In Vivo Functional Effects of *CYP2C9 M1L,* a Novel and Common Variant in the Yup'ik Alaska Native Population[S]

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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 *CYP2C9 Met1Leu* (*M1L*), a novel, relatively common single nucleotide polymorphism hypothesized to confer CYP2C9 poor metabolizer phenotype by disrupting the start codon. *M1L* 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 cytochrome P450 inhibitors and then developed and validated a novel liquid chromatography mass spectrometry method for simultaneous quantification of (*S*)-naproxen, (*S*)-*O*-desmethylnaproxen, and naproxen acyl glucuronide in human urine. The average ratio of (*S*)-*O*-desmethylnaproxen 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 *M1L* 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

The novel *CYP2C9 Met1Leu* variant in Alaska Native people was recently identified. This study validated (*S*)-naproxen as a CYP2C9 probe substrate to characterize the in vivo functional activity of the *CYP2C9 Leu1* variant. The results of this 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 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* (*M1L*) 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 *M1L* gene–transfected HepG2 cells demonstrated that the CYP2C9 Leu1 variant protein does not accumulate in this liver-derived cell line (McDonald et al., 2020). In the Yup'ik AN population, the *M1L* 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

ABBREVIATIONS: AN, Alaska Native; COAG, Clarification of Optimal Anticoagulation through Genetics; EU-PACT, European Pharmacogenetics of Anticoagulant Therapy; HLM, human liver microsome; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography mass spectrometry; *M1L*, *CYP2C9 MetlLeu*; OHSU, Oregon Health & Science University; P450, cytochrome P450; QC, quality control; rs, reference single nucelotide polymorphism; W, University of Washington; YK, Yukon-Kuskokwim.

(total population ~23,000), a 75,000-square-mile area, and all are accessed by air, water, or other nonroad system travel. Communities have health clinics staffed by community health aids, and primary care is offered through five subregional 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 interindividual 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 *M1L* 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 nonsteroidal 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-desmethylnaproxen (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-desmethylnaproxen/(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 *M1L* 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 M1L* genotype was previously determined and who consented to be contacted for future research investigations. Participants were in good health and not taking nonsteroidal 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 and the Yukon-Kuskokwim Health Corporation human studies committee and executive board approved this study. The University of Washington (UW) institutional review board approved the overall research project, as UW was the academic home of the grant funding this research (National Institutes of Health P01-GM116691) and its principal investigators. The study is registered at clinicaltrials.gov (NCT04449471).

After 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. Because of 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 a reference single nucleotide polymorphism (rs) identification 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-desmethylnaproxen-d$_3$ were purchased from Toronto Research Chemicals (ON, Canada). Unlabeled O-desmethylnaproxen, furafylline, sulfaphenazole, and NADPH were purchased from Sigma Aldrich (St. Louis, MO). Pooled human liver microsomes (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 of 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 μl. In experiments using selective P450 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 μM. 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 an (S)-naproxen concentration range of 5–1800 μM. 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-desmethylnaproxen-d$_3$, internal standard, was added. The samples were then centrifuged at 3000g for 10 minutes, decanted into glass culture tubes, dried with nitrogen gas, and resuspended in 50 μl mobile phase. A volume of 20 μl was injected onto the LC/MS.

A P450 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 toward (S)-naproxen. For this experiment, (S)-naproxen was incubated with 10 pmol of each Supersome preparation in 50 mM KH_2PO_4 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*-desmethylnaproxen-d$_3$, internal standard, was added. The samples were centrifuged at 3000g for 10 minutes, decanted into glass culture tubes, dried with N$_2$ gas, and resuspended in 50 μl mobile phase. A volume of 5 μl was injected onto the LC/MS. For P450 enzymes that catalyzed (*S*)-*O*-desmethylnaproxen 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 were normalized for the absolute amount of recombinant P450 added to the incubations (i.e., picomole 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 liquid chromatography tandem mass spectrometry (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 and NADPH (1 mM final concentration), in the presence or absence of 10 μM sulfaphenazole and furafylline, in 50 mM KH$_2$PO$_4$ 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*-desmethylnaproxen into 200 μl of potassium phosphate buffer to generate standard mixtures, with final concentrations of 0.2–10 μM for HLM incubations, 0.1–5 μM for HLM inhibition experiments, and 0.1–30 μM 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 μl) were prepared by adding 100 μl of HPLC-grade water and 100 μl 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 μl of the diluted sample was combined with 100 μl of HPLC-grade water and 100 μl of 1 nmol racemic flurbiprofen acyl glucuronide (internal standard for naproxen acyl glucuronide). For total (*S*)-*O*-desmethylnaproxen detection, urine was diluted 1:4 in blank urine, and then the diluted urine sample (50 μl) was combined with 80 μl HPLC-grade water, 20 μl 6 M HCL, and 100 μl of internal standard (1 nmol racemic *O*-desmethylnaproxen-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*-desmethylnaproxen glucuronide can hydrolyze back to *O*-desmethylnaproxen or isomerize to glucuronidase-resistant iso-glucuronides under alkaline conditions (Davies and Anderson, 1997). All samples were vortexed and centrifuged at 14,000g for 5 minutes; then, 50 μl of sample supernatant was transferred to autosampler vials, and 2 μl was injected onto the LC/MS.

Urine Sample Analysis. To evaluate the effect of *M1L* variation on CYP2C9 function, the ratio of urinary (*S*)-*O*-desmethylnaproxen to unchanged naproxen metabolite to parent 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 × 50 mm × 5 μm) 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 9 minutes. Quantitation was achieved by selected ion monitoring centered on mass-to-charge (m/z) values of 248.1 for (*S*)-naproxen, 251.1 for racemic naproxen-d$_3$, 234.1 for (*S*)-*O*-desmethylnaproxen, 237.1 for racemic *O*-desmethylnaproxen-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, to ensure comparable dose recovery and urine collection compliance. The intraday variation for quantitation of each analyte did not exceed 2% for the low-concentration quality control (QC) for (*S*)-*O*-desmethylnaproxen and did
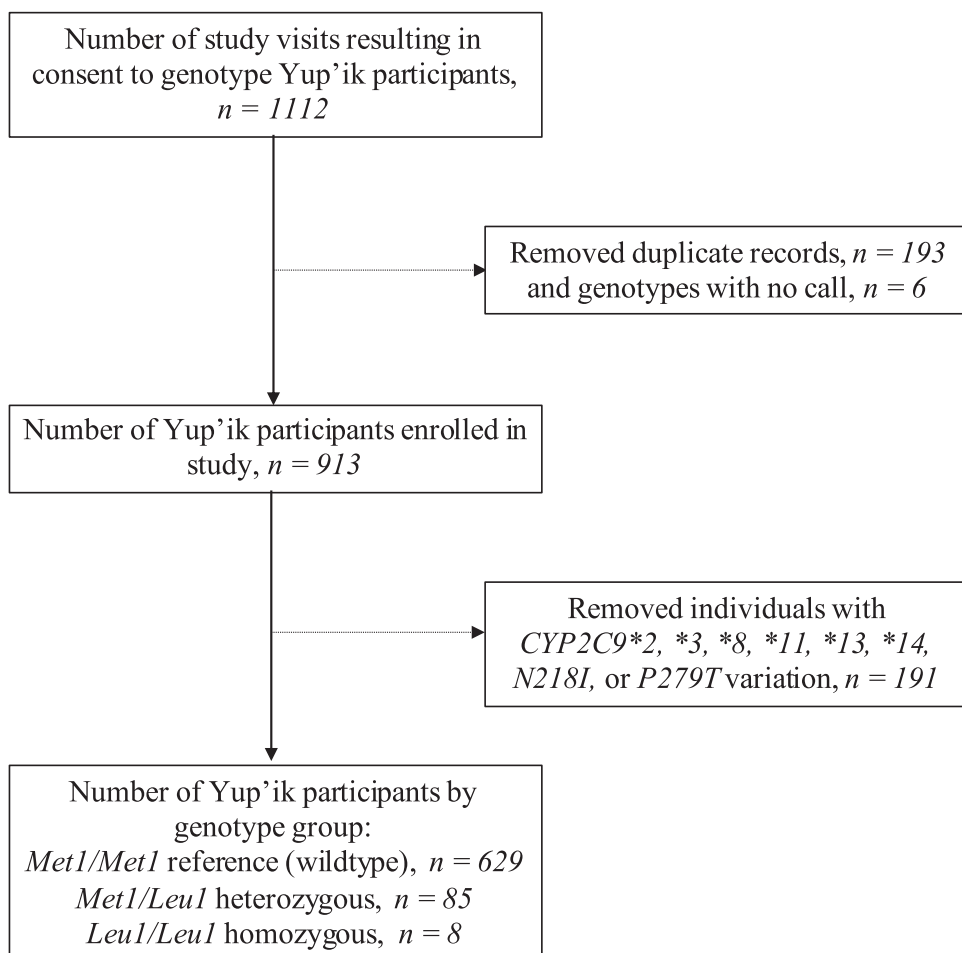
not exceed 6% for the high-concentration QC. The relative errors of the two QC concentrations tested in three independent experiments were within 5% and 8% for the low- and high-concentration QCs, respectively.

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 in which 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 Yukon-Kuskokwim Health Corporation because of the coronavirus disease 2019 (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 the 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.
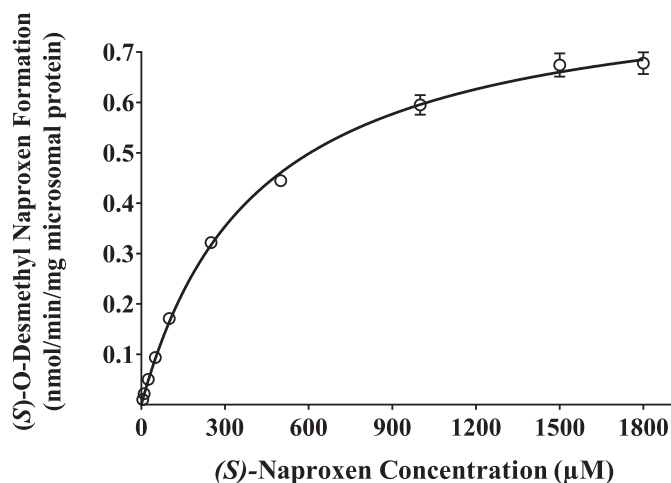
Results

Study Enrollment Based on *M1L* 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 (Fig. 1). Individuals with one or more copies of *CYP2C9*2*, **3*, **8*, **11*, **13*, **14*, **29*, or *N218I* alleles were excluded because of their confounding effects on CYP2C9 activity. A total of eight *Leu1/Leu1* homozygotes were identified from five 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 (wild type), 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*-desmethylnaproxen. A representative Michaelis-Menten plot of (*S*)-*O*-desmethylnaproxen formation in pooled HLMs is shown in Fig. 2, with a mean K$_m$ of 420 ± 2 μM and V$_{max}$ of 0.92 ± 0.06 nmol/min per milligram microsomal protein from three repeated experiments. The P450 Supersome screen showed that (*S*)-*O*-desmethylnaproxen was formed in incubations containing 25 μM naproxen by only three P450 enzymes—CYP2C9, CYP2C8, and CYP1A2 (Fig. 3). The activity of CYP2C9 was much higher than that of CYP1A2 and CYP2C8. A 25 μM concentration is more clinically relevant, considering that (*S*)-naproxen exhibits extensive protein binding (>99%) and its C$_{max}$ is ~250 μM after a single 440-mg dose under fasting conditions (Center for Drug Evaluation and Research, 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, for which the contribution of CYP1A2 increased by over 30-fold and small amounts of product were detected in incubations with several other P450 Supersomes (Fig. 3). In a separate experiment, we evaluated the activity of a vector control Supersome preparation compared with CYP2C9. There was no (*S*)-*O*-desmethylnaproxen product detected in incubations with the vector control at both the 25 and 1000 μM substrate concentrations compared with a robust product formation rate produced by CYP2C9.

Number of study visits resulting in consent to genotype Yup'ik participants, *n = 1112*

Removed duplicate records, *n = 193* and genotypes with no call, *n = 6*

Number of Yup'ik participants enrolled in study, *n = 913*

Fig. 1. Study inclusion based on *CYP2C9 M1L* screening results.

Removed individuals with *CYP2C9*2, *3, *8, *11, *13, *14, N218I,* or *P279T* variation, *n = 191*

Number of Yup'ik participants by genotype group:
Met1/Met1 reference (wildtype), *n = 629*
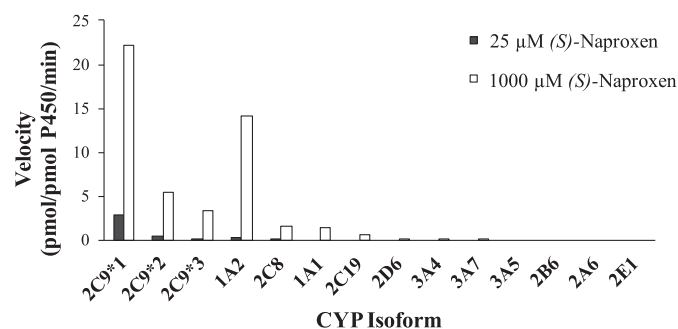*Met1/Leu1* heterozygous, *n = 85*
Leu1/Leu1 homozygous, *n = 8*

Full kinetic experiments were conducted to assess the (*S*)-*O*-desmethylnaproxen intrinsic formation clearances of CYP2C9, CYP1A2, and CYP2C8 Supersomes (Fig. 4). The mean V_{max} values for CYP2C9 and CYP1A2 were 31.7 and 41.7 pmol/min per picomole P450, respectively, whereas 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 per milligram protein (Rowland-Yeo et al., 2004) for CYP2C9, CYP1A2, and CYP2C8, respectively, the average contribution from each of these enzymes to (*S*)-*O*-desmethylnaproxen 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*-desmethylnaproxen in HLMs by CYP2C9 and CYP1A2 from selective
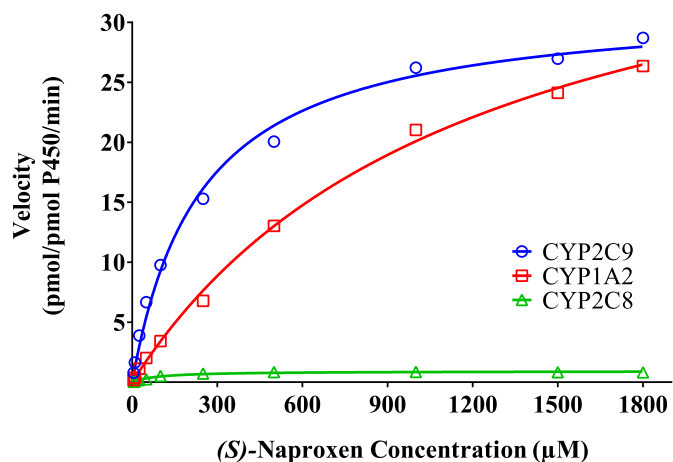

**Fig. 2.** Representative Michaelis-Menten plot of (*S*)-*O*-desmethylnaproxen formation in pooled HLMs. Data are means ± S.D. Individual data points represent means of technical triplicates at a given (*S*)-naproxen concentration from the same experimental replicate, and the solid line reflects the fit of a single-enzyme Michaelis-Menten model to the data.

Fig. 3. P450 Supersome screen at a sub-K_m (*S*)-naproxen concentration (25 μM, closed bars) and a saturating concentration (1000 μM, open bars). Data are mean values across two repeated experiments, each with technical triplicates.

Fig. 4. Michaelis-Menten plot of (*S*)-*O*-desmethylnaproxen 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.

enzyme inhibitor experiments conducted with 20 μM (*S*)-naproxen, a substrate concentration 5-fold below the K_m determined in pooled HLMs. (*S*)-*O*-desmethylnaproxen formation was reduced by 76.9% ± 1.5% with 10 μM sulfaphenazole, a selective CYP2C9 inhibitor; by 21.5% ± 1.6% with 10 μM furafylline, a selective CYP1A2 inhibitor; and by 95.8% ± 2.1% with both sulfaphenazole and furafylline (Fig. 5). The solvents for the inhibitors had negligible effects on the percent inhibition (Fig. 5).

The effect of CYP2C9 and CYP1A2 inhibition on (*S*)-*O*-desmethylnaproxen 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 μM furafylline was greater in the high-CYP1A2 group (39.7% ± 7.0% pmol/mg microsomal protein) compared with the low-CYP1A2 group (23.6% ± 7.6% pmol/mg microsomal protein) (*P* = 0.008), whereas the percent inhibited by 10 μM sulfaphenazole was greater in the low-CYP1A2 group (85.2% ± 11.8% pmol/mg microsomal protein) compared with the high-CYP1A2 group (65.5% ± 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 with the low-CYP1A2 group, the percent inhibited by furafylline was only 1.7-fold greater. Thus, CYP1A2 contribution to (*S*)-*O*-desmethylnaproxen formation in HLMs was always minor in comparison with the CYP2C9 contribution.
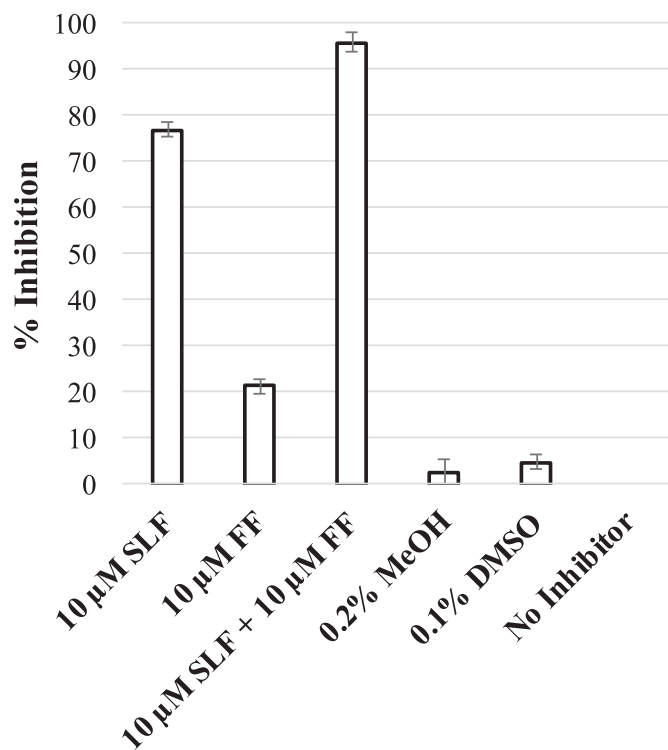
Fig. 5. Effects of selective CYP2C9 and CYP1A2 inhibitors on (*S*)-*O*-desmethylnaproxen formation in pooled HLMs. Sulfaphenazole (SLF) was dissolved in methanol (MeOH), <0.2% final concentration, and furafylline (FF) was dissolved in DMSO, <0.1% final concentration. (*S*)-Naproxen substrate concentration was 20 μM. Percent inhibition was calculated relative to the control containing no inhibitor. Data are means ± S.D. across three repeated experiments, with technical triplicates.

Impact of *M1L* on Urinary Metabolite-to-Parent Ratio. The mean ratio of (*S*)-*O*-desmethylnaproxen to (*S*)-naproxen was greater for the homozygous reference group (18.0 ± 8.0, *n* = 11) compared with the *M1L* variant group (10.3 ± 6.6, *n* = 11), which includes eight *Met1*/Leu1 heterozygotes and three *Leu1/Leu1* homozygotes (Fig. 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 (Supplemental Fig. 1). 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 toward 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).

TABLE 1

Kinetic parameters for *O*-desmethyl (*S*)-naproxen formation by CYP2C9, CYP1A2, and CYP2C8 Supersomes

Reported parameters are the means ± S.D. 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.

P450	K_m ± S.D.	V_{max} ± S.D.	CL_{int} ± S.D.
	μM	*pmol/min per picomole P450*	*μl/min per picomole P450*
CYP2C9	280 ± 8.9	31.7 ± 2.5	0.11 ± 0.10
CYP1A2	1000 ± 97*	41.7 ± 2.4*	0.04 ± 0.003*
CYP2C8	90	0.90	0.01

*P < 0.01.

TABLE 2

Inhibition of (S)-O-desmethylnaproxen formation in single-donor HLMs by sulfaphenazole and furafylline

Data are means ± S.D. 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.

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

*$P < 0.05$; **$P < 0.01$.

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 M1L 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-desmethylnaproxen formation in HLMs, it was predicted that a Leu1 variant group (composed of three Leu1/Leu1 homozygotes and eight heterozygotes) would have a 51% reduction in urinary ratio of (S)-O-desmethylnaproxen to unchanged naproxen compared with 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 drugs with a low narrow therapeutic index, such as warfarin, phenytoin, and tolbutamide, for which 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.

The M1L 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), but 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 that results from a substitution of methionine to valine at the first amino acid position (Ferguson et al., 1998). However, based on data from 1000 genomes, the CYP2C19*4 variant is only found at low frequencies across world populations: 0.8% in a Mexican population (California), 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 United States) (Auton et al., 2015). By contrast, M1L 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.

To characterize the catalytic efficiency of the M1L 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 down-played its utility as a probe substrate because of 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 with 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-desmethylnaproxen 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-desmethylnaproxen 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. Although flurbiprofen could be considered a more CYP2C-selective
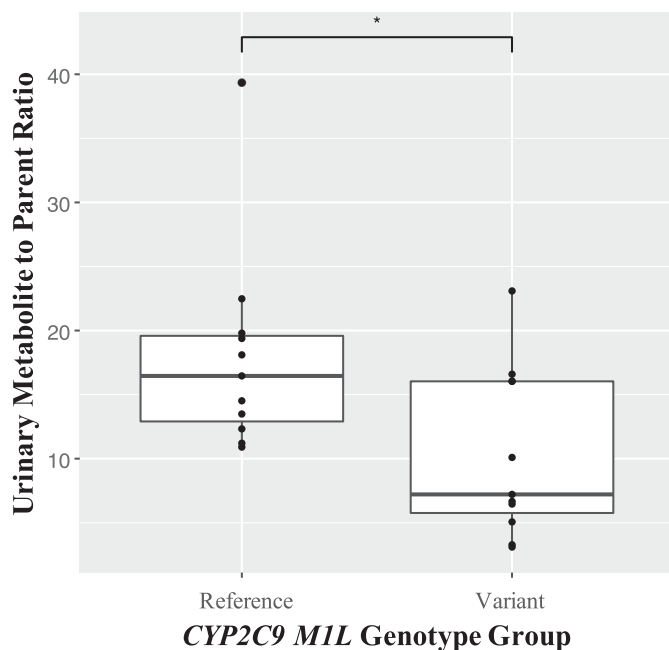
Fig. 6. Urinary metabolite-to-parent ratio of (S)-O-desmethylnaproxen to unchanged (S)-naproxen by M1L genotype. The regression analysis for the comparison between the CYP2C9 Met1/Met1 reference group (n = 11) and Leu1 variant carrier group (combined Met1/Leu1 heterozygotes and Leu1/Leu1 homozygotes) (n = 11) allowed for heteroscedasticity; *$P < 0.05$.

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) 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 Anti-coagulation 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). Although 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, in which the *CYP2C9*2* and *3* frequencies are low and novel reduced or loss-of-function variants, such as *M1L*, are present. Although the loss of CYP2C9 enzyme activity predicted for the *M1L* 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 *M1L* homozygotes to alternative direct oral anticoagulant therapy. Future work should aim to establish the effect of the *M1L* variant on warfarin dose requirement, for example through a prospective study to inform pharmacogenetically guided warfarin dosing algorithms, controlling for other genetic determinants, notably 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.

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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