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Carbamazepine 10,11-epoxidation in human liver microsomes: influence of the *CYP3A5*3* polymorphism

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Carbamazepine (CBZ) is a commonly prescribed antiepileptic drug, and is mainly metabolized to 10,11-CBZ epoxide in humans. Its biotransformation is catalyzed by cytochrome P450 (CYP) enzymes, with the predominant isoforms being CYP3A4 and CYP3A5. In the present study, the effects of the *CYP3A5*3* (rs776746) polymorphism on CBZ 10,11-epoxidation in human liver microsomes genotyped as *CYP3A5*3* were examined using a kinetic analysis. The kinetics for CBZ 10,11-epoxidation fit the Hill model with *n* of approximately 1.9–2.1 in all liver microsomes of the wild-type (*CYP3A5*1/*1*) and heterozygous (*CYP3A5*1/*3*) and homozygous (*CYP3A5*3/*3*) variants. The S_{50}, V_{max}, and CL_{max} values of wild-type liver microsomes were 263–327 μM, 793–1590 pmol/min/mg protein, and 1.51–2.95 μL/min/mg protein, respectively. The V_{max} and CL_{max} values of liver microsomes of the heterozygous variant were approximately 15–40% those of wild-type liver microsomes. On the other hand, the V_{max} and CL_{max} values of liver microsomes of the homozygous variant were more similar to those of the wild-type than the heterozygous variant. These results suggest that the *CYP3A5*3* polymorphism has a negligible effect on CBZ 10,11-epoxidation in an *in vitro* system using human liver microsomes.

1. Introduction

Carbamazepine (CBZ, Fig. 1) is a commonly used anticonvulsant for the treatment of partial and generalized tonic-clonic seizures (Tolou-Ghamari et al. 2013; Kenyon et al. 2014). It is also prescribed for the treatment of psychiatric disorders such as schizophrenia and bipolar disorder (Simhandl and Meszaros 1992; Evins 2003). In humans, CBZ is extensively metabolized in the liver, with less than 5% of the oral dose being excreted as the unchanged form in urine (Eichelbaum et al. 1985). Previous studies reported that CBZ is primarily metabolized to CBZ 10,11-epoxide (Fig. 1), a pharmacologically active metabolite, by cytochrome P450 (CYP) enzymes, and epoxidation is mainly catalyzed by CYP3A subfamily isoforms such as CYP3A4 and CYP3A5 (Kerr et al. 1994; Huang et al. 2004). CBZ 10,11-epoxide is further metabolized by epoxide hydrolase to CBZ 10,11-diol, which is excreted in urine (Tybring et al. 1981; Kerr et al. 1994; Bu et al. 2004).

CYP3A5 has been suggested to represent more than 50% of total CYP3A in some individuals, and substrate specificity for CYP3A5 significantly overlaps that for CYP3A4 (Huang et al. 2004; Kuehl et al. 2001; Williams et al. 2002). These findings indicate that CYP3A5 is quantitatively and functionally important in relation to total CYP3A isoforms, and may play a crucial role in CBZ 10,11-epoxidation. Furthermore, a genetic polymorphism of *CYP3A5* has been reported, and several variants have been identified. The *CYP3A5*3* allele (rs776746) produced by a splicing defect of mRNA has been found to decrease CYP3A5 enzymatic activity (Kuehl et al. 2001; Lamba et al 2002), and this allele has been detected in all ethnic populations at frequencies of 73% of East Asians, 60% of South Asians, 32% of Blacks, 90–93% of Caucasians, and 63% of Hispanics (Xie et al. 2004). Previous studies indicated that the polymorphism of *CYP3A5*3* is associated with CBZ pharmacokinetics in East Asians such as Japanese, Chinese, and Korean epileptic patients (Seo et al. 2006; Park et al. 2009; Zhu et al. 2014).

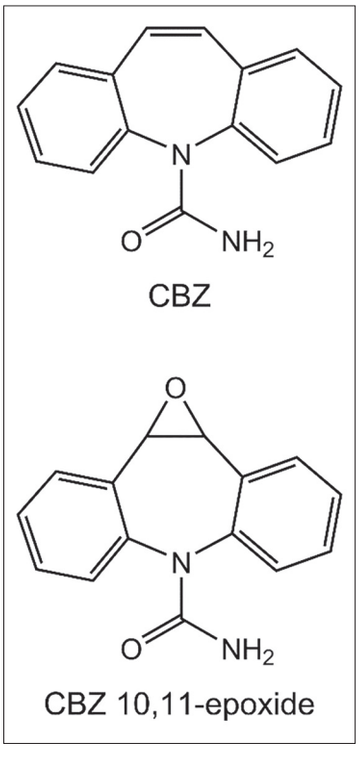

Fig. 1: Chemical structures of CBZ and CBZ 10,11-epoxide

Based on the findings of previous *in vivo* studies, the relationship between the *CYP3A5*3* polymorphism and CBZ metabolism remains controversial and may not be clinically apparent. Furthermore, there is no information from *in vitro* studies on the effects

Table 1: Pooled and individual human liver microsomes used in this study

Donor	Genotype	Sex	Age	Race	Total P450 (pmol/mg protein)	Enzymatic activity (pmol/min/mg protein)		Protein level[c] (pmol/mg protein)	
						CYP2C8[a]	CYP3A4[b]	CYP3A4	CYP3A5
Pooled human liver microsomes									
P1		Female/Male	26–66	Caucasian, African-American, Hispanic, Asian	360	240	5700	86	13
Individual human liver microsomes									
W1	*CYP3A5*1*1*	Male	56	African-American	220	140	5800	170	13
W2	*CYP3A5*1*1*	Female	63	African-American	500	190	11500	320	35
W3	*CYP3A5*1*1*	Male	26	African-American	440	400	4100	110	17
M1	*CYP3A5*1*3*	Hispanic	57	Hispanic	100	42	1300	19	9
M2	*CYP3A5*1*3*	Asian	66	Asian	110	44	1600	23	13
M3	*CYP3A5*3*3*	Caucasian	54	Caucasian	300	210	13600	120	ND

[a]paclitaxel 6α-hydroxylase. [b]testosterone 6β-hydroxylase. [c]Western blot analysis. ND, not detected.

of the *CYP3A5*3* allele on CBZ 10,11-epoxidation. Therefore, the purpose of the present study was to identify whether the *CYP3A5*3* polymorphism affects the metabolism of CBZ. In order to achieve this, the kinetics of CBZ 10,11-epoxidation were examined using human liver microsomes genotyped as *CYP3A5*3*.

2. Investigations and results

2.1. General properties of CBZ 10,11-epoxidation in human liver microsomes

The activities of CBZ 10,11-epoxidation in human liver microsomes were assessed by measuring the formation of CBZ 10,11-epoxide using HPLC. Donor information on pooled and genotyped individual liver microsomes and their enzymatic activities and protein levels of CYP2C8, CYP3A4, and/or CYP3A5 as well as sex, age, and race are listed in Table 1. The activity of CBZ 10,11-epoxidation in pooled liver microsomes (P1) at 300 µM CBZ was 350 pmol/min/mg protein. A typical HPLC chromatogram is shown in Fig. 2.

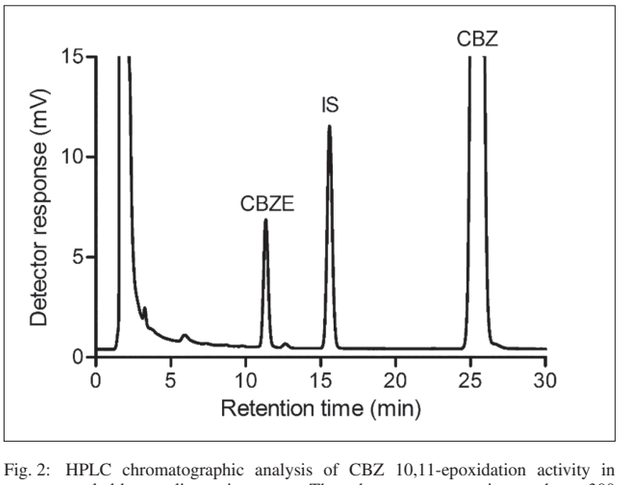

Fig. 2: HPLC chromatographic analysis of CBZ 10,11-epoxidation activity in pooled human liver microsomes. The substrate concentration used was 300 µM. CBZE, CBZ 10,11-epoxide; IS, internal standard (*S*-mephenytoin)

2.2. Kinetics for CBZ 10,11-epoxidation by human liver microsomes

Kinetic analyses on CBZ 10,11-epoxidation were performed using pooled and individual human liver microsomes. The plots (V-[S] and V-V/[S] plots) of the kinetics of liver microsomes genotyped as *CYP3A5*1/*1* (W1), *CYP3A5*1/*3* (M1), and *CYP3A5*3/*3* (M3) as well as pooled liver microsomes (P1) are shown in Fig. 3. The calculated kinetic parameters of all liver microsomes examined are summarized in Table 2. The kinetics fit the Hill model

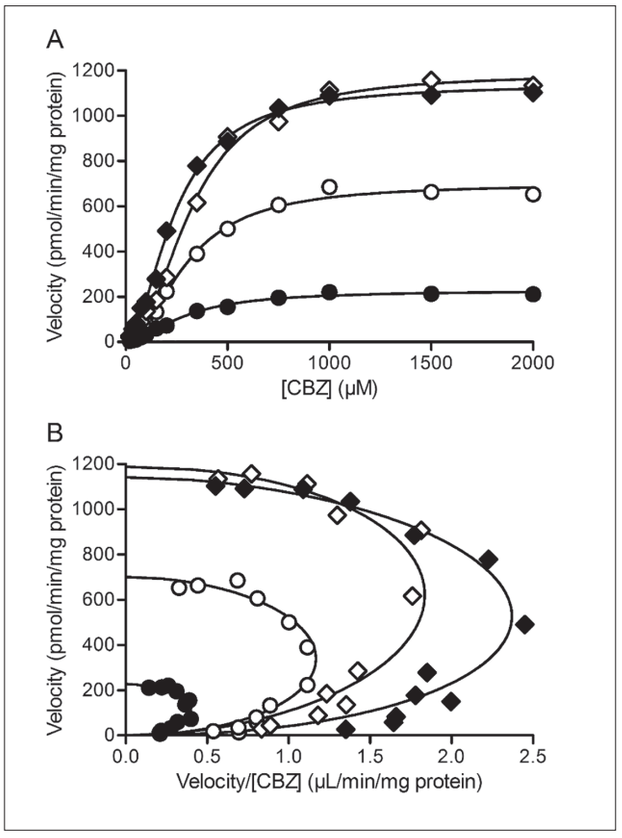

Fig. 3: Kinetics for CBZ 10,11-epoxidation by human liver microsomes. Substrate concentrations were 2–2000 µM. Each point represents the mean of three separate experiments. ™, P1; ¯, W1; ˜, M1; u, M3; (A) V-[S] plots; (B) V-V/[S] plots

with n of approximately 1.9–2.1 in all liver microsomes. The S_{50}, V_{max}, and CL_{max} values of P1 were 307 µM, 703 pmol/min/mg protein, and 1.17 µL/min/mg protein, respectively. Wild-type (*CYP3A5*1/*1*) liver microsomes (W1, W2, and W3) had S_{50}, V_{max}, and CL_{max} values of 263–327 µM, 793–1590 pmol/min/mg protein, and 1.51–2.95 µL/min/mg protein, respectively. In liver microsomes (M1 and M2) of the heterozygous variant (*CYP3A5*1/*3*), V_{max} and CL_{max} values were approximately 15–40% those of wild-type liver microsomes, whereas S_{50} values were similar to those of wild-type liver microsomes. The kinetics for liver microsomes (M3) of the homozygous variant (*CYP3A5*3/*3*) were analyzed further. Kinetic parameters such as S_{50}, V_{max}, and CL_{max} values were more similar to those of liver microsomes of the wild-type than the heterozygous variant.

Table 2: Kinetic parameters for CBZ epoxidation by human liver microsomes

Donor	S_{50} (μM)	V_{max} (pmol/min/mg protein)	n	CL_{max} (μL/min/mg protein)
Pooled human liver microsomes				
P1	307 ± 33	703 ± 92	1.97 ± 0.31	1.17 ± 0.29
Individual human liver microsomes				
W1	327 ± 23	1190 ± 20	2.12 ± 0.35	1.85 ± 0.14
W2	270 ± 13	1590 ± 90	2.03 ± 0.12	2.95 ± 0.22
W3	263 ± 10	793 ± 33	1.97 ± 0.09	1.51 ± 0.05
M1	278 ± 11	226 ± 36	1.81 ± 0.12	0.41 ± 0.05
M2	246 ± 46	296 ± 34	1.90 ± 0.08	0.63 ± 0.11
M3	242 ± 23	1140 ± 80	1.87 ± 0.25	2.39 ± 0.24

Each value represents the mean ± SD of three separate experiments.

3. Discussion

CBZ is a commonly used anticonvulsant in the treatment of epilepsy (Tolou-Ghamari et al. 2013; Kenyon et al. 2014). It undergoes almost complete hepatic biotransformation, and 10,11-epoxide is the principal and active metabolite. Epoxidation has been reported to be catalyzed by CYP3A4, CYP3A5 and CYP2C8, and that CYP3A4 and CYP3A5 especially play the important role (Kerr et al. 1994; Huang et al. 2004). Furthermore, CYP3A5 has quantitatively and functionally been suggested to be the major isoform among the CYP3A subfamily, and a genetic polymorphism has been identified. Among *CYP3A5* alleles, *CYP3A5*3* has been detected in all ethnic populations at high allele frequencies of 32–93% (Xie et al. 2004). Therefore, the effects of the *CYP3A5*3* polymorphism on the metabolism of CBZ need to be clarified for personalized medicine therapy. In the present study, CBZ 10,11-epoxidation was examined in human liver microsomes genotyped as *CYP3A5*3*.

In order to obtain basic information on CBZ 10,11-epoxidation in an *in vitro* system, a kinetic analysis was performed using pooled liver microsomes. The kinetic curves on V-[S] and V-V/[S] plots were sigmoid and hook, respectively, suggesting positive cooperativity. Sakamoto et al. (2013) recently reported that CBZ 10,11-epoxidation in human liver microsomes exhibited sigmoidal kinetics with n of 2.0, and that S_{50} and V_{max} values were 360 μM and 460 pmol/min/mg protein, respectively. Our results from the kinetic analysis of CBZ 10,11-epoxidation were consistent with these findings. The oxidative metabolism of benzodiazepines such as triazolam, flunitrazepam, and diazepam by human liver microsomes has also been reported to exhibit sigmoidal kinetics (Rawden et al. 2005). Based on the present results and previous findings, the CYP -mediated metabolism of drugs with seven-membered rings in humans may be allosterically activated in an *in vitro* system.

Kinetic analyses on CBZ 10,11-epoxidation by individual liver microsomes genotyped as *CYP3A5*3* were subsequently performed. The kinetic model followed the Hill equation in the wild-type and heterozygous and homozygous variants. Although a marked inter-individual difference was not noted in the S_{50} value among liver microsomes examined from the donor, the V_{max} and CL_{max} values of the heterozygous variant were less than 50% those of the wild-type. In contrast to our expectations, the V_{max} and CL_{max} values of the homozygous variant were similar to those of the wild-type; however, commercially available liver microsomes were only obtained from one donor.

In order to investigate the possibility of inconsistencies between the present results and previous *in vivo* findings (Park et al. 2009; Seo et al. 2006; Zhu et al. 2014), correlations between CBZ 10,11-epoxidation activity (V_{max} value) and CYP (CYP2C8 and CYP3A4) activities and protein levels in human liver microsomes described in Table 1 were validated. Correlations were observed between CBZ 10,11-epoxidation activity and CYP3A4 (testosterone 6β-hydroxylase) activity ($r^2 = 0.71$, $p < 0.05$) and protein level ($r^2 = 0.89$, $p < 0.01$). However, a correlation was not found between CBZ 10,11-epoxidation activity and CYP3A5 protein level ($r^2 = 0.22$, $p = 0.29$) or CYP2C8 (paclitaxel 6α-hydroxylase) activity ($r^2 = 0.15$, $p = 0.39$). These results suggest that the contribution of CYP3A5 and CYP2C8 to CBZ 10,11-epoxidation activity is markedly lower than that of CYP3A4; however, several *in vivo* studies reported the importance of CYP3A5 in the metabolism of CBZ. Additionally, the *CYP3A5*3* polymorphism does not appear to have markedly affected CBZ 10,11-epoxidation. Further *in vitro* studies on inter-individual differences in CBZ metabolism using human liver microsomes from a larger number of donors are needed in order to demonstrate the effects of *CYP3A5* including the *CYP3A5*3* polymorphism.

In conclusion, CBZ 10,11-epoxidation in human liver microsomes genotyped as *CYP3A5*3* was examined using a kinetic analysis. The kinetics for CBZ 10,11-epoxidation fit the Hill model in the wild-type and heterozygous and homozygous variants. No marked inter-individual differences were observed in the S_{50} value among liver microsomes examined from the donor. The V_{max} and CL_{max} values of the heterozygous variant were less than 50% those of the wild-type, whereas the V_{max} and CL_{max} values of the homozygous variant were similar to those of the wild-type. These results suggest that the *CYP3A5*3* polymorphism has a negligible effect on CBZ 10,11-epoxidation in an *in vitro* system using human liver microsomes.

4. Experimental

4.1. Materials

CBZ was purchased from Sigma-Aldrich (St Louis, MO, USA). CBZ 10,11-epoxide and *S*-mephenytoin were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Human liver microsomes of pooled (P1) and individual genotyped *CYP3A5*3* (*CYP3A5*1/*1* (wild-type): W1, W2, and W3; *CYP3A5*1/*3* (heterozygous variant): M1 and M2; *CYP3A5*3/*3* (homozygous variant): M3) were obtained from Corning (Corning, NY, USA). β-NADPH was obtained from Oriental Yeast (Tokyo, Japan). All other chemicals and reagents used were of the highest quality commercially available.

4.2. Assay for CBZ 10,11-epoxidation activity

CBZ 10,11-epoxidation activities in human liver microsomes were assessed according to the method of Sakamoto et al. (2013) with some modifications. The incubation mixture contained CBZ (2.0–2000 μM), liver microsomes (500 μg protein/mL), and 1000 μM β-NADPH in a final volume of 200 μL of 50 mM potassium phosphate buffer (pH 7.4). CBZ was dissolved in methanol. The final concentration of methanol in the incubation mixture was 1.0% (v/v). After a preincubation at 37 °C for 2 min, the reaction was initiated by the addition of β-NADPH. An incubation was performed at 37 °C for 30 min. The reaction was terminated by the addition of 200 μL of ice-cold acetonitrile, spiked with 2000 pmol *S*-mephenytoin as an internal standard, and then vortexed. Samples were centrifuged at 12000 × *g* at 4 °C for 10 min. The supernatant was filtered with a polytetrafluoroethylene membrane filter (0.45 μm), and 10 μL of the filtrate was subjected to HPLC with an Inertsil ODS-SP column (5.0 μm, 3.0 mm i.d. × 150 mm; GL Sciences, Tokyo, Japan). The column was maintained at 40 °C. CBZ 10,11-epoxide was isocratically eluted with 0.1% phosphoric/acetonitrile (78:22, v/v) at a flow rate of 0.4 mL/min. UV detection was performed at 210 nm. Standard curve samples spiked with CBZ 10,11-epoxide were prepared in the same manner as incubation samples. Under these conditions, the retention times of CBZ 10,11-epoxide, CBZ, and the internal standard were 11.3, 25.4, and 15.6 min, respectively.

4.3. Data analysis

Kinetic parameters (S_{50}, V_{max}, and n) for CBZ 10,11-epoxidation activities in human liver microsomes were calculated by constructing V-[S] and V-V/[S] plots using SigmaPlot v13 software (Systat Software, San Jose, CA, USA). The kinetic profile was estimated from the respective coefficient of determination and/or Akaike's information criterion values for the Michaelis-Menten, isoenzyme, substrate inhibition, and Hill equations. The *in vitro* clearance value followed by the Hill equation was CL_{max} $(V_{max}/S_{50}{}^{*}(n\text{-}1)/(n(n\text{-}1)^{1/n})$. All values are expressed as the mean±SD of three separate experiments.

Conflict of interest: The authors have no duality of interest to declare.

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