Published in final edited form as:

Biopharm Drug Dispos. 2018 June ; 39(6): 315–318. doi:10.1002/bdd.2135.

***CBR1* rs9024 genotype status impacts the bioactivation of loxoprofen in human liver ^a**

Adolfo Quiñones Lombraña^{1,†}, Nasi Li^{2,†}, Virginia del Solar^{2,†}, G. Ekin Atilla-Gokcumen², and Javier G. Blanco^{1,*}

¹Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York (SUNY), Buffalo, New York

²Department of Chemistry, University at Buffalo, The State University of New York (SUNY), Buffalo, New York

Abstract

Loxoprofen is an anti-inflammatory drug that requires bioactivation into the *trans*-OH metabolite to exert pharmacological activity. Evidence suggests that carbonyl reductase 1 (*CBR1*) is important during the bioactivation of loxoprofen. Here, we examined the impact of the functional single nucleotide polymorphism *CBR1* rs9024 on the bioactivation of loxoprofen in a collection of human liver samples. The synthesis ratios of *trans*-OH loxoprofen/*cis*-OH loxoprofen were 33% higher in liver cytosols from donors homozygous for the *CBR1* rs9024 G allele in comparison to the ratios in samples from donors with heterozygous GA genotypes. Complementary studies examined the impact of *CBR1* rs9024 on the bioactivation of loxoprofen in lymphoblastoid cell lines. *CBR1* rs9024 genotype status impacts the synthesis of the bioactive *trans*-OH metabolite of loxoprofen in human liver.

Keywords

Loxoprofen; *CBR1*; *CBR1* rs9024

1. INTRODUCTION

Loxoprofen is a nonsteroidal anti-inflammatory drug with analgesic and anti-pyretic properties (Naruto, Tanaka, Hayashi, & Terada, 1984; Tanaka, Nishikawa, & Hayashi, 1983; Tanaka, Nishikawa, Matsuda, Yamazaki, & Hayashi, 1984). Loxoprofen was initially marketed as an oral drug, and a topical formulation is also available and widely used in Japan since 2006 (Loxonin® gel 1%, Daiichi Sankyo Co., Ltd, Tokyo, Japan) (Sawamura, Kazui, Kurihara, & Izumi, 2015; Sawamura et al., 2015). Loxoprofen is a prodrug that

^aThis study was supported by the National Institute of General Medical Sciences (award GM073646).

*Corresponding author at: Department of Pharmaceutical Sciences, University at Buffalo, The State University of New York, 470 Kapoor Hall, Buffalo, NY 14214 – 8033, USA, Phone: 716 - 645 - 4820, Fax: 716 - 829 - 6569, jgblanco@buffalo.edu.

[†]Denotes equal contribution

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

undergoes enzymatic reduction into the pharmacologically active *trans*-OH metabolite. The *trans*-OH metabolite of loxoprofen, not the parent drug or the *cis*-OH isomer, is a strong and non-selective inhibitor of pro-inflammatory cyclooxygenase 2 (COX-2) (Riendeau et al., 2004; Sugimoto, Kojima, Asami, Iizuka, & Matsuda, 1991). An early report by Ohara et al. noted that the bioactivation of loxoprofen is catalyzed by hepatic carbonyl reductases (CBRs) (Ohara, Miyabe, Deyashiki, Matsuura, & Hara, 1995). More recently, Sawamura et al. showed that carbonyl reductase 1 (CBR1) is the predominant enzyme involved in the formation of the *trans*-OH metabolite in human skin. The immunodepletion of CBR1 in skin homogenates decreased the reduction of loxoprofen to its *trans*-OH form by more than 80% (Sawamura, Sakurai, et al., 2015).

CBR1 is a cytosolic NADPH dependent enzyme that catalyzes the reduction of endogenous and exogenous carbonyl substrates (Malatkova, Maser, & Wsol, 2010; Shi & Di, 2017). Evidence suggests that genetic polymorphisms in *CBR1* impact the metabolism of carbonyl substrates (Shi & Di, 2017). A functional single nucleotide polymorphism (SNP) in the 3'-untranslated region of *CBR1* (rs9024, G>A) impacts *CBR1* expression in liver and myocardium through mechanisms involving the binding of specific microRNAs to the polymorphic site (Kalabus et al., 2012; Gonzalez-Covarrubias et al., 2009, Quiñones-Lombrana et al., 2014). Liver cytosols with *CBR1* rs9024 homozygous GG genotypes showed higher maximal rates of doxorubicinol synthesis (1.5-fold) compared with samples with heterozygous GA genotypes (Gonzalez-Covarrubias et al., 2009). The variant *CBR1* rs9024 allele exhibits various frequencies in DNA variation panels (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=9024). To date it is not known whether *CBR1* rs9024 impacts the bioactivation of loxoprofen in liver. The aim of this study was to investigate whether *CBR1* rs9024 genotype status impacts the metabolism of loxoprofen in a collection of human liver samples.

2. MATERIAL AND METHODS

2.1. Chemicals

β -NADPH tetrasodium salt hydrate, loxoprofen sodium, and loxoprofen reduced stable isotopes (*cis*-OH form of loxoprofen- d_3 and *trans*-OH form of loxoprofen- d_3) were purchased from Toronto Research Chemicals (Toronto, Canada). The structures of loxoprofen and its *trans*-OH and *cis*-OH metabolites are shown in Figure 1a. Loxoprofen and NADPH stock solutions were prepared in 0.1 M potassium phosphate buffer (pH 7.4).

2.2. Human Liver Samples

The Institutional Review Board of the State University of New York at Buffalo, NY, approved this research. Human liver tissues ($n = 24$) were processed at St. Jude Children's Research Hospital (Memphis, TN) and were provided by the Liver Tissue Procurement and Distribution System (National Institutes of Health Contract N01-DK-9-2310) and by the Cooperative Human Tissue Network (<http://chtn.nci.nih.gov/>). Liver samples were processed following standardized procedures to isolate DNA, RNA, and protein. *CBR1* mRNA expression and *CBR1* rs9024 genotype status of selected liver samples were reported by us (Gonzalez-Covarrubias, Zhang, Kalabus, Relling, & Blanco, 2009). Hepatic CBR1 protein

expression was determined by quantitative immunoblotting with a specific anti-CBR1 antibody and recombinant CBR1 protein standards as previously described (Gonzalez-Covarrubias, et al., 2009).

2.3. Human Lymphoblastoid Cell Lines

Lymphoblastoid cell lines (GM10853, GM10845, GM10857, GM10858, GM10860, GM17240, GM16654, GM16688, and GM16689) derived from donors with European ($N=6$) or Chinese ($N=3$) ancestries were purchased from the Coriell Institute for Medical Research (Camden, NJ). Cultures were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA). Cultures were grown using standard incubation conditions at 37°C, 5% CO₂, and 95% relative humidity.

2.4. Quantification of *trans*- and *cis*-OH forms of loxoprofen

Cytosolic protein extraction was performed with NE-PER Nuclear and Cytoplasmic Extraction Reagents kits (Thermo Fisher Scientific). Validation experiments with liver and lymphoblastoid cell cytosols were performed to determine the oxidation rate of NADPH using a method similar to that reported previously (Gonzalez-Covarrubias, et al., 2009). Our results showed that 150 µM loxoprofen [S] ensured conditions of V_{max} - i.e., maximal CBR activity, zero-order kinetics -. Incubation mixtures (50 µL) containing potassium phosphate buffer (pH 7.4, 0.1 M), cytosolic protein (15 µg), NADPH (400 µM), and loxoprofen (150 µM) were incubated at 37°C for 4 hours (Koo et al., 2005; Mordente et al., 2003). Reactions were quenched by adding 1.1 mL of methanol and incubated again at 37°C for 30 minutes. Samples were then centrifuged, and 1 mL of supernatant was dried under vacuum and resuspended in 100 µL of methanol. Samples were spiked with the *cis*-OH and *trans*-OH forms of loxoprofen-*d*₃ at 1 µM each. The generation of *trans*-OH and *cis*-OH forms of loxoprofen was monitored by liquid chromatography–mass spectrometry (LC-MS) in positive ionization mode ($[M+NH_4]^+$, $m/z = 266.1751$). The LC-MS method is summarized in table 1, and a representative chromatogram is shown in Figure 1a. Calibration curves were linear over the 1.00 – 20.00 pmol range for the *cis*-OH form of loxoprofen-*d*₃ ($r^2 = 0.9998$) and the 0.10 – 20.00 pmol range for the *trans*-OH form of loxoprofen-*d*₃ ($r^2 = 0.9992$). The lower limits of quantification (LLOQ) were 0.50 pmol for the *trans*-OH form and 2.50 pmol for the *cis*-OH form. Quantifications were carried out in triplicates.

2.5. Statistical Analysis

Excel 2007 (Microsoft Office; Microsoft, Redmond, WA) and GraphPad Prism version 4.03 (GraphPad Software Inc., La Jolla, CA) were used for statistical data analysis. The D'Agostino & Pearson test was used to analyze the normality of data sets. The Student's *t* test or the Mann Whitney test were used to compare group means.

3. RESULTS AND DISCUSSION

The relative expression of *CBR1* mRNA in liver samples from donors with *CBR1* rs9024 homozygous GG genotype was significantly higher than in samples from donors with heterozygous GA genotype (*CBR1* mRNA_{rs9024GG} = 6.7 ± 4.7 vs. *CBR1* mRNA_{rs9024GA} =

3.4 ± 2.5. Mann Whitney test, $p = 0.022$. Figure 1b). Livers from donors with *CBR1* rs9024 homozygous GG genotype exhibited higher cytosolic CBR1 protein content than samples from donors with heterozygous GA genotype (CBR1 protein_{rs9024GG} = 9.4 ± 2.1 nmol/g cytosolic protein vs. CBR1 protein_{rs9024GA} = 7.6 ± 2.5 nmol/g cytosolic protein. Student's t test, $p = 0.030$. Figure 1c). On average, the synthesis ratios of *trans*-OH/*cis*-OH forms of loxoprofen were 33% higher in liver cytosols from donors homozygous for the *CBR1* rs9024 GG allele in comparison to the ratios in samples from donors with heterozygous GA genotypes (*CBR1* rs9024 GG_{mole of *trans*-OH form/mole of *cis*-OH form}: 4.85 ± 1.49 vs. *CBR1* rs9024 GA_{mole of *trans*-OH form/mole of *cis*-OH form}: 3.66 ± 1.23. Student's t test $p = 0.044$. Figure 1d). Pearson's correlation analysis showed no linear correlation between CBR1 protein content and *trans*-OH/*cis*-OH loxoprofen ratios ($r_p = 0.258$, $p = 0.224$). The reason for this discrepancy is unclear. It is known that CBR1 is subjected to posttranslational modifications - e.g., *S*-glutathionylation in cysteine 226 - that impact catalytic activity towards specific substrates (Hartmanova et al., 2013). Thus, studies with more robust sample sizes coupled to protein content analysis of CBR1 isoforms are needed to further define the contribution of CBR1 expression to the synthesis of relevant metabolites such as *trans*-OH loxoprofen in human liver.

The impact of *CBR1* rs9024 on the synthesis of *trans*-OH and *cis*-OH forms of loxoprofen was also investigated in lymphoblastoid cell lines with known *CBR1* rs9024 genotype status. None of the lymphoblastoid cell lines synthesized quantifiable levels of the *cis*-OH metabolite (LLOQ_{*cis*-OH loxoprofen}: 2.50 pmol). There was a modest trend towards increased synthesis of *trans*-OH form in cytosols from cells with *CBR1* rs9024 GG genotype in comparison to cells with heterozygous GA and homozygous A genotypes, respectively (*CBR1* rs9024 GG: 1.62 ± 0.06 pmol of *trans*-OH form of loxoprofen/μg of cytosolic protein; *CBR1* rs9024 GA: 1.33 ± 0.22 pmol of *trans*-OH form of loxoprofen/μg of cytosolic protein; *CBR1* rs9024 AA: 1.41 ± 0.39 pmol of *trans*-OH form of loxoprofen/μg of cytosolic protein. Student's t test, GG vs. GA, $p \sim 0.096$, and GG vs. AA, $p \sim 0.405$. Figure 1e).

This study is limited by the lack of samples from individuals homozygous for the *CBR1* rs9024 A allele. While the frequency of the *CBR1* rs9024 A allele is relatively low in individuals of European and African ancestries (e.g., $q = 0.087$ in Europeans, and $q = 0.006$ in Africans), the A allele is relatively more common in individuals of Japanese ancestry ($q = 0.322$. <https://www.pharmgkb.org/variant/PA166155972>). Our data suggest that *CBR1* rs9024 genotype status impacts the hepatic synthesis of bioactive *trans*-OH form of loxoprofen in a manner consistent with the magnitude and direction of previously documented *CBR1* genotype-phenotype associations for the carbonyl substrates menadione and daunorubicin (Gonzalez-Covarrubias, et al., 2009; Quinones-Lombrana et al., 2014). Data from the pilot study in lymphoblastoid cells suggest that *CBR1* rs9024 genotype status may impact the bioactivation of loxoprofen in blood cells. Koo et al. characterized the pharmacokinetics of loxoprofen in rats after intragastric, intravenous, and intramuscular administration (Koo, et al., 2005). The authors concluded that the extrahepatic metabolism of loxoprofen by blood cells could be of pharmacological relevance irrespective of the route of administration (Koo, et al., 2005; Sawamura, Sakurai, et al., 2015). This study provides a foundation to investigate the contribution of *CBR1* rs9024 to the pharmacokinetics and pharmacodynamics of loxoprofen in humans.

References

- Gonzalez-Covarrubias V, Zhang J, Kalabus JL, Relling MV, Blanco JG. Pharmacogenetics of human carbonyl reductase 1 (CBR1) in livers from black and white donors. [Research Support, N.I.H., Extramural]. *Drug Metab Dispos.* 2009; 37(2):400–407. DOI: 10.1124/dmd.108.024547 [PubMed: 19022938]
- Hartmanova T, Tambor V, Lenco J, Staab-Weijnitz CA, Maser E, Wsol V. S-nitrosoglutathione covalently modifies cysteine residues of human carbonyl reductase 1 and affects its activity. *Chem Biol Interact.* 2013; 202(1–3):136–145. S0009-2797(12)00281-5 [pii]. DOI: 10.1016/j.cbi.2012.12.011 [PubMed: 23295225]
- Kalabus JL, Cheng Q, Blanco JG. PLoS One. 2012; 7(11):e48622. Epub 2012 Nov 1. MicroRNAs differentially regulate carbonyl reductase 1 (CBR1) gene expression dependent on the allele status of the common polymorphic variant rs9024. doi: 10.1371/journal.pone.0048622 [PubMed: 23133646]
- Koo TS, Kim DH, Ahn SH, Kim KP, Kim IW, Seo SY, ... Chung SJ. Comparison of pharmacokinetics of loxoprofen and its active metabolites after an intravenous, intramuscular, and oral administration of loxoprofen in rats: evidence for extrahepatic metabolism. *J Pharm Sci.* 2005; 94(10):2187–2197. S0022-3549(16)31871-8 [pii]. DOI: 10.1002/jps.20451 [PubMed: 16136574]
- Malatkova P, Maser E, Wsol V. Human carbonyl reductases. *Curr Drug Metab.* 2010; 11(8):639–658. BSP/CDM/E-Pub/00091 [pii]. [PubMed: 20942781]
- Mordente A, Minotti G, Martorana GE, Silvestrini A, Giardina B, Meucci E. Anthracycline secondary alcohol metabolite formation in human or rabbit heart: biochemical aspects and pharmacologic implications. *Biochem Pharmacol.* 2003; 66(6):989–998. DOI: 10.1016/S0006-2952(03)00442-8 [PubMed: 12963485]
- Naruto S, Tanaka Y, Hayashi R, Terada A. Structural determination of rat urinary metabolites of sodium 2-[4-(2-oxocyclopentylmethyl)phenyl]propionate dihydrate (loxoprofen sodium), a new anti-inflammatory agent. *Chem Pharm Bull (Tokyo).* 1984; 32(1):258–267. DOI: 10.1248/cpb.32.258 [PubMed: 6722952]
- Ohara H, Miyabe Y, Deyashiki Y, Matsuura K, Hara A. Reduction of drug ketones by dihydrodiol dehydrogenases, carbonyl reductase and aldehyde reductase of human liver. *Biochem Pharmacol.* 1995; 50(2):221–227. 0006295295001241 [pii]. [PubMed: 7632166]
- Quinones-Lombrana A, Ferguson D, Hageman Blair R, Kalabus JL, Redzematovic A, Blanco JG. Interindividual variability in the cardiac expression of anthracycline reductases in donors with and without Down syndrome. *Pharm Res.* 2014; 31(7):1644–1655. DOI: 10.1007/s11095-013-1267-1 [PubMed: 24562808]
- Riendeau D, Salem M, Styhler A, Ouellet M, Mancini JA, Li CS. Evaluation of loxoprofen and its alcohol metabolites for potency and selectivity of inhibition of cyclooxygenase-2. *Bioorg Med Chem Lett.* 2004; 14(5):1201–1203. S0960894X03013520 [pii]. DOI: 10.1016/j.bmcl.2003.12.047 [PubMed: 14980665]
- Sawamura R, Kazui M, Kurihara A, Izumi T. Pharmacokinetics of loxoprofen and its active metabolite after dermal application of loxoprofen gel to rats. *Pharmazie.* 2015; 70(2):74–80. DOI: 10.1691/ph.2015.4077 [PubMed: 25997245]
- Sawamura R, Sakurai H, Wada N, Nishiya Y, Honda T, Kazui M, ... Izumi T. Bioactivation of loxoprofen to a pharmacologically active metabolite and its disposition kinetics in human skin. *Biopharm Drug Dispos.* 2015; doi: 10.1002/bdd.1945
- Shi SM, Di L. The role of carbonyl reductase 1 in drug discovery and development. *Expert Opin Drug Metab Toxicol.* 2017; 13(8):859–870. DOI: 10.1080/17425255.2017.1356820 [PubMed: 28712324]
- Sugimoto M, Kojima T, Asami M, Iizuka Y, Matsuda K. Inhibition of prostaglandin production in the inflammatory tissue by loxoprofen-Na, an anti-inflammatory prodrug. *Biochem Pharmacol.* 1991; 42(12):2363–2368. 0006-2952(91)90242-W [pii]. [PubMed: 1764120]
- Tanaka Y, Nishikawa Y, Hayashi R. Species differences in metabolism of sodium 2-[4-(2-oxocyclopentylmethyl)-phenyl]propionate dihydrate (loxoprofen sodium), a new anti-inflammatory agent. *Chem Pharm Bull (Tokyo).* 1983; 31(10):3656–3664. DOI: 10.1248/cpb.31.3656 [PubMed: 6671226]

Tanaka Y, Nishikawa Y, Matsuda K, Yamazaki M, Hayashi R. Purification and some properties of ketone reductase forming an active metabolite of sodium 2-[4-(2-oxocyclopentylmethyl)-phenyl]propionate dihydrate (loxoprofen sodium), a new anti-inflammatory agent, in rabbit liver cytosol. *Chem Pharm Bull (Tokyo)*. 1984; 32(3):1040–1048. DOI: 10.1248/cpb.32.1040 [PubMed: 6744478]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

FIGURE 1.

(a) Ion chromatograms of loxoprofen, *trans*-OH form of loxoprofen, and *cis*-OH form of loxoprofen in human liver. (b) *CBR1* mRNA expression in liver samples (n = 24). (c) CBR1 protein content in liver samples (n = 24). Inset shows a representative immunoblot for cytosolic CBR1, GAPDH, and the recombinant CBR1 standards. (d) Impact of *CBR1* rs9024 genotype status on loxoprofen reductase activity in liver tissue (n = 24), and (e) lymphoblastoid cell lines (n = 9). Each symbol represents the average of individual samples analyzed in triplicates. Horizontal lines indicate group means.

Table 1

LC-MS method for the simultaneous determination of loxoprofen, *trans*-OH form of loxoprofen, and *cis*-OH form of loxoprofen.

LC-MS Method				
HPLC	Agilent 1260			
Mass spectrometer	Agilent 6530 Series Accurate-Mass Quadrupole TOF			
Mobile Phase A	90:10 water/acetonitrile			
Mobile Phase B	10:90 water/acetonitrile			
Additives	0.1% (v/v) formic acid and 5 mM ammonium formate			
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)	Flow Rate (mL/min)
	0 – 3	100	0	0.1
	3 – 20	62.5	37.5	0.5
	20 – 27	0	100	0.5
	27 – 35	100	0	0.5
Column	Gemini C18 reversed-phase column (5 µm, 4.6 mm × 50 mm)			
Injection Volume (µL)	10			