

# CLINICAL AND TOXICOLOGICAL RELEVANCE OF CYP2C9: Drug-Drug Interactions and Pharmacogenetics

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■ **Abstract** CYP2C9 is a major cytochrome P450 enzyme that is involved in the metabolic clearance of a wide variety of therapeutic agents, including nonsteroidal antiinflammatories, oral anticoagulants, and oral hypoglycemics. Disruption of CYP2C9 activity by metabolic inhibition or pharmacogenetic variability underlies many of the adverse drug reactions that are associated with the enzyme. CYP2C9 is also the first human P450 to be crystallized, and the structural basis for its substrate and inhibitor selectivity is becoming increasingly clear. New, ultrapotent inhibitors of CYP2C9 have been synthesised that aid in the development of quantitative structure-activity relationship (QSAR) models to facilitate drug redesign, and extensive resequencing of the gene and studies of its regulation will undoubtedly help us understand interindividual variability in drug response and toxicity controlled by this enzyme.

## OVERVIEW

Cytochrome P450s are a superfamily of oxygen-activating enzymes that carry out an enormous range of metabolic reactions on endogenous and exogenous substrates in processes both beneficial and deleterious to the organism (1, 2). Therapeutically administered drugs, endogenous eicosanoids, steroids, and bile salts, as well as carcinogens and environmental pollutants, are but a few important targets for these versatile catalysts (3). Annotation of the human genome has revealed the presence of some 57 human P450 genes (4), but less than a dozen of these play important roles in the hepatic clearance of drugs (5).

CYP2C9 is a major human liver form of P450 that has drawn considerable attention from researchers owing, in large part, to its role in causing adverse drug reactions (ADRs). ADRs, which are projected to cause hundreds of millions of

dollars in health care costs per year in the United States alone, often result from unanticipated changes in P450 enzyme activity secondary to genetic polymorphisms or metabolically based drug-drug interactions (6, 7). Both mechanisms are highly pertinent to ADRs involving CYP2C9. These can be especially serious because several of the enzyme's substrates exhibit a narrow therapeutic index, therefore the resulting clinical consequences can be severe.

This review summarizes our current knowledge of the enzyme's structure-function relationships, drug-drug interactions, pharmacogenetics, and gene regulation, and it attempts to relate these base-line parameters to key clinical and toxicological features of this important enzyme. Several earlier reviews of CYP2C enzyme properties, function, and genetics may prove to be useful adjuncts to this material (8–11).

## INTRODUCTION

CYP2C9 is one of four functional human CYP2C genes located on the long arm of chromosome 10. Within the CYP2C subfamily, which also comprises CYP2C8, CYP2C18, and CYP2C19, CYP2C9 is arguably the most important member for several reasons. First, it is the largest contributor among these four isoforms to total human liver microsomal P450 content, with estimates of mean microsomal levels ranging from  $40 \pm 10$  pmol/mg (12) to as high as  $89 \pm 9$  pmol/mg (13). Only CYP3A4 is a more quantitatively significant P450 enzyme in human liver. Second, like CYP3A4, CYP2C9 metabolizes a host of clinically important drugs (Table 1). Indeed, it has been estimated that CYP2C9 is responsible for the metabolic clearance of up to 15% of all drugs that undergo Phase I metabolism (5). Third, drug-drug interactions pose therapeutic management problems for several CYP2C9 substrates, especially those involving low therapeutic index substrates, such as (S)-warfarin, tolbutamide, and phenytoin. Fourth, CYP2C9 is subject to significant genetic polymorphism, such that up to 40% of Caucasian populations are carriers of alleles that encode partially defective functional forms of the enzyme. Such gene-drug interactions can exacerbate adverse drug reactions with the same battery of CYP2C9 substrates that display an intrinsically low margin of safety.

### Substrate Specificity: In Vitro and In Vivo Probes for CYP2C9

Three of the most commonly employed substrate probes for determining CYP2C9 activity in crude human tissue fractions are (S)-warfarin (7-hydroxylation), tolbutamide (methylhydroxylation), and diclofenac (4'-hydroxylation). Diclofenac has the advantage that CYP2C9 catalyzes its metabolism with a high turnover number (ca 30/min). Although this is beneficial in allowing for facile, economical HPLC-UV assays to be employed for routine screening in vitro, substrate depletion can prove problematic if this probe is used for determining kinetic

**TABLE 1** Common substrates for CYP2C9 by therapeutic class\*

Class	Antiinflam- matories	Oral hypoglycemics	Oral anticoagulants	Diuretics and uricosurics	Angiotensin II blockers
Substrates	Flurbiprofen Diclofenac Naproxen Piroxicam Suprofen Ibuprofen Mefenamic acid Celecoxib	Tolbutamide Glyburide Glipizide Glimepiride	(S)-Warfarin (S)-Acenocoumarol (Phenprocoumon)**	Torsemide Ticrynafen** Sulfinpyrazone sulfide	Losartan Irbesartan Candesartan
Class (con't.)	Antiasthmatics	Anticonvulsants	Anticancer agents	Endogenous compounds	Miscellaneous
Substrates (con't.)	Zafirlukast (Zileuton)	Phenytoin (Phenobarbital) (Trimethadione)	Cyclophosphamide (Tamoxifen)	Arachidonic acid 5-Hydro- xytryptamine Linoleic acid	Mestranol Fluvastatin $\Delta^9$ -Tetrahydro- cannabinol (Benzopyrene) (Pyrene) (Fluoxetine) (Sildenafil) (Rosiglitazone)

\*This listing is not intended to be exhaustive.

\*\*Parentheses indicates that (where known) other P450s or metabolic pathways play a major role in clearance.

parameters. Conversely, turnover of (S)-warfarin by CYP2C9 is extremely slow, with a  $k_{cat}$  of only  $\sim 0.2/\text{min}$ . However, 7-hydroxywarfarin fluoresces strongly, and this specific metabolite is readily quantitated from microsomal incubations by HPLC with fluorescence detection. Tolbutamide is a convenient compromise between these extremes, and confidence in its use is enhanced by the observation of excellent correlations between rates of tolbutamide methylhydroxylation and several other prototypical CYP2C9 activities in human liver microsomes (14).

Two of the best in vivo probes for CYP2C9 activity are tolbutamide and flurbiprofen (15, 16). Importantly, the clearance of each of these biomarkers is affected in a predictable fashion by carriers of the *CYP2C9*\*3 allele (see sections below), thereby providing a pharmacogenetic validation for the in vivo probe. Interestingly, diclofenac is not a useful in vivo probe for the enzyme. Glucuronidation of the parent is an important component of clearance, and the acyl glucuronide itself is a substrate for CYP2C8, which then forms the 4'-hydroxy acyl glucuronide (17). Consequently, CYP2C9-dependent formation of total (free plus conjugated) 4'-OH diclofenac in urine appears to be a modest contributor to the overall clearance of the drug. Safety concerns initially prevented the use of racemic warfarin as an in vivo probe in healthy individuals. However, concomitant administration of warfarin with vitamin K abrogates its pharmacodynamic effect and has permitted its use in "cocktail" form to evaluate global P450 activity when administered with probes for the other major P450 forms (18).

## Inhibitor Specificity: Ligand-Based Models for CYP2C9

Dangerous drug-drug interactions can arise when a CYP2C9 inhibitor is added to a therapeutic regime that includes low therapeutic index drugs like (S)-warfarin, tolbutamide, or phenytoin (7). In cases like these, patients can risk life-threatening bleeding episodes, hypoglycemia, and neurotoxicity as a result of the diminished CYP2C9 enzyme activity. Although considerable efforts have been expended to construct computational P450 models directed toward predicting active-site structure or sites of substrate metabolism (19–21), an important rationale for developing predictive inhibitor-based models of CYP2C9 is that new therapeutic agents with significant potential for drug-drug interactions may be identified early in the discovery process and appropriate structural modification initiated. It is axiomatic to state that the study of a drug's functional group characteristics that impart high affinity for a target enzyme or receptor also reveal complementary information about the protein's binding pocket, and so these types of studies perform a dual function.

Even cursory examination of the structural features of the drugs listed in Table 1 demonstrates that CYP2C9 exhibits selectivity for acidic compounds, as exemplified by the large number of arylacetic acid or arylpropionic acids that are substrates for the enzyme. Early studies, reviewed in Reference 22, established the basic CYP2C9 pharmacophore of a hydrogen bond donor heteroatom and/or anionic moiety in the ligand located 7–8 Å from the substrate metabolism site and separated by an intervening hydrophobic zone (23–25). As more substrates and inhibitors for CYP2C9 have emerged, it is evident that neutral compounds also bind to this enzyme with high affinity (8, 26). However, for two very similar compounds, such as warfarin (ring closed and not an anion in the CYP2C9 active site) and phenprocoumon (an anion at physiological pH), the anion is the tighter binder to CYP2C9 (27).

The first quantitative structure-activity relationship (QSAR) for CYP2C9 inhibitors overlaid 19 coumarin derivatives; 5 carboxylate-containing drugs, including a number of NSAIDs; 2 sulfonamides, and phenytoin with  $K_i$  values ranging from 100 nM–30  $\mu$ M (28). The resulting partial least squares model, based on the Comparative Molecular Field Analysis (CoMFA) program, had a standard error of the estimate of 0.17 log units. Subsequently, this QSAR model was able to predict the binding affinity of 14 structurally diverse compounds, with a mean error of approximately 6  $\mu$ M (29). Other three-dimensional (3D)-QSAR efforts have focused on alignment-independent techniques that facilitate examination of more structurally diverse training sets. In this regard, CYP2C9 inhibitor models developed with the program Catalyst generally predicted  $K_i$  within 1 log residual (30), and a conformer- and alignment-independent method predicted  $K_i$  for 11 of 12 structurally unrelated compounds within 0.5 log units (31).

A new generation of very-high-affinity CYP2C9 inhibitors is based on a 2-alkyl, 3-benzoyl benzofuran template (32) (see Figure 1). This core structure is found in the antiarrhythmic agent, amiodarone, one of the most commonly

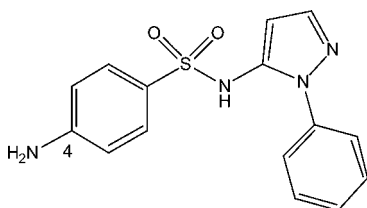
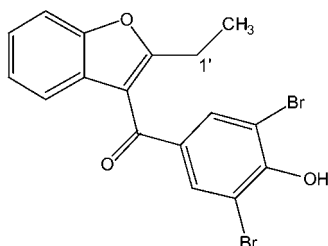
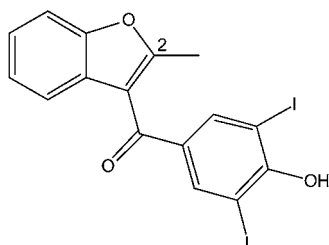
coprescribed drugs with warfarin in the treatment of atrial fibrillation. Amiodarone exhibits a clinically important drug-drug interaction with warfarin by inhibiting P450-mediated clearance of both its enantiomers (33). Much of this effect may be attributable to the major metabolite, desethylamiodarone, which has a  $K_i$  against CYP2C9 of 2.3  $\mu\text{M}$  compared with 95  $\mu\text{M}$  for amiodarone itself (34). Comedication with amiodarone generally results in a decrease of the maintenance dose of warfarin by 25%–30% (35). The prototype in this series of new inhibitors is benzbromarone, a uricosuric agent used in Europe and Japan. Its ADR liability was first revealed with reports of a drug-drug interaction that occurs between warfarin and benzbromarone, wherein the anticoagulant effect of warfarin is potentiated (36). Follow-up studies found that benzbromarone's  $K_i$  for inhibition of (S)-warfarin 7-hydroxylation by human liver microsomes was  $\sim 10$  nM, and the in vivo clearance of (S)-warfarin in humans was reduced by approximately 50% upon coadministration of the two drugs (37). In retrospect, given the potency of CYP2C9 inhibition by benzbromarone, this drug-drug interaction is not unexpected.

Almost two dozen benzbromarone analogs have now been synthesized and 2-methyl-3-(3',5'-diiodo-4'-hydroxybenzoyl)benzofuran (Figure 1) has emerged as the most potent inhibitor of CYP2C9, with a  $K_i$  of 1 nM (26). The pharmacophore obtained from CoMFA analysis, using a structurally diverse training set ( $n = 58$ ) that straddled four orders of magnitude of inhibitor potency, retained a number of the earlier models' features, but also reflected important new interactions. The most striking of these is the identification of a region near the 1' position of the benzofuran ring (see Figure 1) that exhibits negative steric interactions. The relative affinities of the various classes of CYP2C9 inhibitors, the benzbromarones, acyclic warfarins, sulfaphenazoles, and the cyclic hemi-ketal warfarins, are all well predicted based on a combination of this new steric interaction, the degree of negative charge(s) at a specific location(s) in the substrate, and the lipophilicity of the hydrophobic zone. The reciprocal interactions with the CYP2C9 active site are considered below.

## CYP2C9 Structure: Site-Directed Mutagenesis and Crystallization Studies

Because CYP2C9 mainly selects for substrates and inhibitors that are lipophilic and weakly acidic, it would be expected that complementary interactions with both hydrophobic and hydrogen bond donor or acceptor amino acids would occur in the active site of the enzyme. Site-directed mutagenesis efforts from many groups have identified numerous amino acids important to CYP2C9 function, including Arg97, Arg108, Phe114, Arg144, Asp293, Ser286, Asn289, Ile359, Ser365, and Phe476 (38–43).

In particular, there is a strong support for a role for F114 and F476 in substrate orienting interactions with (S)-warfarin and diclofenac because removal of these aromatic residues substantially alters product regioselectivity (39, 43). The crystal structure of CYP2C9 with (S)-warfarin bound confirms a central role for these two

Sulfaphenazole  $K_i = 200$  nMBenzbromarone  $K_i = 20$  nM2-Methyl-3-(3',5'-diiodo-4'-hydroxybenzoyl)benzofuran  $K_i = 1$  nM**Figure 1** High-affinity CYP2C9 inhibitors.

aromatic residues in binding of this ligand (44). In contrast, Arg97 plays a more important role in binding heme, whereas Asp293 appears to have dual functions in controlling product regioselectivity as well as maintaining holo-enzyme stability (42, 44–46). Ser286 and Asn289 are I-helix residues important in conferring substrate specificity toward the NSAIDs, ibuprofen, and diclofenac (38), whereas Ser365 appears to be the target nucleophile adducted by activation of tienilic acid (43). Arg144 and Ile359 together largely determine the genetic background that confers a wild-type phenotype (see following section).

Although significant progress was made prior to the initial crystallization of CYP2C9 in mapping hydrophobic active site residues of CYP2C9 by mutagenesis, the nature of the anionic binding site in CYP2C9 remained elusive. Early homology modeling efforts gave rise to several predictions for charged amino acids that could

be involved in polar interactions with CYP2C9 ligands, including Arg97, Arg105, Arg108, and Asp293 (19, 20, 29, 41, 47). Recent mutagenesis and crystallization studies now identify Arg108, specifically, in the NSAID substrate selectivity of CYP2C9 (46, 48).

Interestingly, the first crystal structure of CYP2C9, with (S)-warfarin bound, does not implicate Arg108 in active-site interactions with this ligand (44), and the second, with flurbiprofen bound, does not implicate F476 (48). However, with the solution of several crystal structures for CYP2C5, it has become apparent that the binding of different ligands to a mammalian P450 can involve multiple substrate binding modes (49, 50). Moreover, it is speculated by the authors of the first crystal structure for CYP2C9 that (S)-warfarin is not bound in a catalytically productive orientation (44). If this is the case, cocrystallization of CYP2C9 with multiple ligands representative of the multiple binding pockets inferred from atypical kinetic studies (see below) will be required to provide a detailed picture of the CYP2C9 active site and the enzyme's interactions with structurally diverse ligands.

### Atypical CYP2C9 Kinetics

A recently appreciated complicating factor in the prediction of drug-drug interactions and drug clearance from in vitro data is the atypical kinetic behavior exhibited by several mammalian P450 enzymes (51, 52). Although CYP3A4 is the most extensively studied human P450 in this regard, an increasing allosteric literature has accumulated for CYP2C9 over the past five years (53). Indeed, a recent systematic study of some 1500 structurally diverse compounds identified more than 30 activators of CYP2C9 activity from which a heteroactivation pharmacophore for the enzyme was generated (54). Just as  $\alpha$ -naphthoflavone has emerged as the prototypical effector molecule for CYP3A4, dapsone is the best documented activator of CYP2C9—exhibiting heterotropic and homotropic positive cooperativity (55). Recent mechanistic studies suggest that dapsone activation is accompanied by a change in the partition between flurbiprofen hydroxylation and uncoupling (56). More efficient catalysis in the presence of the activator may reflect a closer approach of the substrate to the heme iron in the presence of the effector, as revealed recently by NMR (57). Although many scenarios might be envisioned for the molecular basis underlying these phenomena (58), P450 activation kinetics is generally held to result from multiple ligand occupancy in the active-site of the isoform involved. Strong support for this view is derived from structural studies of the soluble enzyme P450eryF. Spectral analysis demonstrated cooperative binding of androstenedione and 9-amino-phenanthrene to P450eryF (Hill coefficients of  $\sim 1.3$ ), and crystallization of the protein with either of the ligands bound showed that two molecules were present in the active site at the same time (59). No such direct structural data are available for mammalian P450s that exhibit cooperative ligand binding based on analysis of steady-state kinetics. However, site-directed mutagenesis of CYP3A4 designed to crowd the active site of the enzyme (inferred from homology modeling) has been shown to abolish cooperativity (60) and the

crystal structures of CYP2C9 are indicative of a large active site that might readily accommodate more than one ligand (44, 48).

Although CYP2C9 activation is now a well-documented phenomenon *in vitro*, it remains to be seen whether it has clinical relevance. Few studies have been performed as yet, but Tracy and coworkers did report a modest, yet statistically significant, increase (11%) in flurbiprofen clearance following cotreatment with dapsone *in vivo* (61). It is worth noting that an *in vivo* significance for such allosteric phenomena with P450s is not established even for the much more intensively studied CYP3A4 enzyme. Therefore, for the foreseeable future CYP2C9 activation may remain an *in vitro* curiosity, albeit one that promotes new ideas about the elasticity of the P450 active site.

## CYP2C9 Pharmacogenetics

The first indications of polymorphism in the *CYP2C9* gene arose when multiple cDNAs were cloned in the late 1980s and early 1990s. Subsequently, a systematic investigation of possible sites of allelic variation confirmed the existence of the *CYP2C9*\*2 and *CYP2C9*\*3 variants at significant frequencies (close to 10%) in a Northern European population (62). Population studies by several other groups extended these findings and it is now clear that up to 40% of Caucasians possess one or more variant *CYP2C9* allele (63). This high frequency has prompted numerous studies aimed at determining the functional effects of these common CYP2C9 variants.

The *CYP2C9*\*2 allele reflects a missense mutation in exon 3 that causes a nonconservative Arg→Cys substitution at amino acid 144. The consensus view from *in vitro* studies conducted with the recombinantly expressed CYP2C9.2 is that this mutation causes a small decrement in  $V_{\max}$  (0%–35%) and little or no change in the  $K_m$  for substrate catalysis (64). *In vivo* studies have generally been difficult to interpret owing to the paucity of CYP2C9\*2/\*2 homozygotes available for study, but recent clinical investigations that did include this test group also suggest modest decreases in drug clearance attributable to this mutation (15, 65). Arg144 maps to helix C, which is located on the exterior of the protein and forms part of the putative P450 reductase binding site (66). Loss of activity may reflect altered affinity for the coenzyme P450 reductase, which appears to bind reversibly to positively charged surface amino acids on P450s (67).

The *CYP2C9*\*3 allele arises from a missense mutation in exon 7 that causes an Ile→Leu substitution at amino acid 359. *In vitro* and *in vivo* experiments consistently demonstrate substantial loss of enzyme activity owing to this mutation (64). In fact, we recently identified five \*3/\*3 homozygotes in a Caucasian anticoagulation clinic population and were able to demonstrate that this mutation is associated with low warfarin dose and increased risk of bleeding during the warfarin stabilization phase (68). Loss of activity for CYP2C9.3 reflects a combination of decreased  $V_{\max}$  and increased  $K_m$  for CYP2C9 substrates (40). Recent (unpublished) studies from our group confirm that the spectral binding constant,



$K_s$ , is increased substantially for CYP2C9.3 relative to the wild-type enzyme. The structural basis for diminished P450 activity as a consequence of the \*3 allele is not yet clear, as new 3D information for CYP2C9 places this residue outside the active site of the enzyme, some distance from the heme (44, 48). Ile359 is situated below the SRS-6 region (70), which contains the important orienting amino acid F476. The physical proximity of the \*3 locus and this active-site region could conceivably result in global conformational changes that secondarily diminish binding affinity. However, more studies are needed to better explain the functional deficit attributable to this important CYP2C9 polymorphism.

Extensive resequencing of *CYP2C9* in ethnically diverse populations demonstrates that this gene is highly polymorphic (71, 105) (<http://egp.gs.washington.edu>). At the time of writing (April, 2004), a total of 12 CYP2C9 coding-region alleles were listed on the P450 Allele Web Site (<http://www.imm.ki.se/CYPalleles>), and to our knowledge, all but the \*4 and \*7 alleles have been independently verified by multiple research groups. In our own laboratory, resequencing across ~60 kb of *CYP2C9* in 192 warfarin patients of Caucasian origin revealed a total of 129 single nucleotide polymorphism (SNP) sites (105). The prevalence of coding-region mutations in this study population (allele frequency in parentheses) decreased in the following order: \*2 (11%), \*3 (6%), \*11 (1%), and \*12 and \*9 (0.5%). Consideration of sequence variation in the *CYP2C9* gene allowed us to infer 23 haplotypes, 10 of which are represented at a frequency of >3% (105). In another study, resequencing of DNA from 92 individuals across three different racial groups predicted at least 21 haplotypes (71). The \*2 and \*3 alleles are each isolated on one major haplotype background, and both appear to be more significant contributors to variability in warfarin maintenance dose than any of the other eight major haplotypes (105). A qualitatively similar situation has been reported for the warfarin analog acenocoumarol (72).

*CYP2C9* polymorphisms vary dramatically between different ethnic populations. An early genotyping study by Goldstein's group demonstrated that the *CYP2C9*\*2 and *CYP2C9*\*3 variants that are common in Caucasians were represented in African-Americans but at much lower allele frequencies (1%–2%) (73). *CYP2C9*\*6 was detected by the same group in one African-American patient who had an adverse reaction to phenytoin. This rare, null allele is devoid of activity owing to a splicing mutation that results in a truncated protein (74). *CYP2C9*\*5, D360→E, is selectively expressed in African-Americans at an allele frequency of ~1% (75, 76). Recombinant CYP2C9.5 exhibited a large increase in  $K_m$  for several substrates, but little change in  $V_{max}$ , and we have suggested that this was predictive of a decrease in the in vivo catalytic efficiency of the enzyme. However, the infrequency of this variant complicates further in vivo studies. Genotyping studies in Korean and East Asian populations have not detected the *CYP2C9*\*2 polymorphism, although the *CYP2C9*\*3 allele is present at low frequencies (1%–2%) (77). Similar to the situation with *CYP2C9*\*6, a rare polymorphism *CYP2C9*\*4 (I359→T), has been reported in one Japanese patient who had an adverse reaction to phenytoin (78). Not surprisingly, recombinant CYP2C9.4 exhibited defective

metabolism of several substrates in vitro (79). Recently, four new *CYP2C9* SNPs were reported in a Hong Kong Chinese anticoagulation clinic population, I181→L, H184→P, Q192→P, and L208→V, but on closer examination these appear to be spurious and likely a consequence of improper primer design (80). Further studies are required to determine the frequency of occurrence of the newer ethnic-specific *CYP2C9* alleles, establish haplotypes in these populations, and delineate their functional consequences.

## Gene Regulation of CYP2C9

Whereas the great majority of drug-drug and pharmacogenetic interactions involving *CYP2C9* result in an exacerbated clinical response, induction of *CYP2C9* is associated with enhanced metabolic clearance and possible loss or diminution of therapeutic activity. Consideration of in vivo drug-drug interaction data indicates that *CYP2C9* is significantly induced by rifampin (81), and to a lesser extent by phenobarbital and phenytoin (8). These observations can be replicated at the mRNA and protein levels in primary human hepatocytes (82), thereby providing a platform for detailed studies of *CYP2C9* induction.

Several mechanisms exist for the upregulation of P450 genes, but enzyme induction most often involves transcriptional activation by nuclear receptors that bind to *cis*-regulatory elements in the gene's promoter (83). The *CYP2C9* promoter contains at least four important regulatory elements: an HNF4 $\alpha$  site located at -139 to -125, a glucocorticoid responsive element at -1676 to -1662, a PXR site at -1818 to -1802, and a CAR/PXR element at -2898 to -2882 (84-86). The PXR responsive element at -1818 appears to be the major contributor to rifampin induction of *CYP2C9* (87). Several other putative regulatory pathways have been suggested, including those involving C/EBP $\alpha$  (88) and vitamin D (89), but the extent to which these and other regulatory mechanisms contribute to constitutive expression of the gene remains to be established.

5'-Flanking polymorphisms of *CYP2C9* are increasingly well documented. Eleven SNPs have been reported in the first 2 kB of the promoter region in Japanese and Caucasians, some of which may be associated with altered gene transcription (90, 91). Marked allele frequency differences were noted between these two ethnic groups that did not explain the population difference in (S)-warfarin clearance reported between the two populations (92). Nineteen promoter SNPs out to -2.7 kB are listed on the Environmental Genome Project Web site (<http://egp.gs.washington.edu/data/cyp2c9>), and a further 40 SNPs have been identified out to -10 kB, thereby permitting a high resolution description of the haplotype structure of the gene (vide infra). The extent to which these 5'-flanking polymorphisms contribute to interindividual variability in *CYP2C9* status in different ethnic groups is currently an active area of research.

Finally, the developmental expression pattern of *CYP2C9* has recently been established (93). Levels of *CYP2C9* were 1%-2% of mature values in the first trimester, increasing substantially in late fetal life to approximately 30% of adult

values. As for constitutive expression of the gene, further studies are needed to evaluate upstream regulatory sequences and establish basic mechanisms of ontogenic regulation.

## CYP2C9 and Endogenous Metabolism

Although members of the CYP 1–3 families are predominantly involved in the metabolic clearance of drugs and other xenobiotics, pronounced extrahepatic expression in many cases has stimulated questions about their role in the metabolism of endogenous substances. CYP2C9 protein expression has been demonstrated in a wide variety of human extrahepatic tissues, both by Western blotting of tissue microsomes and in situ immunohistochemistry (94, 95). CYP2C9 has also been demonstrated to metabolize the endogenous compounds 5-hydroxytryptamine and linoleic acid in vitro (96, 97); however, it is arachidonic acid that has garnered most interest as an endogenous substrate for the enzyme.

Cytochromes P450, together with cyclooxygenase and lipoxygenase enzymes, convert arachidonic acid to a plethora of products that exhibit critical pharmacological effects. CYP2 enzymes, in particular, have been implicated in the formation of vasoactive epoxyeicosatrienoic acids (EETs) within the vascular system. EETs relax vascular smooth muscle by opening potassium channels and hyperpolarizing smooth muscle cells. As such, EETs are prime candidates for the endothelial-derived hyperpolarization factor (EDHF), a vasodilation pathway that remains after inhibition of nitric oxide and prostacyclin-mediated responses. CYP2C9 generates primarily 14,15-EET and 11,12-EET (98), and the enzyme is clearly expressed at the protein level in a variety of endothelial cells (95).

CYP2C9 has also been linked with the putative EDHF synthase on the basis of the finding that sulfaphenazole inhibited the EDHF-mediated response in pig coronary arteries, as did antisense oligonucleotides against CYP2C8/9 (99). Moreover, nifedipine, an inducer of CYP2C enzymes, enhanced both endothelial mRNA expression of CYP2C and 11,12-EET production, also in pig coronary arteries (100). However, given the lack of specificity of the molecular probes used in these studies and species differences in isoform expression, identification of CYP2C9 as an EDHF synthase in human coronary vascular beds cannot be made with certainty.

Nonetheless, examination of the potential for CYP2C(9)-dependent vasoactivity to modulate cardiovascular disease is now an active area of investigation, and one that extends interest in this enzyme to the new arena of disease pathology. Recently, Yasar et al. concluded that possession of the more common genetic variants of CYP2C9 and CYP2C8 was associated with a modest increase in the risk of acute myocardial infarction, at least in females (101). In the same study, no statistically significant associations were made between different CYP2C genotypes and hypertension. However, other P450 isoforms such as CYP2J2, as well as non-P450 enzymes, such as soluble epoxide hydrolase, are also strongly implicated in determining EET levels in humans (102). Future pharmacogenetic association studies aimed at evaluating the role of these drug-metabolizing enzymes in complex diseases states such as hypertension will need to be multivariate in design.

In conclusion, in the 20 plus years since CYP2C9 was first identified in human liver (103), this isoform has become one of the most studied human P450s owing largely to its quantitative significance in oxidative drug metabolism, role in adverse drug reactions, and pharmacogenetic variability. CYP2C9 is also the first human P450 enzyme crystallized, and this pivotal event can be expected to propel future structural, biochemical, biophysical, and clinical studies aimed at a fuller understanding of this enzyme's role in xenobiotic and endobiotic disposition.

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