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Human Tissue Distribution of Carbonyl Reductase 1 Using Proteomic Approach With Liquid Chromatography-Tandem Mass Spectrometry

Wenyi Hua, Hui Zhang*, Sangwoo Ryu, Xin Yang, Li Di*

Department of Pharmacokinetics, Dynamics and Metabolism, Pfizer, Inc., Groton, Connecticut

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A B S T R A C T

Human tissue distribution of carbonyl reductase 1 (CBR1) is quite controversial in the literature. To understand the differences, CBR1 protein abundance in human intestine, liver, and kidney has been determined using a proteomic approach with liquid chromatography-tandem mass spectrometry. The results show that CBR1 distribution in the 3 tissues is relatively similar, within 2- to 3-folds of each other. Intestine has the highest CBR1 enzyme level (106 pmol/mg protein) followed by liver (76 pmol/mg protein) and kidney (39 pmol/mg protein). The high abundance of CBR1 in the intestine and kidney suggests the critical role of this enzyme in gut first-pass metabolism and extra-hepatic clearance. CBR1 is also detected, for the first time, in the microsomal fractions with 5- to 17-fold lower amounts than cytosols of the corresponding tissues. The average CBR1 protein amount is 14 pmol/mg protein in human hepatocytes. Individual variability of CBR1 is about 4-fold based on 13 lots of cryopreserved human hepatocytes. The relative expression factor between human liver cytosol and human recombinant CBR1 enzyme (hr-CBR1) is 0.0042. The relative activity factor between human liver cytosol and hr-CBR1 is 0.040, suggesting that significant amount of CBR1 protein in the hr-CBR1 has no catalytic activity.

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Introduction

Carbonyl reductase 1 (CBR1) is one of the most important enzymes in the CBR family and it plays a critical role in the detoxification of certain carbonyl-containing xenobiotics. CBR1 is a cytosolic enzyme and it uses nicotinamide adenine dinucleotide phosphate hydrogen as a cofactor for its reductase function. CBR1 has broad substrate specificity compared to its paralog, CBR3, which has very narrow substrate specificity and plays a minor role in drug metabolism.[1] CBR1 is involved in the metabolism of several clinically important drugs, such as doxorubicin, daunorubicin, dolasteron, nabumetone, haloperidol, pentoxifylline, and bupropion.[2] Inhibitors of CBR1 are promising agents for adjuvant therapy of anthracyclines to prolong the anticancer efficacy by minimizing formation of the cardiotoxic alcohol metabolite.[3,4] Therefore, full characterization of CBR1 will provide useful information on drug discovery and development to predict clearance, assess drug-drug interaction (DDI) potential, and understand the impact of pharmacogenomics, individual variability, and disease state of the enzyme. CBR1 is inducible through the aryl hydrocarbon receptor[5,6] and strong inducers of aryl hydrocarbon receptor (e.g., β-naphthoflavone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) show concomitant up-regulation of CBR1 mRNA, protein, and activity.[5] High individual variability of CBR1 expression has been reported to be about 20-fold based on mRNA levels, and about 10-fold based on protein abundance.[7-10] The variability may reflect the impact of environmental induction factors rather than the individual status of CBR1 gene variants.[8] Tissue distribution of CBR1 in humans is quite controversial in the literature. Some studies show that liver is the major organ of CBR1 with minimal amount in other tissues,[8,11] whereas others report that intestine has the highest expression and liver and kidney contain lower levels, but still quite significant.[12] The understanding of CBR1 tissue distribution is important to scale clearance from *in vitro* to *in vivo*, anticipate potential first-pass effect, and evaluate extra-hepatic contribution to clearance. In this study, we quantify the CBR1 protein abundance in human liver, intestinal and kidney cytosols and microsomes, human hepatocytes, and human recombinant CBR1 enzyme (hr-CBR1) using a proteomic approach with liquid chromatography-tandem mass spectrometry (LC-MS/MS) to understand CBR1 tissue distribution at the protein level. The CBR1 tissue distribution is further verified by functional activity of the enzyme using doxorubicin as a substrate in the cytosols. Relative expression factor (REF) and relative activity factor (RAF) are determined. The data will be useful to

Abbreviations used: CBR, carbonyl reductase; DDI, drug-drug interactions; DMF, dimethylformamide; f_m, fraction metabolized; IDA, information-dependent acquisition; ISEF, intersystem extrapolation factors; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PK, pharmacokinetics; RAF, relative activity factor; REF, relative expression factor; SRM, selected reaction monitoring; TOF, time-of-flight.

* *Correspondence to:* Hui Zhang (Telephone: 860-686-0449; Fax: 860-715-9843) and Li Di (Telephone: 860-715-6172).

E-mail addresses: Hui.Zhang3@Pfizer.Com (H. Zhang), Li.Di@Pfizer.Com (L. Di).

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model pharmacokinetics (PK) and DDI mediated by CBR1 using physiological-based PK approaches, develop intersystem extrapolation factor values, understand fraction metabolized (f_m) by CBR1, and scale clearance for CBR1 substrates.

Experimental Section

Materials

Pools of 3-12 mixed gender donors were obtained from XenoTech (Kansas City, KS) for human intestine (prepared from duodenal and jejunal tissues) and kidney cytosols and microsomes. Human liver microsomes were purchased from Corning Life Sciences (Tewksbury, MA) and human liver cytosols from BioreclamationIVT (Baltimore, MD). Recombinant human CBR1 (Lot# GR150388-2) was purchased from Abcam (Cambridge, MA). Cryopreserved human hepatocytes of individual donors were obtained from multiple vendors (BioreclamationIVT; In Vitro ADMET Laboratories, Malden, MA; Life Technologies, Carlsbad, CA) or custom pooled with more than 10 donors of mixed gender (BioreclamationIVT). Male CD-1 mouse liver microsomes of 338 donors (control matrix) were obtained from Corning Life Sciences. Stable heavy isotope-labeled (purity >95% and isotopic purity >99%) and light standard peptides (purity >95%, peptide purity was determined by HPLC and identified by mass spectral analysis; amino acid analysis determined net peptide content; and concentrations of synthesized peptides were corrected by purity for quantitation.) were obtained from New England Peptide (Gardner, MA). Amino acid residue arginine (*R*) was labeled for both VVN and LSF peptides. RapiGest SF was from Waters (Milford, MA) and MS-grade trypsin was purchased from Thermo Scientific™ (Guilford, CT). Doxorubicin, iodoacetamide, dithiothreitol, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Doxorubicinol was obtained from Fisher Scientific (Pittsburgh, PA).

Sample Preparation for Peptide Mapping, Selection, and Quantitation

Peptide mapping and selection were initially performed with 0.5 mg protein of human microsome and cytosol samples. The samples were mixed with 4 volumes of 50 mM ammonium bicarbonate buffer (pH 8.5) with 0.1% of RapiGest SF in a 1-mL 96-well LoBind Plate (Eppendorf, Hauppauge, NY). The samples were heated at ~80°C for 5 min in a water bath (VWR, Radnor, PA). The proteins were reduced by 5 mM dithiothreitol at 37°C for 30 min followed by 10 mM iodoacetamide for 30 min in the dark. The final solution was incubated with trypsin at 20:1 (protein/trypsin) overnight at 37°C with mild agitation. The tryptic peptide solution was acidified (to pH <2) with formic acid and transferred to a clean 1-mL 96-well LoBind plate followed by brief centrifugation (Beckman Coulter, Indianapolis IN) to allow the solution to settle at the bottom of the plate. Acetonitrile (20 μL) was added to the samples so that there was 5% final organic solvent.

For quantitation purpose, 0.5 mg protein of samples (3 replicates of each sample) was processed using the same procedure as described above. Pooled mouse liver microsomes were used as control matrix to match the total protein amount when needed. Stock solutions of light and heavy isotope-labeled peptides were prepared at a concentration of 200 μM in dimethylformamide (DMF). For standard curves, the combined standard working solutions of the 2 peptides were prepared in the range of 0.025-50 μM in 1:1 DMF/water and 10 μL of the standards were spiked into the digested control matrix after acidification. In total, 10 μL of 1:1 DMF:water was spiked into all other samples (except standard curves) to compensate for the volume difference between the standard curve and samples. About 10 μL of the combined isotope-labeled peptide solution at 3 μM in the same diluent was spiked after sample digestion as internal standards.

Table 1
LC Method for Peptide Mapping Using IDA/SWATH Acquisition

UPLC pumps	Agilent 1290 Infinity Binary Pump		
Autosampler	CTC PAL Autosampler		
Autosampler needle wash	Wash 1: 0.1% formic acid in acetonitrile Wash 2: 50:50 methanol/water		
Mobile phase A	Water with 0.1% formic acid		
Mobile phase B	Acetonitrile with 0.1% formic acid		
Flow rate	0.25 mL/min		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.01	98	2
	2.00	98	2
	55.00	65	35
	60.00	5	95
	65.00	5	95
	67.00	98	2
	72.00	98	2
Column	XBridge BEH C18 2.5 μm, 130 Å, 100 × 2.1 mm		
Injection volume (μL)	20		

UPLC, ultra performance liquid chromatography.

Peptide Selection and Quantitation Method Development

Detailed LC and MS/MS methods for peptide mapping using information-dependent acquisition (IDA) and SWATH™ are summarized in Tables 1 and 2. Briefly, 20 μL of the digested samples were injected onto a BEH C18 (XBridge 130 Å, 100 × 2.1 mm, 2.5 μm; Waters) by the CTC PAL autosampler (Leap Technologies, Carrboro, NC). A triple Time-Of-Flight (TOF) 6600 mass spectrometer (Sciex, Toronto, ON) was used for data acquisition. An IDA experiment was first performed to obtain peptide identification using ProteinPilot™ Software 5.0 (Sciex) with Paragon database search algorithm (proteome library: Uniprot_Homo_sampiens, updated on February 2015). Both biological modifications and amino acid substitutions were searched in “Thorough” mode. The samples were then subjected to SWATH acquisition on the same LC-triple TOF MS. The resulting protein pilot.group file from IDA acquisition was used to generate the ion library to guide SWATH data processing. It was processed using the SWATH Acquisition

Table 2
MS/MS Method for Peptide Mapping Using IDA/SWATH Acquisition

Mass spectrometer	Sciex Triple TOF-6600-Electrospray (+)
Data collection software/version	Analyst TF 1.7.1, PeakView® 2.1 with MicroApp 1.0
Ion source temperature	600°C
IonSpray voltage floating	5500 V
Declustering potential	20 V
TOF MS scan (with IDA)	350 *m*/*z* to 1600 *m*/*z* with accumulation time of 0.1 s
IDA switch criteria	For ions >350 *m*/*z* and <1250 *m*/*z* With charge state 2-5 For intensity exceeding 150 cps Exclude former target ions for 2 s and after 1 repeat Maximum number of candidate ions/cycle: 30 spectra
IDA advanced	With rolling collision energy
Product ion IDA scan	250 *m*/*z* to 1600 *m*/*z* with accumulation time of 0.025 s
TOF MS scan (with SWATH)	350 *m*/*z* to 1600 *m*/*z* with accumulation time of 0.05 s
TOF product ion (SWATH)	35 consecutive 26.7 Da isolation windows for precursor ions from 350 *m*/*z* to 1250 *m*/*z* Product scan from 350 *m*/*z* to 1600 *m*/*z* with accumulation time of 0.065 s

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