhibitor, and by $95.8 \pm 2.1\%$ with both sulfaphenazole and furafylline

(Figure 5). The solvents for the inhibitors had negligible effects on the percent inhibition (Figure 5).

The effect of CYP2C9 and CYP1A2 inhibition on (*S*)-O-desmethyl naproxen formation by HLMs was also assessed in two groups of single donor HLMs, high CYP1A2 expressors ($n = 5$) with an average CYP1A2 content of 31.2 ± 10.8 pmol/mg microsomal protein, and low CYP1A2 expressors ($n = 5$) with an average of 2.8 ± 2.3 pmol/mg microsomal protein. CYP2C9 content was 53.2 ± 13.3 and 36.6 ± 6.3 pmol/mg microsomal protein in the high and low CYP1A2 groups, respectively. As predicted, the percent inhibited by 10 μ M furafylline was greater in the high CYP1A2 group ($39.7 \pm 7.0\%$ pmol/mg microsomal protein), compared to the low CYP1A2 group ($23.6 \pm 7.6\%$ pmol/mg microsomal protein) ($P = 0.008$), while the percent inhibited by 10 μ M sulfaphenazole was greater in the low CYP1A2 group ($85.2 \pm 11.8\%$ pmol/mg microsomal protein), compared to the high CYP1A2 group ($65.5 \pm 4.1\%$ pmol/mg microsomal protein) ($P = 0.017$) (Table 2). Although the CYP1A2 content was 11-fold greater in the high CYP1A2 group, compared to the low CYP1A2 group, the percent inhibited by furafylline was only 1.7-fold greater. Thus, CYP1A2 contribution to (*S*)-O-desmethyl naproxen formation in HLMs was always minor, in comparison to the CYP2C9 contribution.

Impact of *MIL* on Urinary Metabolite to Parent Ratio

The mean ratio of (*S*)-O-desmethyl naproxen to (*S*)-naproxen was greater for the homozygous reference group (18.0 ± 8.0 , $n = 11$), compared to the *MIL* variant group (10.3 ± 6.6 , $n = 11$), which includes 8 *Met1/Leu1* heterozygotes and 3 *Leu1/Leu1* homozygotes (Figure 6). Pairwise comparison (allowing for heteroscedasticity) was significant ($P = 0.011$), indicating reduced activity for the *Leu1* variant. The mean metabolite to parent ratios for heterozygotes and

Leu1/Leu1 homozygotes were 9.7 ± 5.6 and 12.1 ± 10.1 , respectively (Figure S1). One of the three *Leu1/Leu1* homozygote participants reported using tobacco products, which may have induced their CYP1A2 activity and skewed that result and mean for a small sample size. There was no evidence of significant metabolic shifting towards the parent glucuronide elimination pathway in *Leu1* carriers, as the mean urinary metabolite to parent ratio for the conjugate in carriers of the *Leu1* allele (32.3 ± 12.9) was similar to that of the reference group (34.5 ± 9.4).

Discussion

Alaska Native people are under-represented in genetic research, but have unique pharmacogene variation that may critically impact their response to drug therapy. This is the first study to characterize prospectively the *in vivo* functional effect of the novel, relatively common, *CYP2C9 MIL* single nucleotide polymorphism identified in Yup'ik and other AN people. The results suggest that a change in the start codon conferred complete loss of function, with no protein synthesis. Given the mean contributions of CYP2C9 (80%) and CYP1A2 (20%) to (*S*)-O-desmethyl naproxen formation in HLMs, it was predicted that a *Leu1* variant group (comprised of three *Leu1/Leu1* homozygotes and eight heterozygotes) would have a 51% reduction in urinary ratio of (*S*)-O-desmethyl naproxen to unchanged naproxen, compared to the reference group. The observed 43% reduction in the *Leu1* variant group is in good agreement with this prediction. A loss of enzyme activity with the *Leu1* variant has clinical implications, particularly for low narrow therapeutic index drugs such as warfarin, phenytoin and tolbutamide, where carriers of the variant would be more likely to experience an exaggerated drug response. In

addition, failure to include this variant in a pharmacogenetic test panel, if implemented to guide drug dose selection, could result in phenotypic misclassification in the Yup'ik population.

While the *MIL* variant is a novel *CYP2C9* impaired function variant found in the Yup'ik population (and at a lower frequency in other AN groups) (Fohner et al., 2015), it is not the only example of loss of the translation start codon conferring poor metabolizer status in the P450 2C subfamily. *CYP2C19*4* (rs28399504) is a loss-of-function allele, resulting from a substitution of methionine to valine at the first amino acid position (Ferguson et al., 1998). However, based on 1000 genomes data, the *CYP2C19*4* variant is only found at low frequencies across world populations: 0.8% in a Mexican population (California, USA), 0.5% in a Han Chinese population (Beijing, China), and the allele was not detected in Europeans (Utah residents with Northern and Western European ancestry) or in African Americans (Southwestern USA) (Consortium et al., 2015). By contrast, *MIL* is present at a relatively high minor allele frequency of 6.3% in the Yup'ik population and, thus, can contribute to variability in the clearance of *CYP2C9* substrates and the associated pharmacological responses.

In order to characterize the catalytic efficiency of the *MIL* variant, this study first had to establish the use of (*S*)-naproxen as an over the counter probe substrate to assess *CYP2C9* enzyme activity. Earlier studies characterizing the *in vitro* metabolism of (*S*)-naproxen downplayed its utility as a probe substrate due to involvement of *CYP1A2* (Miners et al., 1996; Rodrigues, 2005), and because an *in vivo* study in a Korean population did not observe a difference in the mean plasma concentration-time profile of (*S*)-naproxen in *CYP2C9*1/*3*, compared to *CYP2C9* reference individuals (Bae et al., 2009). However, lack of change in (*S*)-naproxen concentration alone, does not provide evidence for the absence of a pharmacogenetic-pharmacokinetic relationship between *CYP2C9* genotype and naproxen metabolism because (*S*)-

naproxen is eliminated primarily by direct glucuronidation (60% of the dose) (Vree et al., 1993). Only 20% of the dose is eliminated as (*S*)-O-desmethyl naproxen and its secondary glucuronide and sulfate metabolites (Sugawara et al., 1978; Kiang et al., 1989; Vree et al., 1993; Davies and Anderson, 1997). Therefore, to detect the effect of *CYP2C9* variation on (*S*)-naproxen, it is necessary to consider both the unchanged (*S*)-naproxen as well as its metabolites that are cleared through a *CYP2C9*-mediated pathway, as was done in the current study. Furthermore, the *in vitro* experiments conducted here demonstrate that at physiologically relevant concentrations, *CYP2C9* is the major enzyme responsible for naproxen O-dealkylation and that *CYP1A2* only plays a minor role. Moreover, the results of inhibitor experiments conducted in single donor HLMs demonstrate that the overall contribution of *CYP1A2* to (*S*)-O-desmethyl naproxen formation does not increase substantially with increasing *CYP1A2* protein abundance (Table 2). Thus, elevated *CYP1A2* expression and activity, due to genotype (Thorn et al., 2012) or xenobiotic exposure (Zevin and Benowitz, 1999; Dobrinas et al., 2011), is not expected to significantly impact *CYP2C9*'s predominant role in the O-demethylation of (*S*)-naproxen *in vivo*. While flurbiprofen could be considered a more *CYP2C*-selective *in vivo* probe than (*S*)-naproxen, it was not deemed superior for this study, because of concerns with using a drug available only by prescription in communities without local physician oversight and a recommendation by our community advisors that we use a probe drug familiar to the population (available over-the-counter) in order to enhance recruitment.

The identification of a novel *CYP2C9* variant that impairs enzyme function, and is unique to a population under-represented in biomedical, and especially genetic research (Popejoy and Fullerton, 2016), illustrates the importance of population-specific pharmacogenetic studies to guide medication therapy. A pharmacogenetic algorithm that is based on polymorphisms from a

specific subset of the global population may not be as clinically beneficial for populations in which the frequency of variant alleles is markedly different or if enzyme activity is determined by uncharacterized genetic variation. This was demonstrated by the conflicting results published by two randomized clinical trials, the European Pharmacogenetics of Anticoagulant Therapy (EU-PACT) (Pirmohamed et al., 2013), and the Clarification of Optimal Anticoagulation through Genetics (COAG) (Kimmel et al., 2013) trials. The EU-PACT trial showed a benefit for genotype-guided warfarin dosing over standard clinical care, but the COAG trial did not find a significant difference between the two groups (Kimmel et al., 2013; Pirmohamed et al., 2013). Variation in the ethnicities and genetics of the sample populations likely contributed to the different results (Scott and Lubitz, 2014). While the EU-PACT participants were primarily European, the COAG study population included 27% African-Americans, who have lower frequencies of *CYP2C9**2 and *3 (the only *CYP2C9* variant alleles considered in the pharmacogenetic algorithm), but higher frequencies of other reduced function *CYP2C9* variants (e.g., *5, *6, *8, and *11) (Limdi et al., 2015). Similarly, current pharmacogenetic warfarin dose algorithms would likely not optimize warfarin dosing for the Yup'ik population, where the *CYP2C9**2 and *3 frequencies are low and novel reduced or loss of function variants such as *MIL* are present. While the loss of *CYP2C9* enzyme activity predicted for the *MIL* variant may be greater than with *CYP2C9**3 (80% decrease in enzymatic activity (Takanashi et al., 2000), dosing recommendations for the *CYP2C9**3 variant can provide some clinical guidance. The Gage pharmacogenetic algorithm recommends a warfarin dose reduction of 33% per *CYP2C9**3 allele (Gage et al., 2008). The most conservative guidance would be to switch *MIL* homozygotes to alternative direct oral anticoagulant therapy. Future work should aim to establish the effect of the *MIL* variant on warfarin dose requirement, for example through a prospective study to

inform pharmacogenetically-guided warfarin dosing algorithms, controlling for other genetic determinants, notably *VKORC1* (vitamin K epoxide oxidase reductase complex subunit 1) genotype (Rieder et al., 2005). The Yup'ik population may benefit from the consideration and inclusion of population-specific genetic variation in clinical decisions surrounding personalized medication therapy. Clearly, an understanding of genetic variation in under-represented minority populations is essential if pharmacogenetic testing is to reach its optimal clinical utility in patients of all ethnicities.

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Authorship Contributions

Participated in research design: Henderson, Hopkins, Boyer, Thornton, Rettie, Thummel

Conducted experiments: Henderson, Hopkins, Boyer

Performed data analysis: Henderson, Thornton

Wrote or contributed to the writing of the manuscript: Henderson, Hopkins, Boyer, Thornton, Rettie, Thummel

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Footnotes

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- b. The authors declare no conflicts of interest.
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Figure Legends

Figure 1. Study inclusion based on *CYP2C9* *MIL* screening results.

Figure 2. Representative Michaelis-Menten plot of (*S*)-*O*-desmethyl naproxen formation in pooled HLMs.

Data are means \pm S.D.s. Individual data points represent means of technical triplicates at a given (*S*)-naproxen concentration from the same experimental replicate and the solid line reflects the fit of a single-enzyme Michaelis-Menten model to the data.

Figure 3. CYP Supersome™ screen at a sub- K_m (*S*)-naproxen concentration (25 μ M, closed bars) and a saturating concentration (1000 μ M, open bars).

Data are mean values across two repeated experiments, each with technical triplicates.

Figure 4. Michaelis-Menten plot of (*S*)-*O*-desmethyl naproxen formation by CYP2C9, CYP1A2, and CYP2C8 Supersomes™.

The displayed results are from a representative experiment. Individual data points represent the means of technical duplicates at a given (*S*)-naproxen concentration and the solid lines reflect the fit of a single-enzyme Michaelis-Menten model to the data.

Figure 5. Effects of selective CYP2C9 and CYP1A2 inhibitors on (*S*)-*O*-desmethyl naproxen formation in pooled HLMs.

Sulfaphenazole (SLF) was dissolved in methanol (MeOH) <0.2% final concentration, and furafylline (FF) was dissolved in DMSO, <0.1% final concentration. (*S*)-naproxen substrate

concentration was 20 μ M. Percent inhibition was calculated relative to the control containing no inhibitor. Data are means \pm S.D.s across three repeated experiments, with technical triplicates.

Figure 6. Urinary metabolite to parent ratio of (S)-O-desmethyl naproxen to unchanged (S)-naproxen by *MIL* genotype.

The regression analysis for the comparison between the *CYP2C9* *Met1/Met1* reference group ($n = 11$) and *Leu1* variant carrier group (combined *Met1/Leu1* heterozygotes and *Leu1/Leu1* homozygotes) ($n = 11$) allowed for heteroscedasticity; $*P < 0.05$.

Tables

Table 1. Kinetic parameters for O-desmethyl (S)-naproxen formation by CYP2C9, CYP1A2, and CYP2C8 Supersomes™.

Reported parameters are the means \pm S.D.s across three repeated experiments for CYP2C9 and CYP1A2 Supersomes™, (duplicate experiments for CYP2C8), each with technical duplicates. Comparisons between CYP2C9 and CYP1A2 were assessed using two-tailed unpaired t-tests allowing for heteroscedasticity. The asterisks denote statistical significance when compared with the same kinetic parameter in CYP2C9 Supersomes™; * $P < 0.01$.

P450	K_m \pm S.D. (μM)	V_{max} \pm S.D. (pmol/min/pmol P450)	CL_{int} \pm S.D. (μL/min/pmol P450)
CYP2C9	280 \pm 8.9	31.7 \pm 2.5	0.11 \pm 0.10
CYP1A2	1000 \pm 97*	41.7 \pm 2.4*	0.04 \pm 0.003*
CYP2C8	90	0.90	0.01

Table 2. Inhibition of (S)-O-desmethyl naproxen formation in single donor HLMs by sulfaphenazole and furafylline.

Data are means \pm S.D.s across three repeated experiments. Comparisons between low ($n = 5$) and high ($n = 5$) CYP1A2 expressors were assessed using two-tailed unpaired t-tests allowing for heteroscedasticity. The asterisks denote statistical significance when compared with percent inhibition in low CYP1A2 expressors; * $P < 0.05$ and ** $P < 0.01$.

CYP1A2 Expression	Average CYP1A2 \pm S.D. (pmol/mg microsomal protein)	Average CYP2C9 \pm S.D. (pmol/mg microsomal protein)	Inhibition by Furafylline \pm S.D. (%)	Inhibition by Sulfaphenazole \pm S.D. (%)
Low	2.8 \pm 2.3	36.6 \pm 6.3	23.6 \pm 7.6	85.2 \pm 11.8
High	31.2 \pm 10.8	53.2 \pm 13.3	39.7 \pm 7.0**	65.5 \pm 4.1*

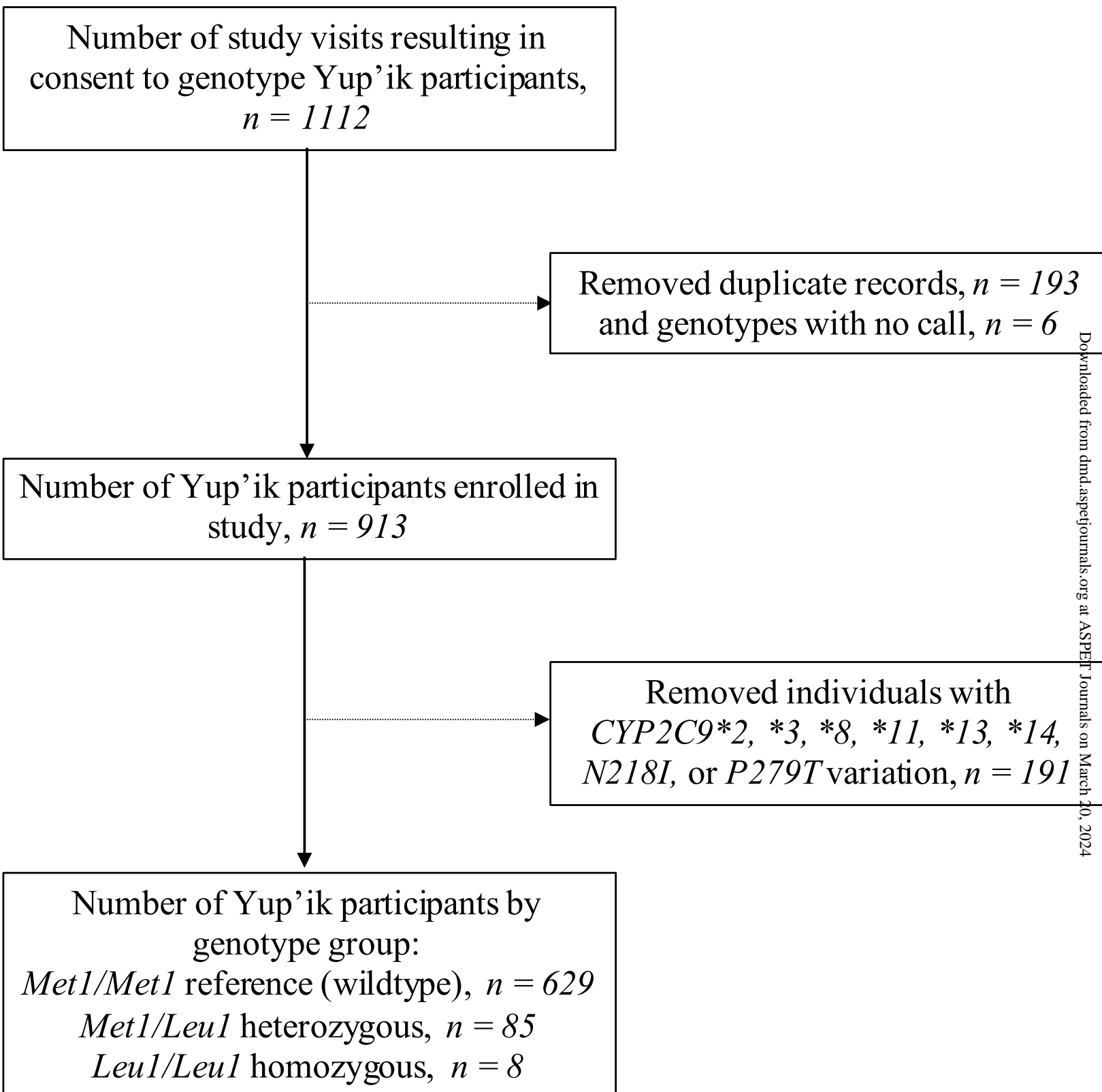


Figure 1.

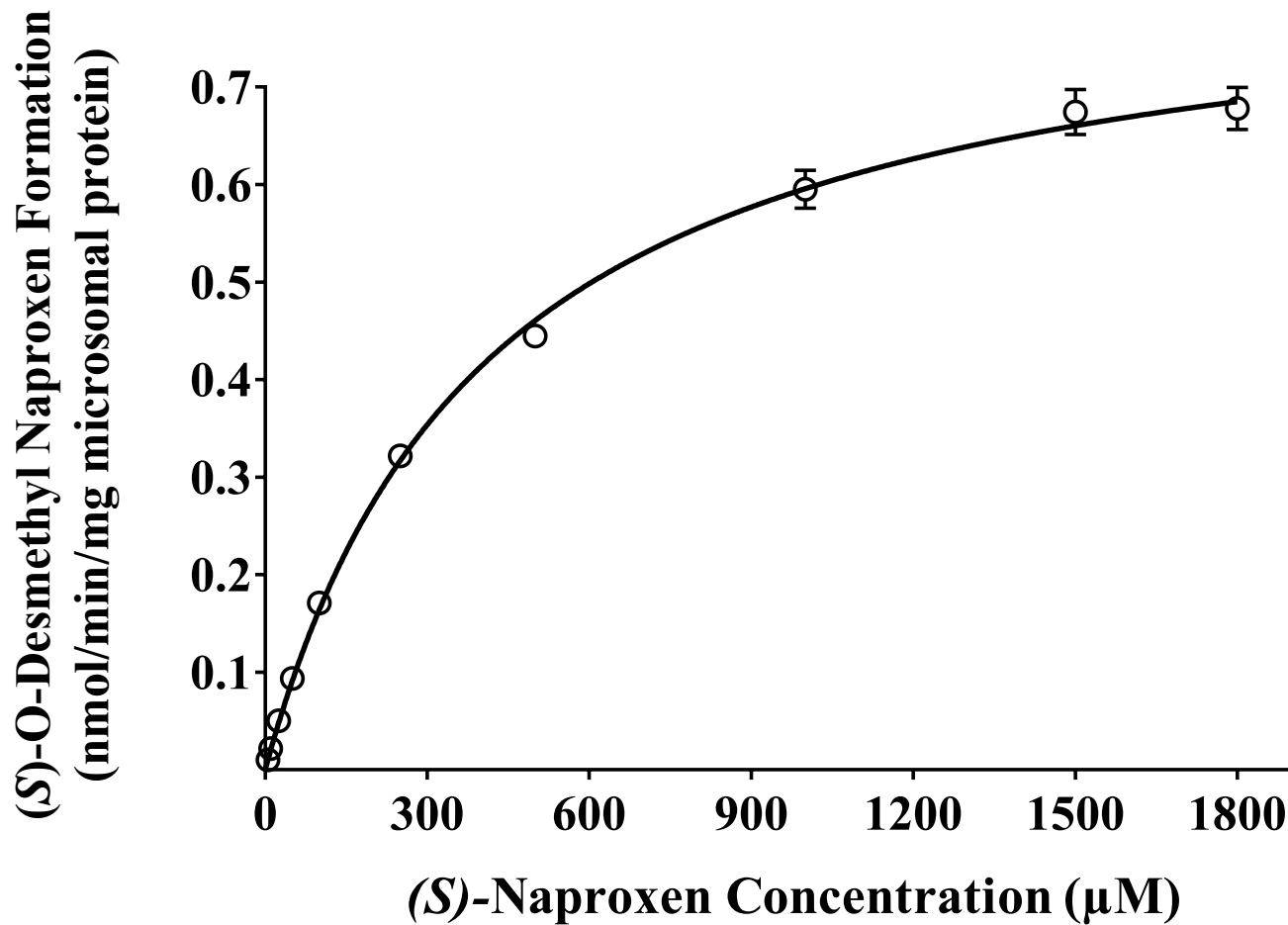


Figure 2.

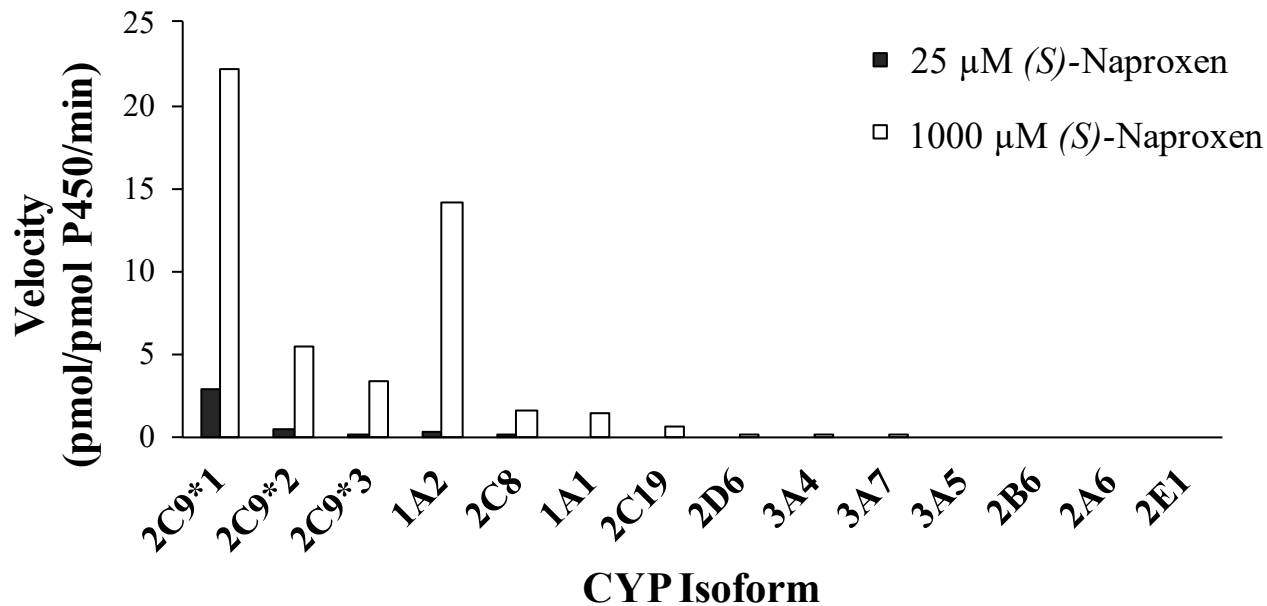


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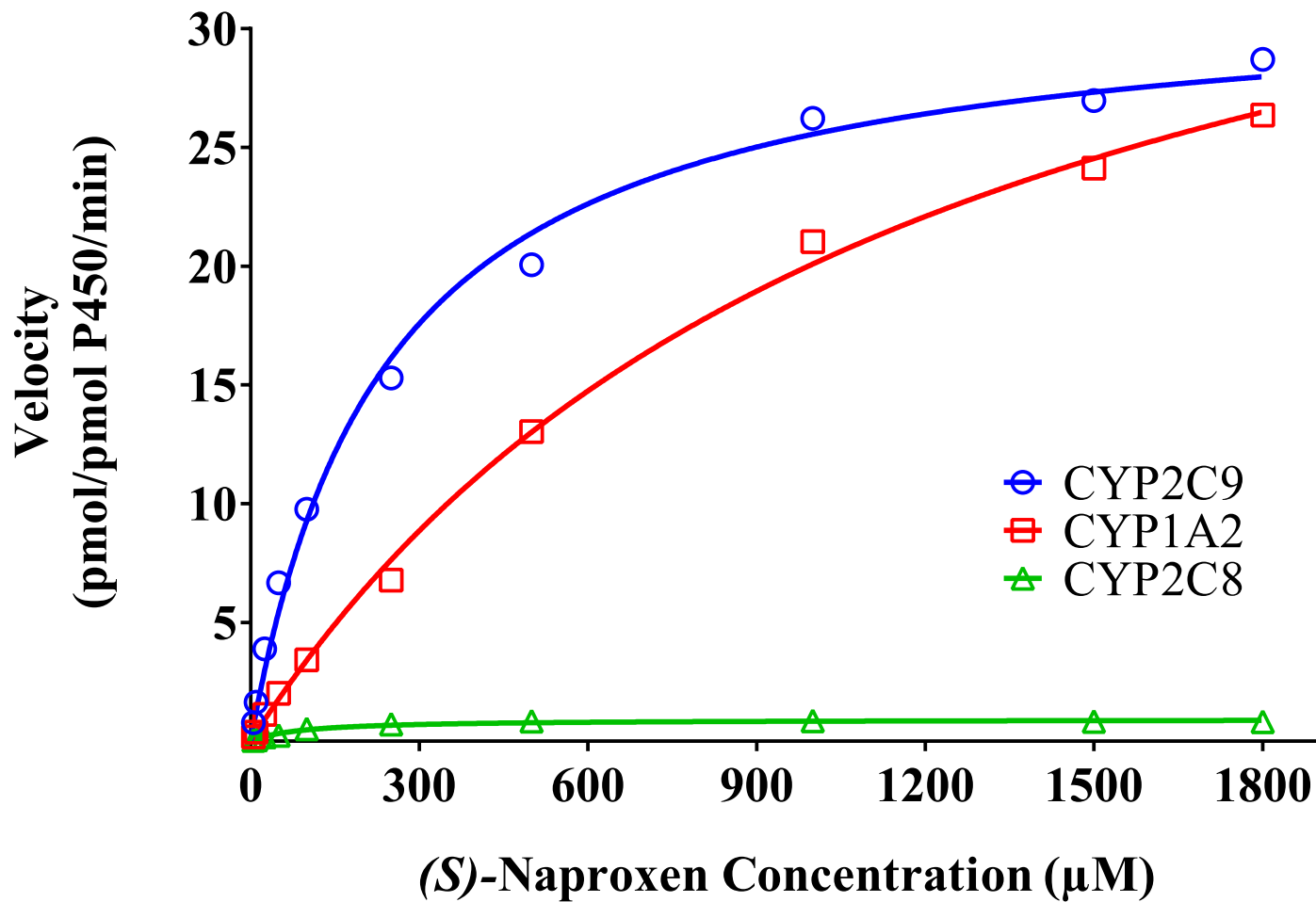


Figure 4.

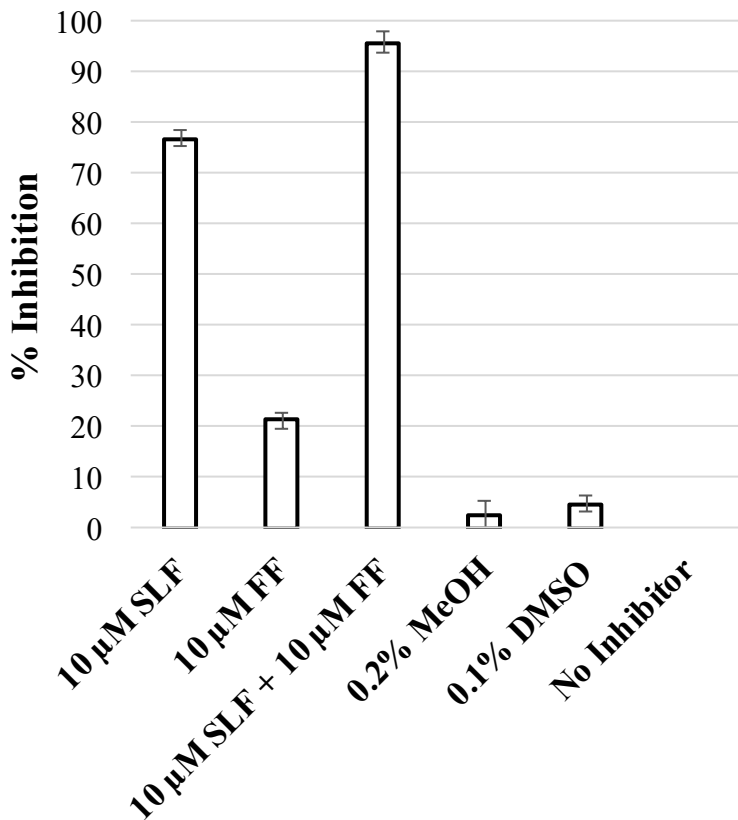


Figure 5.

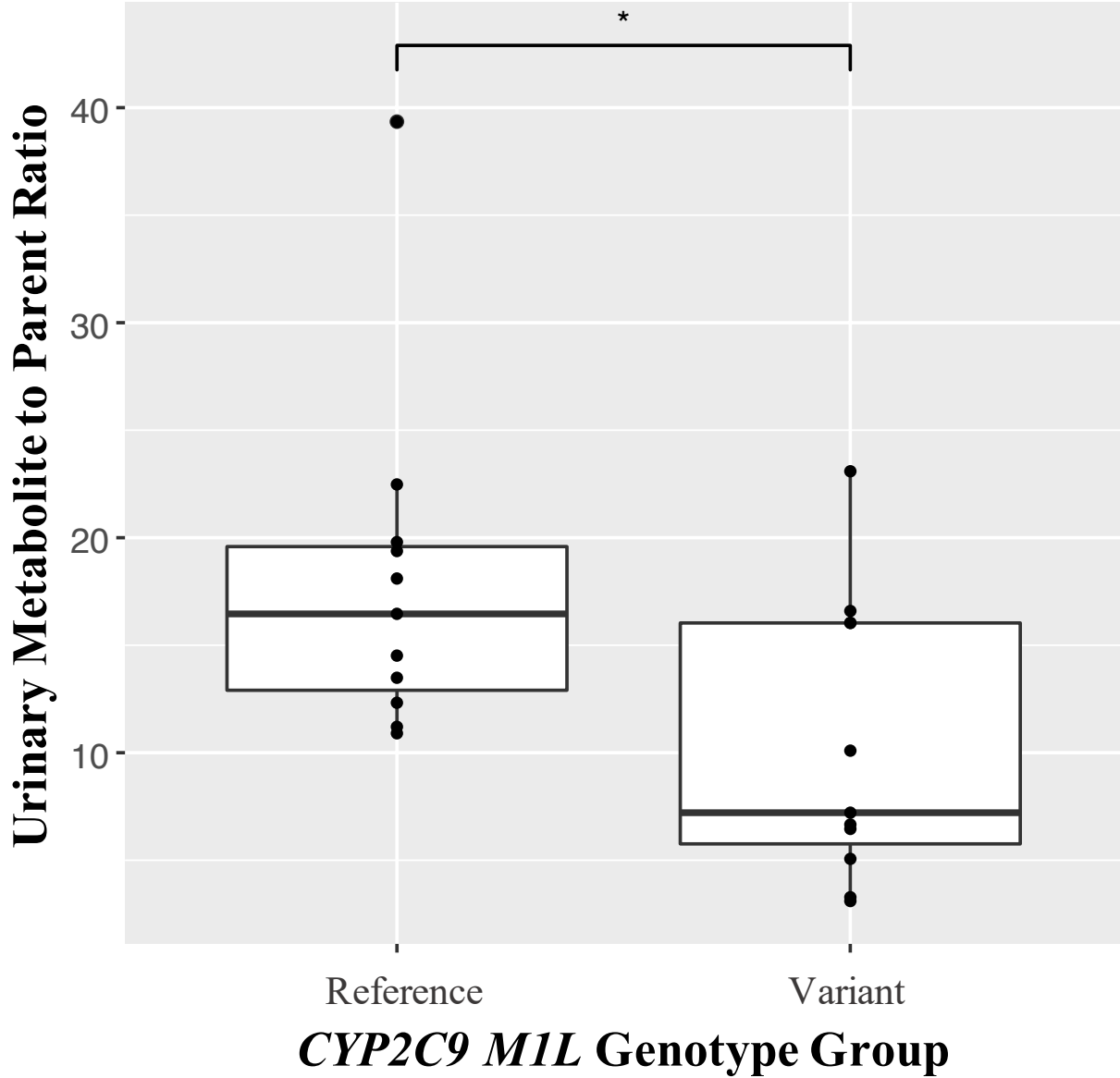


Figure 6.