Effect of Gemfibrozil on the Metabolism of Brivaracetam in vitro and in Human Subjects

J.M. Nicolas, H. Chanteux, M. Rosa, S. Watanabe, A. Stockis

UCB Pharma, Braine-l’Alleud, Belgium (JMN, HC, MR, SW, AS)
Running Title: Effect of gemfibrozil on brivaracetam disposition

Corresponding author:

Jean-Marie Nicolas, UCB Pharma, Chemin du Foriest, Braine-l’Alleud B-1420, Belgium (e-mail Jean-Marie.Nicolas@ucb.com).

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Abbreviations: AUC, area under the plasma curve; BRV, brivaracetam; BRV-AC, carboxylic acid derivative of BRV; BRV-OH, hydroxylated metabolite of BRV; BRV-OHAC, hydroxy acid metabolite of BRV; CL/F, oral plasma clearance; Cmax, maximum plasma concentration; CYP, cytochrome P450; fe, percentage of the dose excreted in urine; tmax, time to Cmax
Abstract

Brivaracetam (BRV) is a new high affinity synaptic vesicle protein 2A ligand in Phase III for epilepsy. Initial studies suggested that the hydroxylation of BRV into BRV-OH is supported by CYP2C8. Other metabolic routes include hydrolysis into a carboxylic acid derivative (BRV-AC), which could be further oxidized into a hydroxy-acid derivative (BRV-OHAC). The aim of the present study was to investigate the effect of gemfibrozil (CYP2C9 inhibitor) and its 1-O-β-glucuronide (CYP2C8 inhibitor) on BRV disposition both in vivo (healthy participants) and in vitro (human liver microsomes and hepatocytes). In a two-period randomized crossover study, 26 healthy male participants received a single oral dose of 150 mg BRV alone or at steady-state of gemfibrozil 600 mg bid. Gemfibrozil did not modify plasma and urinary excreted BRV, BRV-OH or BRV-AC. The only observed change was a modest decrease (ca. -40%) in plasma and urinary BRV-OHAC. In human hepatocytes and/or liver microsomes, gemfibrozil potently inhibited the hydroxylation of BRV-AC into BRV-OHAC (Ki = 12 µM) while having a marginal effect on BRV-OH formation (Ki ≥ 153 µM). Gemfibrozil-1-O-β-glucuronide had no relevant effect on both reactions (Ki > 200µM). In conclusion, gemfibrozil did not influence the pharmacokinetics of BRV and its hydroxylation into BRV-OH. Overall, in vitro and in vivo data suggest that CYP2C8 and CYP2C9 are not involved in BRV hydroxylation while hydroxylation of BRV-AC to BRV-OHAC is likely to be mediated by CYP2C9.
INTRODUCTION

Brivaracetam (BRV, (2S)-2-[(4R)-2-oxo-4-propylpyrrolidinyl] butanamide) is an investigational compound that displays a high and selective affinity for SV2A, a protein involved in synaptic vesicle exocytosis and neurotransmitter release (Gillard, et al., 2011). In addition, BRV has inhibitory effects on voltage-dependent sodium currents (Zona, et al., 2010). In adequate well-controlled trials completed to date, adjunctive BRV demonstrated efficacy and good tolerability in adults with focal epilepsy (French, et al., 2010; Werhahn, et al., 2010; van Paesschen and Brodsky, 2007).

BRV exhibits a linear and predictable pharmacokinetic profile with rapid and complete absorption after oral administration, low plasma protein binding and 7-8 h elimination half-life (Sargentini-Maier, et al., 2007; Sargentini-Maier, et al., 2008; Rolan, et al., 2008). BRV is mainly cleared by metabolism. The major pathway involves hydrolysis of the acetamide side chain giving rise to a carboxylic acid derivative BRV-AC. The latter reaction has a wide tissue distribution, and involves amidase rather than cytochrome P-450 (CYP). In addition, both BRV and BRV-AC can be hydroxylated to BRV-OH and BRV-OHAC, respectively. Up to 97% of an oral dose is eliminated in the urine, with 9, 16, 34, and 15 % recovered as BRV, BRV-OH, BRV-AC, and BRV-OHAC, respectively (for complete structure determinations, see Sargentini-Maier, et al., 2008).

Initial in vitro phenotyping assays suggested that BRV hydroxylation into BRV-OH was primarily supported by CYP2C8 with some involvement of other isoforms (Whomsley, et al., 2007). CYP2C8 accounts for 6% of total CYP (Rostami-Hodjegan...
and Tucker, 2007) and is involved in the metabolism of many endogenous substances (e.g., arachidonic acid and retinoid acid) and clinically important drugs. Its activity has a wide intraindividual variability probably resulting from genetic polymorphism and drug-mediated induction or inhibition (Lai, et al., 2009). Altered pharmacokinetics of the CYP2C8 substrate cerivastatin was associated with potential fatal rhabdomyolysis which led to the withdrawal of the drug from the market in 2001 (Farmer, 2001). This finding and other severe drug interactions focused attention on CYP2C8 which is now listed in the updated version of the US Food and Drug Administration guidance document discussing drug interaction studies (Huang, et al., 2008).

Gemfibrozil is a lipid-lowering agent found to be a potent in vitro inhibitor of CYP2C9 (Ki 5.8 µM) with much weaker effect on the other isoforms (Ki of 24, 69, 82, >300 µM on CYP2C19, CYP2C8, CYP1A2 and CYP3A4 respectively) (Hinton, et al., 2008; Wen, et al., 2001). Contrasting with the in vitro findings, gemfibrozil-mediated pharmacokinetic interactions in vivo are mostly reported with CYP2C8 substrate drugs as exemplified by cerivastatin (5.6-fold increase in parent drug AUC) (Backman, et al., 2002), montelukast (4.5-fold) (Karonen, et al., 2010), atorvastatin (1.4-fold) (Whitfield, et al., 2011), pioglitazone (3.4-fold) (Deng, et al., 2005), loperamide (2.9-fold) (Niemi, et al., 2006), rosiglitazone (2.3-fold) (Niemi, et al., 2003), and repaniglide (7.0-fold) (Tornio, et al., 2008). For a large part, these interactions observed in vivo are not caused by gemfibrozil itself but by its 1-O-β-glucuronide metabolite, which is a specific CYP2C8 mechanism-based inhibitor with a K_i of 20-52 µM and a k_{inact} of 0.21 min^{-1} (Ogilvie, et al., 2006; Jenkins, et al., 2011). The glucuronide provides plasma peak concentrations close to the parent drug (Tornio, et al., 2008), shows lower plasma protein binding (88.5 and 99.4%,...
respectively)(Shitara, et al., 2004), and is reported to accumulate in the liver (Sabordo, et al., 1999). Considering all these properties, the average unbound hepatic concentrations for unchanged gemfibrozil and for its glucuronide after a standard 600 mg gemfibrozil dose were estimated to be 3 and 89 µM, respectively (Hinton, et al., 2008). These figures together with the \textit{in vitro} inhibitory constants reported above predict massive inhibition of CYP2C8-mediated reactions (via the glucuronide) with a more modest effect on CYP2C9 (via parent drug). The FDA has recommended using gemfibrozil as a model CYP2C8 inhibitor for \textit{in vivo} drug interaction studies (Huang, et al., 2007).

The present study investigated the \textit{in vitro} and \textit{in vivo} effects of gemfibrozil on the pharmacokinetics of BRV and its biotransformation into BRV-OH, BRV-AC, and BRV-OHAC.
METHODS

Pharmacokinetic Interaction Study in Healthy Participants

Study Design and Participants

This was a single-center, open-label, randomized, 2-sequence, 2-period, 2-treatment crossover study in 26 male healthy participants, who received the following 2 treatments: (A) a single dose of BRV 150 mg (as 3x50mg tablets, UCB Pharma SA, Brussels, Belgium); and (B) gemfibrozil 600 mg every 12 hours for 7 days (Gevilon, Pfizer Pharma GmbH, Berlin, Germany), with a single dose of BRV 150 mg given on the fourth day of gemfibrozil dosing. There were at least 14 days between the end of dosing for the first period and the start of dosing for the second period. Gemfibrozil was administered daily at 7am and 7pm, and BRV was administered at 8am. Meals were served half an hour after gemfibrozil intake except on the morning of BRV administration when fasting was maintained until four hours post BRV-dosing.

Mandatory confinement was from the evening before the first administration until the morning of day 4 for treatment A; for treatment B from the evening before the first administration until the morning of day 1 and from the evening of day 3 until the morning of day 7, and any additional confinement was at the discretion of the investigator or at the request of the individual subject. The first morning dose of gemfibrozil (day 1) was taken at the clinical center, and the subjects were instructed to take the remaining tablets until the morning tablet of day 3 at home. During the non-confinement period, the investigator’s staff gave phone calls to every subject at the appropriate times. The dates and times of intake were recorded in personal diaries and in the case record forms. From the evening of day 3 until release from
the unit in the morning of day 8, subjects were confined again and medication was given under supervision.

Inclusion and exclusion criteria

To enter the study, individuals were required to have a body mass index between 18 and 28 and were required to be in good health, as determined by medical history, physical examination, vital signs, electrocardiogram (ECG), and clinical laboratory measurements. All subjects were males aged 18 to 55 years. Subjects with hepatic, renal or gastrointestinal dysfunction or any other significant medical condition were excluded from the study. Subjects were also excluded if they had donated blood or participated in a drug study during the preceding two months. The consumption of grapefruit and grapefruit-derived products was prohibited from 14 days prior to the study. Alcohol, xanthine-containing preparations (coffee, chocolate, tea, cola) and herbal therapies (including St John’s Wort) were also prohibited from 48 h prior to each drug treatment period.

The final protocol and informed consent documentation were reviewed and approved by the Medical Ethics Committee of the Faculty of Medicine of the University of Liege (Belgium) prior to the start of any study procedures. The study was performed in accordance with Good Clinical Practice regulations and Declaration of Helsinki.

Blood Sampling

Venous blood samples for pharmacokinetic analysis were drawn at the following times on the days that BRV was administered: pre-dose, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 6, 9, 12, 24, 36, 48 and 72 h post-dose. Blood samples were collected in tubes
containing lithium heparin, and the plasma was separated in a refrigerated centrifuge during 10 min at 1500g within 30 minutes after sampling. The samples were stored at –20°C prior to analysis.

Blood samples were taken for determination of gemfibrozil trough levels before the morning dose on Days 4, 5, 6 and 7. The corresponding plasma samples were stored at -80°C prior to analysis.

All urine emissions were collected in the following time intervals: 0-12, 12-24, 24-48 and 48-72 h after each BRV administration. They were stored in separate, labeled vessels and were kept refrigerated at ca 4-8°C until each fraction was completed. The total urine collected in each time interval was mixed thoroughly and the total volume was recorded. Aliquots were stored at ca -20°C prior to analysis.

Determination of Analyte Concentrations

BRV and its three metabolites (BRV-OH, BRV-AC, BRV-OHAC) were determined in plasma and urine using a validated LC-MS/MS method (Sargentini-Maier, et al., 2012). The quantification range was 10 to 2000 ng/mL in plasma and 100 to 20000 ng/mL in urine for the parent compound, and 2 to 2000 ng/mL in plasma and 20 to 20000 ng/mL in urine for the metabolites. Metabolites concentrations were expressed in μg-equivalents of brivaracetam. Intermediate precision was ≤13% and relative error was 8% or less for all analytes. Gemfibrozil was determined by Parexel International (Poitiers, France) using a proprietary HPLC method with fluorescence detection, with a lower quantification limit of 100ng/mL.

Pharmacokinetics
The following pharmacokinetic parameters were derived for BRV and each metabolite: Cmax (maximum observed plasma concentration), tmax (time to Cmax), AUC(0-t) (AUC from time 0 to the last quantifiable concentration), AUCinf (AUC from time 0 extrapolated to infinity), t1/2 (apparent terminal half-life) and fe (percentage of the dose excreted in the urine from time 0 to 72 h). CL/F (oral plasma clearance) was derived for BRV only. Values for pharmacokinetic parameters were determined using WinNonlin 4.0.1 (Pharsight Corp., Mountain View, CA, USA) with the linear trapezoidal method for calculation of AUC values. AUCinf was calculated as AUC(0-t) + Ct/λz, where Ct is the last quantifiable concentration (observed) and λz is the slope of linear regression of the natural logarithm (ln) of concentration against time during the terminal phase of the concentration–time profile. Apparent terminal t1/2 was calculated as ln(2)/λz. CL/F was obtained as the ratio of dose to AUCinf. Actual sample collection times were used for the pharmacokinetic analysis. Concentrations below the limit of quantification were not used.

For gemfibrozil, the morning trough levels on days 4, 5, 6 and 7 were reported.

**Statistical Analysis**

To assess the effects of gemfibrozil on the pharmacokinetics of BRV and its metabolites, natural log-transformed Cmax, AUC(0-t), AUCinf, t1/2 and Ae(0-72h) values were analyzed with a crossover analysis of variance (ANOVA) model consisting of subject, period, and treatment as fixed effects and subject within sequence as a random effect. Least squares mean differences between test (BRV with gemfibrozil) and reference (BRV alone) treatments and corresponding 90% confidence intervals (CI) were estimated from this model and back-transformed to
derive estimates of the mean ratios (MR, test/reference) of least squares geometric means (LSM) and the 90% CIs for these ratios.

Lack of pharmacokinetic interaction of gemfibrozil on BRV and its metabolites would be concluded if the 90% CIs for the MR (test/reference) of LSMs were fully contained within the 80% to 125% interval for the primary parameters Cmax, AUC(0-t) and AUCinf.

The sample size calculation was based on a type-I error of 5% and on an intra-subject coefficient of variation of 23%, based on previous studies. It was estimated that 24 subjects would provide at least 90% power for the ratio of pharmacokinetic parameters to be within the range of 0.80-1.25. In order to account for dropouts, 26 subjects were enrolled.

All statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA) or StatXact version 7.0 (Cytel Software, Cambridge, MA, USA)

**Chemicals and Reagents**

BRV (2S-2-[(4R)-2-oxo-4-propylpyrrolidin-1-yl]butanamide; brivaracetam), and its three metabolites BRV-OH, BRV-AC and BRV-OHAC (Figure 1) were synthesized at UCB (Braine-l'Alleud, Belgium). Gemfibrozil was obtained from Sigma-Aldrich (Bornem, Belgium) and gemfibrozil-1-O-β-glucuronide from Toronto Research Chemicals (Ontario, Canada). NADPH-regenerating system was obtained from BD Gentest (Woburn, USA). All other chemical reagents were of analytical grade.

Human liver microsomes (HLM, pool of 50 donors) were purchased from Xenotech (Lenexa, KS, USA). Cryopreserved human hepatocytes (pool of 10 donors) were obtained from Cellzdirect (Durham, UK).
BRV and BRV-AC were added to the \textit{in vitro} incubates as a solution in potassium phosphate buffer to which gemfibrozil and gemfibrozil-1-\textit{O}-\textit{β}-glucuronide were added as a solution in water/acetonitrile (50/50, v/v) or DMSO, respectively. Final solvent concentration in incubates was \(\leq 1\%\) and 0.2\% for acetonitrile and DMSO, respectively.

\textbf{In Vitro Assays}

Preliminary assays were conducted in HLM and hepatocytes to determine kinetic parameters of BRV and BRV-AC hydroxylation reactions. This was followed by a first inhibition assay to quantify the IC\textsubscript{50} of gemfibrozil on both reactions. All these data (not shown) were combined to set-up the substrate and inhibitor concentration range to explore for proper Ki determination.

\textit{Incubations with human liver microsomes}

All incubations with HLM were carried out in polypropylene containers at ca 37°C in a shaking water bath, with 50 mmol/L potassium phosphate buffer (pH 7.4), containing a NADPH regenerating system [NADP (1.3mmol/L), glucose 6-phosphate (3.3mmol/L), magnesium chloride (3.3mmol/L) and glucose 6-phosphate dehydrogenase (0.4U/mL)]. Incubations were performed in triplicate.

HLM concentration and incubation times were selected in order that initial rate conditions for BRV and BRV-AC hydroxylation were respected (HLM concentrations of 0.5 and 0.2 mg/mL and incubation times of 20 and 30 min, for BRV and BRV-AC, respectively). Both reactions were determined using varying concentrations of substrate (from 0.5 to 6 mM ) and of gemfibrozil (0-750 µM) to allow Ki determination.
Gemfibrozil-1-O-β-glucuronide was examined for its potential to act as a
metabolism-dependent inhibitor of BRV and BRV-AC hydroxylation. Gemfibrozil-1-O-
β-glucuronide (200 µM) was pre-incubated in triplicate with HLM and NADPH
regenerating system for 30 min. After this 30 min pre-incubation, BRV and BRV-AC
(2 and 1.5 mM, respectively) were added and the incubation resumed to measure
residual activity. Incubations were also performed without pre-incubation in order to
evaluate the direct inhibitory potential of gemfibrozil-1-O-β-glucuronide.

Reactions were stopped by ice-cold acetonitrile. The tubes were then thoroughly
mixed using a vortex mixer and centrifuged (ca 10000g, ca 4°C) for 10 min. Clear
supernatants were analyzed by LC-MS/MS.

**Incubations with human hepatocyte suspensions**

Cryopreserved hepatocytes were thawed accordingly the provider’s information.
Viability was assessed by trypan blue exclusion (>90% viability). Incubations
(ca 0.5x10^6 cells/mL) were carried out in polypropylene containers placed on a rotor
agitator, at 37°C, with Hank’s balanced Salt Solution buffer. BRV (1.5-12 mM) was
incubated for 30 min in the presence of 0-2.5 mM gemfibrozil. BRV-AC (0.25-2 mM)
was incubated for 60 min in the presence of 0-30 µM gemfibrozil.

Reactions were stopped by the addition of ice-cold acetonitrile. The tubes were then
thoroughly mixed using a vortex mixer and centrifuged (ca 14,000g, ca 4°C) for
10 min.

**In vitro sample analysis**

The amount of BRV-OH and BRV-OHAC formed was determined by LC-MS/MS.
The HPLC system used was an UPLC Aquity instrument (Waters, Milford, USA)
coupled with a Quattro Premier mass spectrometer. The analytical column was an
Acquity UPLC HSS T3 (50 x 2.1mm, 1.8µm), operated at 30°C. Analyses were performed with a gradient method. Eluent A was water containing 0.1% formic acid and eluent B was acetonitrile containing 0.1% formic acid. Multiple reaction monitoring in positive-ion mode was used for all analytes using electrospray ionization. Data acquisition and the analytical parameters including the selection of ions for each compound were performed by the application software, MassLynx 4.1 (Waters Ltd., Hertfordshire, UK). Deuterated (d6) internal standards (IS) were used for the quantification of both analytes. The mass-to-charge transition (m/z) of precursor ions and product ions for each compound was identified as follows: m/z 229.10 ->184.00 for BRV-OH, m/z 235.27->190.03 for d6-BRV-OH (IS), m/z 230.00 ->184.00 for BRV-OHAC, m/z 236.05->190.06 for d6-BRV-ACOH (IS). The concentrations were determined by the peak area ratio method.

**In vitro data analysis**

Ki values were determined by non-linear regression analysis using Galileo (version 3.0.0.01, Thermo Corporation, USA).
RESULTS

Pharmacokinetic interaction study in healthy participants

Trough plasma levels of gemfibrozil were in the range of 0.8-1.2 µg/mL, in line with literature data (Karonen, et al., 2011).

The plasma-concentration time curves (Figure 2) and pharmacokinetic parameters (Table 1) for BRV, BRV-OH and BRV-AC were similar whether BRV was given alone or in combination with gemfibrozil at steady-state (ie. < 20% changes). The only significant gemfibrozil-induced changes were restricted to the BR-OHAC metabolite. Gemfibrozil decreased BR-OHAC Cmax (-48%), AUCinf (-34%), fraction excreted in urine (-40%), and prolonged its elimination half-life (1.4-fold). All these effects were significant as the corresponding 90% confidence intervals on the ratios were entirely outside of the no effect 80-125% boundaries.

Treatment-emergent adverse events (mild or moderate dizziness and fatigue) were consistent with the known safety profile of BRV. There were no clinically-relevant changes in laboratory tests, vital signs, physical examination and ECG data. One participant was discontinued on the 6th day of gemfibrozil intake during the 1st period, due to the appearance erythematous pruritic rash. Recovery was uneventful. All other participants completed both treatment periods.

Inhibitory effects of gemfibrozil on the in vitro metabolism of BRV

The two hydroxylation reactions, i.e., BRV into BRV-OH and BRV-AC into BR-OHAC, were monitored in human liver microsomes and in hepatocytes using varying substrate and gemfibrozil concentrations (Figure 3). Gemfibrozil weakly inhibited the hydroxylation of BRV into BRV-OH, in either human liver microsomes (Ki(SD) = 153 ± 33 µM) or human hepatocytes (Ki(SD) = 284 ± 74 µM)(Table 2).
Gemfibrozil showed a more pronounced effect on the hydroxylation of BRV-AC into BRV-OHAC with Ki of 11.8 ± 1.8 and 11.5 ± 0.7 µM, in microsomes and hepatocytes respectively.

Non-linear regression analysis resulted in better goodness-of-fit parameters when using different mechanisms of inhibition (mixed, competitive, non-competitive) for the two reactions and test systems. These observed differences were however considered minor and were not further explored.

As explained earlier, the in vivo effects of gemfibrozil on CYP2C8 are mediated by a reactive form of its 1-O-β-glucuronide. Thus, in a subsequent assay, gemfibrozil 1-O-β-glucuronide was tested for its potential to inhibit BRV metabolism, either directly or after prior incubation to allow metabolism-based inhibition (Table 3). The two hydroxylation reactions remained poorly affected by gemfibrozil 1-O-β-glucuronide, irrespective the incubation protocol (≤ 25% inhibition at 200 µM).
DISCUSSION

CYP2C8 was previously identified as the major CYP isoform responsible for the hydroxylation of BRV into BRV-OH. Other contributors (i.e., 2C19, 2B6 and 3A4) were identified but were thought to play a minor role (Whomsley et al, 2007). Only ca. 16% of an oral dose of BRV is excreted as BRV-OH (Sargentini-Maier, et al., 2008). As a consequence, a total inhibition of that pathway would result in <20% increase of parent AUC. However, this figure might be underestimated as BRV-OH could possibly be transformed into BRV-OHAC, or other minor metabolites. The purpose of the present study was to investigate the effects of gemfibrozil, a potent and well recognized 2C8 inhibitor, on the pharmacokinetics and metabolism of BRV.

In healthy participants, 600 mg gemfibrozil twice daily for 7 days did not affect the formation of BRV-OH (i.e., there was no change in its AUC, C_max or fe). Similarly, the pharmacokinetics of parent BRV remained unchanged (i.e., no effect on its AUC, C_max, t_1/2, or fe). Although encouraging for the program, these findings were mostly unexpected. Indeed, after such a dosing regimen of gemfibrozil, the hepatic free concentration of gemfibrozil 1-O-β-glucuronide is estimated at 89 µM (Hinton, et al., 2008). The latter value combined with published values of K_i (20 µM), k_inact (0.21 min^{-1}; maximal rate of inactivation), and k_e (0.0008 min^{-1}; rate constant describing enzyme degradation) predict an almost complete inhibition of CYP2C8-mediated reactions. The impact on the AUC of parent drug can be derived using the equation below (Ogilvie, et al., 2006; Ogilvie, et al., 2006):

\[
\frac{AUC_i}{AUC} = \frac{1}{\left(\frac{km}{fn} + \frac{k_inact}{Ki} + \frac{ke}{1} + \frac{1}{fm}\right)}
\]
where I is the hepatic free gemfibrozil 1-O-β-glucuronide concentration, and fm the fraction of BRV dose eliminated by CYP2C8. On the basis of this simulation, gemfibrozil was predicted to inhibit over 99% of BRV-OH formation with a minimum 19% increase in the AUC of BRV, which contradicts the actual data.

The effect of gemfibrozil on BRV hydroxylation was thus explored in vitro using both NADPH-fortified human liver microsomes and intact hepatocytes. Indeed, the inhibitory potential of gemfibrozil towards CYP2C8 was reported to vary according to the test system used. The inhibition is weaker in microsomes when compared to hepatocytes (IC₅₀ of 120-150 versus 2-64 µM, respectively) (Parkinson, et al., 2010). This difference is due to the ability of hepatocytes to activate gemfibrozil to gemfibrozil 1-O-β-glucuronide through a UGT2B7-mediated reaction (Mano, et al., 2007). The glucuronide metabolite acts as a potent irreversible metabolism-dependent CYP2C8 inhibitor, a property not shared by gemfibrozil itself. In the present work, gemfibrozil inhibited BRV-OH formation with the same, if not lower, potency in hepatocytes than in microsomes. In addition, the measured inhibition in hepatocytes appeared at gemfibrozil concentrations ca. 10-fold higher than those reported to inhibit 2C8 in the same model. A subsequent in vitro assay showed that gemfibrozil 1-O-β-glucuronide was not inhibitory against BRV-OH formation (≤25% inhibition at 200 µM), even after preincubation to allow metabolism-based inhibition to occur.

All together the above in vivo and in vitro findings strongly suggest that BRV-OH formation is not primarily supported by CYP2C8. Several similar reports have been published where in vitro CYP phenotyping data did not match clinical findings, especially within the CYP2C family. The antidiabetic agent glibenclamide is primarily transformed in vitro by CYP3A4 with some minor contribution of CYP2C19 (van
Giersbergen, et al., 2002; Naritomi, et al., 2004; Zhou, et al., 2010). This finding contrasts with clinical data showing that glibenclamide \textit{in vivo} clearance is primarily mediated by CYP2C9 (Niemi, et al., 2002; Yin, et al., 2005). Another example is chlorpropamide which is oxidized \textit{in vitro} by both CYP2C9 and CYP2C19, whereas its \textit{in vivo} disposition is influenced by CYP2C9, but not by CYP2C19 genetic polymorphism (Shon, et al., 2005). Overall, it has been suggested that CYP phenotyping might be particularly difficult for low turnover compounds with multiple CYPs involved (Zhang, et al., 2007).

The initial identification of CYP2C8 as the main isoform supporting BRV-OH formation was primarily driven by chemical inhibition assays (Whomsley et al, 2007). The reaction was most strongly inhibited by quercetin, a frequently used reference CYP2C8 inhibitor (Nebot, et al., 2010). Retrospective literature analysis suggested that quercetin is not strictly specific to CYP2C8 and could also inhibit CYP2C9 and CYP2C19 with IC50 values in the low µmolar range (Zou, et al., 2002). The interpretation of the above-mentioned chemical inhibition assay was further complicated by the large effect observed with the vehicle alone (ca. 50% inhibition with methanol 1% vol/vol). Immunoinhibition assays and incubations with recombinant CYPs were carried out in parallel and revealed the simultaneous involvement of several isoforms, i.e., CYP 2C8, 2C9, 2C19 and 3A4. Further, data obtained with recombinant CYPs were not adjusted for relative CYP abundance, nor for any other correction factors (eg. ISEFF). It can be tentatively hypothesized that all together these technical difficulties led to wrong CYP assignment of BRV hydroxylation.

A standard gemfibrozil dosing regimen is also expected to impact CYP2C9-mediated pathways, although to a much lower extent than CYP2C8.
concentration of unchanged gemfibrozil was estimated to reach 3 µM, close to its Ki on CYP2C9 (5.8 µM)(Hinton, et al., 2008). In vitro/in vivo extrapolation would predict a maximal 34% decrease in a 2C9-mediated reaction (1/(1+I/Ki)), with much less effect, if any, on the other isoforms. Consistent with these predictions, gemfibrozil increases the AUC of some CYP2C9 substrates such as nateglinide (+47%; gemfibrozil coadministered with itraconazole)(Niemi, et al., 2005), glimepiride (+23%)(Niemi, et al., 2001) and rosuvastatin (+88%) (Schneck, et al., 2004). The larger effect on rosuvastatin is thought to be due to additional non-CYP-related mechanisms (ie.. inhibition of OATP1B1-mediated hepatic uptake). The absence of any effect of gemfibrozil on BRV-OH formation in human participants suggest that the reaction does not primarily involve CYP2C9. Supporting this hypothesis, gemfibrozil has a very marginal inhibitory effect on BRV-OH formation in human liver microsomes (Ki of 153 µM where 5.8 µM is reported for CYP2C9-mediated reactions)(Hinton, et al., 2008).

In the present study, the effect of gemfibrozil was restricted to a modest in vitro and in vivo inhibition of a secondary metabolic pathway of BRV, i.e., its hydroxylation into BRV-OHAC. The reaction does not appear to be supported by CYP2C8 since it is not inhibited in vitro by gemfibrozil 1-O-glucuronide. On the other hand, the measured Ki of gemfibrozil (12 µM) on that pathway is in the same range as the published value describing its inhibitory effect on CYP2C9 (5.8 µM), suggesting involvement of this latter isoform in the reaction. In addition, the amplitude of the interaction observed in vivo (ie. 30-40% reduction in BRV-OHAC AUC and excretion) is consistent with the expected effect on a 2C9 reaction (34%, see above).
In conclusion, in healthy participants, gemfibrozil had no effect on the hydroxylation of BRV into BRV-OH. Together with the in vitro inhibition assays, these findings suggest that neither CYP2C8 nor 2C9 are involved in the reaction, which contradicts the previously reported in vitro CYP phenotyping data. The reasons underlying this discrepancy remain to be investigated. Of interest, a recently reported study in healthy Japanese participants suggested the involvement of CYP2C19 in the hydroxylation of BRV. Plasma exposure and excretion of BRV-OH was indeed decreased 10-fold in subjects with CYP2C19 poor metabolizer genotype while the clearance of parent BRV was merely decreased by 30% (Stockis, et al., abstracts of the annual symposium of the American Epilepsy Society, Baltimore, MD, USA, December 2011).

In the present clinical interaction study, the only measurable effect of gemfibrozil was a modest but significant decrease in a secondary metabolic route, i.e., hydroxylation of BRV-AC into BRV-OHAC. Based on the measured in vitro inhibitory constants, CYP2C9 is possibly involved in that reaction. In any case, gemfibrozil did not affect the pharmacokinetic parameters of unchanged BRV suggesting that BRV can be safely coadministered with CYP2C8 or CYP2C9 inhibitors.
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Authorship contributions

Participate in research design: Nicolas, Chanteux, Rosa, Watanabe, Stockis
Conducted experiments: Chanteux, Rosa, Watanabe
Contributed new reagents or analytical tools: Chanteux, Rosa
Performed data analysis: Nicolas, Chanteux, Rosa, Watanabe, Stockis
Wrote or contributed to the writing of the manuscript: Nicolas, Chanteux, Stockis
References


Figure legends

Figure 1. Chemical structures of Brivaracetam and its hydroxy (BRV-OH), acid (BRV-AC) and hydroxyacid (BRV-OHAC) metabolites

Figure 2. Geometric mean (SD) plasma concentrations of BRV (A), BRV-OH (B), BRV-AC (C) and BRV-OHAC (D) in 26 healthy volunteers after a single oral of 150 mg BRV given alone (solid symbols) or with gemfibrozil 600 mg twice daily (open symbols).

Figure 3. Inhibitory effect of gemfibrozil on the hydroxylation of BRV to BRV-OH (A, C) and of BRV-AC to BRV-OHAC (B, D), in human liver microsomes (A, B) and in human hepatocytes (C, D). T. Experimental data are shown as means of triplicate determinations. Curves were obtained by simultaneous nonlinear regression. Gemfibrozil concentrations (µM) are given for each inhibition curve.
Table 1
Geometric mean (CV%) pharmacokinetic parameters of BRV and its three major metabolites in healthy participants after a single oral dose of 150 mg BRV given alone or with gemfibrozil 600 mg twice daily.

<table>
<thead>
<tr>
<th>Variable</th>
<th>BRV alone (n=25)</th>
<th>BRV + Gemfibrozil (n=26)</th>
<th>Mean ratio % (90% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>4.15 (24.3)</td>
<td>4.16 (26.2)</td>
<td>101 (94-108)</td>
</tr>
<tr>
<td>AUC(0-t) (µg.h/mL)</td>
<td>41.3 (14.7)</td>
<td>39.1 (16.6)</td>
<td>95 (93-97)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (µg.h/mL)</td>
<td>41.4 (14.8)</td>
<td>39.2 (16.6)</td>
<td>95 (93-97)</td>
</tr>
<tr>
<td>CL/F (L/h)</td>
<td>3.62 (14.8)</td>
<td>3.83 (16.6)</td>
<td>-</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>8.09 (13.4)</td>
<td>7.64 (14.8)</td>
<td>94 (93-96)</td>
</tr>
<tr>
<td>fe (%)</td>
<td>8.33 (28.8)</td>
<td>8.47 (20.8)</td>
<td>103 (95-112)</td>
</tr>
<tr>
<td><strong>BRV-OH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>0.154 (52.3)</td>
<td>0.183 (49.0)</td>
<td>119 (113-124)</td>
</tr>
<tr>
<td>AUC(0-t) (µg.h/mL)</td>
<td>4.12 (44.9)</td>
<td>4.87 (47.0)</td>
<td>118 (114-122)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (µg.h/mL)</td>
<td>4.18 (44.6)</td>
<td>4.94 (45.4)</td>
<td>118 (114-122)</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>9.58 (13.4)</td>
<td>9.25 (15.5)</td>
<td>97 (94-99)</td>
</tr>
<tr>
<td>fe (%)</td>
<td>12.9 (43.1)</td>
<td>14.6 (46.4)</td>
<td>113 (106-120)</td>
</tr>
<tr>
<td><strong>BRV-AC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>0.244 (20.1)</td>
<td>0.285 (21.8)</td>
<td>117 (108-127)</td>
</tr>
<tr>
<td>AUC(0-t) (µg.h/mL)</td>
<td>3.23 (22.3)</td>
<td>3.55 (22.0)</td>
<td>111 (106-116)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (µg.h/mL)</td>
<td>3.29 (22.5)</td>
<td>3.61 (22.0)</td>
<td>110 (105-116)</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>8.59 (17.9)</td>
<td>7.99 (16.8)</td>
<td>93 (90-96)</td>
</tr>
<tr>
<td>fe (%)</td>
<td>27.2 (20.6)</td>
<td>30.0 (26.3)</td>
<td>111 (102-121)</td>
</tr>
<tr>
<td><strong>BRV-OHAC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>0.0476 (25.9)</td>
<td>0.0247 (44.3)</td>
<td>52 (46-58)</td>
</tr>
<tr>
<td>AUC(0-t) (µg.h/mL)</td>
<td>0.905 (22.9)</td>
<td>0.553 (42.0)</td>
<td>61 (55-67)</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (µg.h/mL)</td>
<td>0.966 (21.8)</td>
<td>0.634 (33.3)</td>
<td>66 (61-71)</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>11.2 (9.2)</td>
<td>15.7 (31.1)</td>
<td>139 (125-155)</td>
</tr>
<tr>
<td>fe (%)</td>
<td>14.2 (19.6)</td>
<td>8.55 (41.9)</td>
<td>60 (53-67)</td>
</tr>
</tbody>
</table>
Table 2
*In vitro* inhibitory effect of gemfibrozil on brivaracetam metabolic pathways in human liver microsomes and hepatocytes. Fitted Ki values are expressed ± standard error.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Human liver</th>
<th>Ki (µM)</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRV hydroxylation into BRV-OH</td>
<td>microsomes</td>
<td>153 ± 33</td>
<td>Mixed ($\alpha$ = 2.95)</td>
</tr>
<tr>
<td></td>
<td>hepatocytes</td>
<td>284 ± 74</td>
<td>Mixed ($\alpha$ = 10.5)</td>
</tr>
<tr>
<td>BRV-AC hydroxylation into BRV-OHAC</td>
<td>microsomes</td>
<td>11.8 ± 1.8</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td>hepatocytes</td>
<td>11.5 ± 0.7</td>
<td>Non competitive</td>
</tr>
</tbody>
</table>
Table 3

*In vitro* inhibitory effect of gemfibrozil 1-O-glucuronide on brivaracetam metabolic pathways in human liver microsomes. Results expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Test system</th>
<th>% Inhibition at 200 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRV hydroxylation into BRV-OH</td>
<td>direct</td>
<td>23 ± 2</td>
</tr>
<tr>
<td></td>
<td>with preincubation</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>BRV-AC hydroxylation into BRV-OHAC</td>
<td>direct</td>
<td>14 ± 3</td>
</tr>
<tr>
<td></td>
<td>with preincubation</td>
<td>16 ± 8</td>
</tr>
</tbody>
</table>
Figure 1

Brivaracetam, BRV

BRV-OH

BRV-AC

BRV-OHAC
Figure 2C
Figure 3B

This graph shows the relationship between BRV-AC concentration (mM) and enzyme activity (V, pmol/min/mg protein). The data points are indicated by different symbols, each corresponding to a specific concentration of an inhibitor. The curves represent the enzyme's activity at various inhibitor concentrations, allowing for the visualization of inhibition kinetics.
Figure 3C

$V$ (pmol/min/10^6 hepatocytes)

$\text{BRV (mM)}$

0 3 6 9 12

0 5 10 15

0 100 300 1000 2500

\[ V = \frac{V_{	ext{max}} [\text{BRV}]}{K_{	ext{m}} + [\text{BRV}]} \]
Figure 3D