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Drug metabolism by CYP2C8.3 is determined by substrate dependent interactions with cytochrome P450 reductase and cytochrome b5☆

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ABSTRACT

Genetic polymorphisms in *CYP2C8* can influence the metabolism of important therapeutic agents and cause interindividual variation in drug response and toxicity. The significance of the variant *CYP2C8*3* has been controversial with reports of higher *in vivo* but lower *in vitro* activity compared to *CYP2C8*1*. In this study, the contribution of the redox partners cytochrome P450 reductase (CPR) and cytochrome b5 to the substrate dependent activity of CYP2C8.3 (R139K, K399R) was investigated in human liver microsomes (HLMs) and *Escherichia coli* expressed recombinant CYP2C8 proteins using amodiaquine, paclitaxel, rosiglitazone and cerivastatin as probe substrates. For recombinant CYP2C8.3, clearance values were two- to five-fold higher compared to CYP2C8.1. CYP2C8.3's higher k_{cat} seems to be dominated by a higher, but substrate specific affinity, towards cytochrome b5 and CPR (K_D and $K_{m,red}$) which resulted in increased reaction coupling. A stronger binding affinity of ligands to CYP2C8.3, based on a two site binding model, in conjunction with a five fold increase in amplitude of heme spin change during binding of ligands and redox partners could potentially contribute to a higher k_{cat}. In HLMs, carriers of the *CYP2C8*1/*3* genotype were as active as *CYP2C8*1/*1* towards the CYP2C8 specific reaction amodiaquine *N*-deethylation. Large excess of cytochrome b5 compared to CYP2C8 in recombinant systems and HLMs inhibited metabolic clearance, diminishing the difference in k_{cat} between the two enzymes, and may provide an explanation for the discrepancy to *in vivo* data. *In silico* studies illustrate the genetic differences between wild type and variant on the molecular level.

1. Introduction

Cytochrome P450 2C8 (CYP2C8) accounts for 7% of the total hepatic CYP content [1,2] and is estimated to be involved in the metabolism of at least 5% of drugs cleared by phase I oxidative processes [3]. CYP2C8 is predominantly involved in the drug metabolism of amodiaquine (AQ), amiodarone, cerivastatin (CER), montelukast, paclitaxel (PAC), repaglinide, rosiglitazone (RG), and troglitazone [3,4]. CYP2C8 also contributes to the metabolism of endogenous substrates such as the epoxidation of arachidonic acid [5] and hydroxylation of *all trans* retinoic acid [6]. From the at least 17 genetic variants described for *CYP2C8* (http://www.cypalleles.ki.se/cyp2c8.htm), *CYP2C8*3* is the most common in Caucasians (allele frequency of up to 0.15) [7] but seems to be absent in African Americans and attracted particular attention vis-à-vis its effect on drug metabolism.

*CYP2C8*3* encodes a protein with two amino acid substitutions at R139K and K399R, which are highly linked (>95%) [8]. Reports investigating the effect of these mutations on the clearance of CYP2C8 substrates *in vivo* were recently reviewed [9]. The inheritance of one or more alleles of *CYP2C8*3* is correlated with an increased clearance *in vivo* but overall, studies failed to consistently connect the *CYP2C8*3* allele to alteration in pharmacokinetics (see [9] and citations within). Discrepancies were mostly explained by other contributing CYP enzymes, diverse study design and other metabolic pathways depending on substrate.

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Abbreviations: CYP, cytochrome P450; CPR, cytochrome P450 reductase; CER, cerivastatin; AQ, amodiaquine; PAC, paclitaxel; RG, rosiglitazone; DEAQ, *N*-de ethyl amodiaquine; DLPC, L-α-dilauryl-sn-glycero-3-phosphocholine; HLMs, human liver microsomes; SIR EI^+, selective ionization reaction positive ionization; MRM EI^+, multiple reaction mode positive ionization.

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Reports investigating the metabolic activity of the expressed variant protein CYP2C8.3 *in vitro* have also been controversial. Several investigators contradict *in vivo* findings with lower metabolic rates for AQ *N*-de-ethylation [10,11], a slightly lower rate of PAC 6α-hydroxylation [7,12] and arachidonic acid epoxidation [13] *in vitro*. Other studies report also slightly higher metabolic rates for PAC and 13-*cis*-retinoic acid in partially purified recombinant CYP2C8.3 [14]. Each study used a different expression system such as *E. coli*, or partially purified *E. coli* membrane systems, without the addition cytochrome b5 [7,14,15], yeast [11] or HepG2 cells [12]. In contrast, recombinant CYP2C8.3 reconstituted with CPR and cytochrome b5 demonstrated a higher activity towards CER [8], a finding that is consistent with the above mentioned *in vivo* data. Interestingly, both amino acid mutations (R139K and K399R) in the corresponding CYP2C8.3 protein are located on the proximal site of the heme in the binding region of cytochrome b5 and CPR [16] and may suggest an involvement of the redox-partners in the variable activity of CYP2C8. The activation of CYP enzymes by cytochrome b5 is well established [17] for CYP3A4 [18], CYP2B4 and variants [19], CYP2A6 [20], CYP2C9 [21] and CYP2C19 [22]. Further, cytochrome b5 is also reported to decrease reaction uncoupling [23] and increase k_{cat}.

This investigation aimed to assess the effect of cytochrome b5 and CPR on a particular CYP2C8 genetic variant of clinical significance. For this purpose, metabolic activities of genotypes *CYP2C8*1* and *CYP2C8*3* were tested using a range of substrates *in vitro* in HLMs derived from individual human donor livers with pre-determined *CYP2C8* genotypes, CYP2C8 protein, CPR and cytochrome b5 content. These studies revealed either a slightly higher or equal metabolic activity of the *CYP2C8*3* genotype which is consistent with reported data for other drugs such as repaglinide *in vivo* [24]. Interestingly CYP2C8 activity was inversely associated with CYP b5 content. These data, however, do not align with published data for recombinantly expressed CYP2C8.3 protein. Consequently, CYP2C8.1 and CYP2C8.3 were engineered and expressed and kinetically evaluated towards the metabolic activity of probe substrates, ligand binding and affinity towards cytochrome b5 and CPR. The interaction of CYP2C8.3 protein with its redox partners was further rationalized *in silico* to address the structural differences caused by these two point mutations.

2. Materials and methods

2.1. Materials

All chemicals including terfenadine and chloroquine were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated, and used without further purification. CER sodium salt ((3R,5S,6E)-7-[4-(4-Fluorophenyl)-5-(methoxymethyl)-2,6-bis(1-methylethyl)-3-pyridinyl]-3,5-dihydroxy-6-heptenoic Acid Sodium Salt), hydroxy CER (M23, 6-[(1S)-2-hydroxy-1-methylethyl]-metabolite), desmethyl CER (M1, 5-(hydroxyl-methyl)-metabolite), RG, *N*-desmethyl RG, and 5-hydroxy RG were purchased from Toronto Research Chemicals (North York, ON, CA). Fluvastatin was a gift from em. Professor W. Trager (University of Washington). 6-Hydroxy PAC and DEAQ were purchased from Santa Cruz Chemicals (Santa Cruz, CA). Protein test kit was purchased from Pierce Biotechnology (Rockford, IL). Acetonitrile, methanol, water, zinc sulfate, ammonium formate and formic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). L-α-Dilauryl-sn-glycero-3-phosphocholine (DLPC) was from Avanti Polar Lipids Inc., (Alabaster, AL, USA). Human liver samples were obtained from the University of Washington School of Pharmacy Human Tissue Bank (Seattle, WA) (*24, 25*).

2.2. Expression of cytochrome P450 reductase, cytochrome b5 and CYP2C8.1 (wild type) and CYP2C8.3

Rat cytochrome P450 reductase and cytochrome b5 were expressed using previously established protocols [25,26]. The two base pair changes rs11572080 and rs10509681 for genotype *CYP2C8*3* were introduced into CYP2C8 (wild type) using site directed mutagenesis and the wild type and variant were expressed as previously described [13]. P450-CO difference spectra, gel electrophoresis, pyridine hemochromogen analysis and Lowry protein determination assay were used to evaluate CYP-enzyme quality and quantity [27,28]. Time of flight mass spectrometry (Waters Micromass High-Definition MS System, Quadrupole/Triwave™/Orthogonal Acceleration Time-of-Flight Tandem Hybrid Mass Spectrometer QToa – TOF MS/IMS/MS) was used to characterize the molecular mass of CYP2C8 proteins using the separation method of Cheesman et al. [29]. Mass determination resulted in a MW of 56,815 ± 13 for CYP2C8.1 and 56,837 ± 10 for CYP2C8.3 (calc. MW for both CYP2C8.1 and CYP2C8.3 protein 56835.77).

2.3. Characterization of human liver microsomes

Samples of human liver ($n = 56$) from Caucasian donors were obtained from the University of Washington School of Pharmacy Human Tissue Bank (Seattle, WA) according to previously published protocols [30]. Protein concentrations were determined by the method of Lowry et al. [31]. Genotype of *CYP2C8*3* and corresponding CYP2C8 protein content were previously determined [32].

2.4. In vitro assays for recombinant CYP2C8 and human liver microsomes

DLPC micelles (2.5 mM) were prepared by repeated sonication and supplemented in the order of CYP2C8, cytochrome P450 reductase, and cytochrome b5 in the ratio 160:1:2:1 [33] – the ratio CYP:DLPC was constant for all kinetic evaluations. The mixture was incubated on ice for 20 min and diluted with potassium phosphate buffer (100 mM KPi, pH 7.4) to reach final assay concentration of 50 pmol mL^{-1} for AQ, CER, and RG, and for PAC metabolism. For enzyme kinetics, 0.2 mg mL^{-1} of human liver microsomes or 10 pmol P450 mL^{-1} of recombinant CYP2C8 were used as final concentrations in the assay mixture.

AQ and RG metabolism: Assay mixtures were supplemented with substrate at final concentrations 0.1–200 μM (for kinetics), pre-incubated for 5 min at 37 °C in a shaking water bath. Reactions were initiated by adding 10 μL of 10 mM NADPH to 90 μL assay mixture for a total volume of 100 μL. The AQ incubations were quenched with 10 μL 10% trichloracetic acid after 5 min; the assay for the RG metabolism was terminated after 5 min incubation by adding 10 μL of 15% zinc sulfate. Subsequently, internal standard (10 μL to achieve a final concentration of 0.26 μM chloroquine for AQ and 0.5 μM terfenadine for RG) was added. Samples were immediately vortexed, centrifuged at 10,000 × *g* for 10 min at room temperature and 10 μL (15 μL for RG) of supernatant was injected into LC–MS for analysis. DEAQ, *p*-hydroxy RG (PHRG), and demethyl RG (DMRG) were quantified by using a standard calibration curve ranging from 0.005 to 2.5 μM.

PAC metabolism: 450 μL of 100 mM KPi buffer with PAC (concentration range 0.1–100 μM) and reconstituted CYP enzyme with above mentioned concentrations were pre-incubated for 5 min at 37 °C and the assay was initiated by adding 50 μL of 10 mM NADPH. Reactions were quenched after 30 min by adding 2 mL ice-cold ethyl acetate containing internal standard (25 nM 10-deacetyl baccatin III), extracted and the organic phase removed. Extraction was repeated twice with 2 mL ethyl acetate; the organic phases were pooled for a total of 6 mL, evaporated to