Characterization of inhibitory effect of carbapenem antibiotics on the de-conjugation of valproic acid glucuronide

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Running title: Carbapenem irreversibly inactivates de-conjugation of VPA-G

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Abbreviations

VPA  valproic acid
VPA-G  valproic acid glucuronide
VPAGase  putative valproic acid glucuronide de-conjugation enzyme
GABA  γ-aminobutyric acid
CBPM  carbapenem antibiotics
DRPM  doripenem
PAPM  panipenem
MEPM  meropenem
BIPM  biapenem
IPM  imipenem
SL
saccharic acid 1,4-lactone

UGT
uridine 5'-diphosphate glucuronosyl transferase

UDP-GA
uridine 5'-diphosphate glucuronic acid

4-MU
4-methylumbelliferone

4-MUG
4-methylumbelliferyl-β-D-glucuronide

MFA-G
mefenamic acid glucuronide

$k_{\text{inact}}$
the maximum inactivation rate constant

$K'_{\text{app}}$
the apparent dissociation constant

$k_{\text{obs}}$
the apparent inactivation rate constant

$v_{\text{syn}}$
the enzyme synthesis rate

$k_{\text{deg}}$
the enzyme degradation rate constant

$E$
the amount of active enzyme

$E_{\text{ss}}$
the amount of active enzyme at steady state

$E_0$
the trough amount of active enzyme after inactivated by CBPM

$I$
concentration of carbapenem antibiotics

$K_p$
liver-to-blood concentration ratio

$k_{el}$
the elimination rate constant

$V_d$
distribution volume

$MW$
molecular weight

LC-MS/MS
liquid chromatography-tandem mass spectrometry

MRM
multiple reaction monitoring

ESI
electrospray ionization

SD
Sprague-Dawley
Abstract

Serum concentrations of valproic acid (VPA) are markedly decreased by co-administration of carbapenem antibiotics (CBPM). Although inhibition of de-conjugation of VPA-glucuronide (VPA-G) to VPA by CBPM has been proposed as one of the mechanisms to account for this drug-drug interaction, little information is available on the mode of inhibition. In the present study, we characterized the enzyme involved in the de-conjugation of VPA-G by using human and rat liver cytosol. It is suggested that 1) de-conjugation activity inhibited by CBPM may be selective for VPA-G, 2) de-conjugation of VPA-G may be mediated by enzyme(s) other than β-glucuronidase, and 3) the irreversible inactivation may be responsible for the inhibition of de-conjugation of VPA-G by CBPM. Finally, the kinetic parameters for inactivation ($K'_{app}$ and $k_{inac}$) were determined for four CBPMs of diverse structure from *in vitro* experiments. Based on the results of simulation analyses with these parameters and the degradation rate constant of the putative VPA-G de-conjugation enzyme obtained from experiments using rats, it is probable that the de-conjugation enzyme for VPA-G in the liver is rapidly and mostly inactivated by these CBPMs under clinical situations.
Introduction

Valproic acid (VPA) is a widely used drug with antiepileptic activity. By inhibiting γ-aminobutyric acid (GABA) transaminase, VPA elevates the level of GABA and suppresses epileptic seizures (Peterson and Naunton, 2005). Orally administered VPA is almost completely absorbed in the small intestine and predominantly metabolized in the liver by glucuronidation, oxidation and hydroxylation, and then finally excreted into urine and bile. The glucuronidation reaction is mediated by uridine 5’-diphosphate glucuronosyl transferase (UGT), which requires uridine 5’-diphosphate glucuronic acid (UDP-GA) as a cofactor. It has been believed that the glucuronidation of VPA is reversible and VPA-G is de-conjugated to VPA by β-glucuronidase expressed in the whole body and enterobacteria (Williams et al., 1992; Slattum et al., 1995). Because the effective and toxic concentrations of VPA are relatively close and there is a large inter-individual difference in the relation between dose and serum concentration of VPA (Beydoun et al., 1997), its serum concentrations need to be monitored.

Concerning the disposition of VPA, the presence of drug-drug interaction between VPA and carbapenem antibiotics (CBPM) is well established and was initially reported by Nagai et al. (1997). Co-administration of CBPM causes a rapid decrease in serum concentrations of VPA and increases the risk of epileptic seizures (Yamagata et al., 1998). This drug-drug interaction occurs rapidly and continues for as long as several days after withdrawal of CBPM, although the half-life of CBPM is quite short.

Concerning the interaction between VPA and CBPM, the following mechanisms have been proposed: [1] inhibition of intestinal VPA absorption by CBPM (Torii et al., 2001; Torii et al., 2002), [2] interruption of enterohepatic circulation of VPA by CBPM (Kojima et al., 1998), [3] increased partition of VPA into erythrocytes...
by CBPM (Omoda et al., 2005; Ogawa et al., 2006), [4] elevation of UDP-GA levels by CBPM (Yamamura et al., 1999; Yamamura et al., 2000), [5] induction of UGT by CBPM (Mori and Mizutani, 2007), and [6] inhibition of de-conjugation of VPA-glucuronide (VPA-G) by CBPM (Nakajima et al., 2004; Nakamura et al., 2008). However, most of the previously proposed mechanisms (mechanism [1] – [5]) may not solely explain the interaction observed under clinical conditions because of the following reasons: with mechanism [1], the interaction observed after intravenous administration of VPA (Clause et al., 2005; Coves-Orts et al., 2005; Spriet et al., 2007) cannot be accounted for. For mechanism [2], the interaction in bile cannulated rats, where enterohepatic circulation of VPA was negligible (Yamamura et al., 1999), cannot be accounted for. For mechanism [3], the mass balance calculation indicates that the increase in partition of VPA into erythrocytes cannot sufficiently account for the decrease in serum VPA concentration in clinical situations. For mechanisms [4] and [5], it is difficult to explain the selectivity of the interaction between VPA and CBPM, and also the rapid effect of CBPM on the plasma concentrations of VPA. In addition to the previously described mechanisms, Nakajima et al. (2004) showed that the de-conjugation activity of VPA-G in rat liver cytosol was inhibited by doripenem (DRPM), and also demonstrated that de-conjugation clearance of VPA-G calculated from rat \textit{in vivo} studies was significantly decreased during co-administration of DRPM. Nakamura et al. (2008) showed that de-conjugation of VPA-G in human liver cytosol was also completely inhibited by saccharic acid 1,4-lactone (SL), a classical β-glucuronidase inhibitor. However, it is unlikely that β-glucuronidase is the predominant enzyme involved in de-conjugation of VPA-G and the inhibition target of CBPMs that induces decrease in serum concentration of VPA. This is because there has been no report showing such an evident drug-drug interaction with CBPMs other
than that observed between VPA and CBPMs, although β-glucuronidase is involved in de-conjugation of a broad range of glucuronic acid-conjugates.

In the present study, we characterized the de-conjugation activity involving VPA-G in human and rat liver cytosol in a series of in vitro and ex vivo experiments. Firstly, we examined substrate specificity and inhibitor sensitivity. Then, the mode of inhibition by CBPMs was examined. Finally, we simulated time profiles of the activity of VPA-G de-conjugation in human liver after administration of CBPM.
Materials and methods

Chemicals

Human liver pooled cytosol prepared from 150 donors was purchased from BD Biosciences (Bedford, MA). Panipenem (PAPM), meropenem (MEPM), DRPM and biapenem (BIPM) were purchased from Daiichi-Sankyo (Tokyo, Japan), Dainippon-Sumitomo Pharma (Osaka, Japan), Shionogi (Osaka, Japan) and Meiji-Seika Kaisha (Tokyo, Japan), respectively (Fig. 1). VPA, hexanoic acid and mefenamic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). VPA-G was obtained from Toronto Research Chemicals (Ontario, Canada). Bile samples after intravenous infusion of mefenamic acid into rats was used as mefenamic acid glucuronide (MFA-G). Only MFA-G was detected but mefenamic acid was not detected in the collected bile. SL and 4-methylumbelliferone (4-MU) were obtained from Sigma Aldrich (St. Louis, MO). 4-MU-β-D-glucuronide (4-MUG) was purchased from Nacalai Tesque (Kyoto, Japan).

Animals

Male Sprague-Dawley (SD) rats were obtained from SLC Inc. (Shizuoka, Japan). All animals used in this study were housed in temperature- and humidity-controlled animal cages with a 12-h dark/light cycle and with free access to water and standard animal chow (MF, Oriental Yeast, Tokyo, Japan). Experiments involving rats were conducted using protocols approved by the Animal Studies Committee of the University of Tokyo (approval number: P08-088).

Glucuronide de-conjugation experiment in vitro

After 15 min pre-incubation of a reaction mixture (72 μl) consisting of 1.6
mg/mL human or rat liver cytosol and inhibitors (50 μM MEPM or 2 mM SL in 100 mM potassium phosphate buffer, pH 7.4 at 37°C), enzyme reactions were initiated by adding 8 μl of substrates: final 50 μM VPA-G, 100 μM 4-MUG, or 200 μM MFA-G. Concentrations of MEPM and SL were selected to completely inhibit the de-conjugation reaction of MEPM (see Results) and β–glucuronidase (Oleson and Court, 2008), respectively. After incubation at 37°C in a shaking water bath for 40 min, the reaction was terminated by transferring the 20 μl aliquot to another tube containing 70 μl water, 10 μl HClO₄, and 10 μl 100 μM hexanoic acid (internal standard) and then 10 μl 2M KHCO₃ was added for neutralization. De-conjugated compounds from glucuronides were quantified by LC-MS/MS as described in the next section. Data are represented as the means ± S.D. of triplicate experiments otherwise indicated.

Quantification by LC-MS/MS

LC/ESI-MS/MS multiple reaction monitoring (MRM) analyses were conducted on a Quattro Premier XE Tandem Quadrupole Mass Spectrometer coupled to an ACQUITY Ultra Performance LC System with integral autoinjector (Waters, Milford, MA). The Premier XE was run in ESI (negative)-MS/MS MRM mode at a source temperature of 120°C and a desolvation temperature of 350°C by monitoring the following mass transitions (parent to daughter ion): m/z 142.9 to 142.9 for VPA, m/z 174.77 to 132.69 for 4-MU, m/z 240.23 to 195.50 for mefenamic acid and m/z 114.76 to 114.76 for hexanoic acid. Since VPA is known not to be further fragmented (Ji et al., 2003), VPA and hexanoic acid were quantified by non-reactive transition to reduce background interference. The cone voltage was set at 30 V for VPA, 47 V for 4-MU, 30 V for mefenamic acid, and 25 V for hexanoic acid. Collision energy was set at 7 V for VPA, 26 V for 4-MU, 30 V for mefenamic acid, and 7 V for hexanoic acid.
Incubation product mixtures were separated on an ACQUITY Ultra Performance LC bridged ethyl hybrid 1.7 μm, 2.1×100-mm C_{18} HPLC column (Waters) by using 2.5 μl of each specimen with a flow rate of 0.3 ml/min and a binary solvent system of water containing 5 mM ammonium acetate (solvent A) and liquid chromatography-grade methanol (Nacalai Tesque) containing 5 mM ammonium acetate (solvent B). Chromatography of VPA and hexanoic acid (internal standard) was performed using a method as follows: t = 0 min % solvent B = 20, t = 1 min % solvent B = 20, t = 1.5 min % solvent B = 65, t = 2.5 min % solvent B = 65, t = 2.6 min % solvent B = 95, t = 4.5 min % solvent B = 95, t = 4.6 min % solvent B = 20 (total run time was 6 min). The retention times for VPA and hexanoic acid were 3.1 and 2.6 min, respectively. Chromatography of 4-MU and hexanoic acid (internal standard) was performed using a method as follows: t = 0 min % solvent B = 20, t = 1 min % solvent B = 20, t = 3.0 min % solvent B = 60, t = 3.1 min % solvent B = 95, t = 4.0 min % solvent B = 95, t = 4.1 min solvent B = 20 (total run time was 6 min). The retention times for 4-MU and hexanoic acid were 2.6 and 2.9 min, respectively. Chromatography of mefenamic acid and hexanoic acid (internal standard) was performed using a method as follows: t = 0 min % solvent B = 20, t = 1 min % solvent B = 20, t = 1.5 min % solvent B = 70, t = 2.5 min % solvent B = 70, t = 2.6 min % solvent B = 90, t = 4.5 min % solvent B = 90, t = 4.6 min solvent B = 20 (total run time was 6 min). The retention times for mefenamic acid and hexanoic acid were 3.6 and 2.6 min, respectively. Data analyses were carried out using MassLynxNT software (ver. 4.1) and quantified using sample peak area.

**Determination of $K_m$ for VPA-G de-conjugation activity**

VPA-G de-conjugation experiments were performed by the method described previously using 12.5 μM, 25 μM, 50 μM and 100 μM VPA-G. Incubation was
performed for 5, 10, or 20 min to confirm the linearity of the activity versus the incubation time. Experiments were also performed in the presence of 50 μM MEPM or 5 mM SL.

**Examination of irreversible inactivation *in vitro***

The pre-incubation time and CBPM concentration-dependence of VPA-G de-conjugation inhibition were then examined. Pre-incubation was initiated by mixing 6.4 μl 20 mg/ml human liver cytosol with different concentrations of CBPM (PAPM, MEPM, DRPM, or BIPM). After pre-incubation, the enzyme reaction was initiated by adding 72 μl VPA-G to produce a final concentration of 50 μM. After incubation at 37°C in a shaking water bath for 40 min, the reaction was terminated and de-conjugated VPA was quantified by the method described above. Conditions involving the CBPM concentrations and pre-incubation times are summarized in Table 1. Data are presented as the means ± S.D. of triplicate experiments.

Kinetic parameters for irreversible inactivation were obtained as reported elsewhere (Ito et al., 1998). The logarithm of the remaining enzyme activity, which was determined as the formation rate of VPA, was plotted against the pre-incubation time, and the apparent inactivation rate constant ($k_{o,b}$) was determined from the slope of the initial three points. Then, the value of $k_{o,b}$ was plotted against CBPM concentrations ([I]), and the parameters ($k_{inact}$, $K'_{app}$) were obtained by the nonlinear least-squares regression method (MULTI) (Yamaoka et al., 1981) according to the following equation:

$$k_{obs} = \frac{k_{inact} \times I}{K'_{app} + I}$$

(1)

where $k_{inact}$ and $K'_{app}$ represent the maximum inactivation rate constant and the apparent
dissociation constant between VPA-G de-conjugation enzyme and VPA, respectively.

**Calculation of rat $k_{\text{deg}}$ in vivo**

The enzyme is continuously synthesized and degraded. The change in enzyme amount can be described by the following equation:

$$\frac{dE}{dt} = v_{\text{syn}} - k_{\text{deg}}E$$

(2)

where $E$, $v_{\text{syn}}$ and $k_{\text{deg}}$ are the amount of active enzyme, the rate of enzyme synthesis and the degradation rate constant, respectively. At steady-state, $v_{\text{syn}}$ equals the degradation rate ($k_{\text{deg}}E_{ss}$), where $E_{ss}$ is the amount of enzyme at steady-state. Moreover, when the rate of enzyme synthesis is constant, the change in enzyme amount can be also described by the following equation:

$$\frac{dE}{dt} = k_{\text{deg}}E_{ss} - k_{\text{deg}}E$$

(3)

By solving differential Eq. 3, active enzyme amount ($E(t)$) can be explained by the following equation:

$$E(t) = E_{ss} - (E_{ss} - E_0) \cdot \exp(-k_{\text{deg}}t)$$

(4)

where $t$ and $E_0$ are the time after administration of inhibitor and the trough amount of active VPA-G deconjugation enzyme after being inactivated by MEPM in vivo.

From Eq. 4, the $k_{\text{deg}}$ of rat VPAGase was calculated by monitoring the recovery of VPA-G de-conjugating activity after inactivation by MEPM under in vivo conditions. Rats were lightly anesthetized by diethyl ether and a bolus dose of MEPM (100 mg/kg) was injected through the jugular vein to inactivate VPAGase. Rats were housed under the above mentioned conditions after recovery from anesthesia. Then, 3, 12, 24, 48 and 72 hr after dosing, rats (n = 2) were sacrificed by bleeding via central artery under anesthesia with diethyl ether and blood was removed completely by a chilled saline
perfusion. The liver was quickly removed and homogenized in four volumes of 100 mM potassium phosphate buffer (pH 7.4). Liver cytosol was prepared by centrifugation of the liver homogenate at 9,000 g for 10 min at 4°C followed by ultracentrifugation at 100,000 g for 60 min at 4°C. The supernatant was kept frozen at -80°C until use. The protein concentration of each cytosol sample was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

VPA-G de-conjugation activity of each cytosol \(E(t)\) and control cytosol \(E_{ss}\) were evaluated by the method mentioned previously and the parameters \(k_{deg}\) and \(E_0\) were obtained by the nonlinear least-squares regression method (MULTI) according to the following equation:

\[
\frac{E(t)}{E_{ss}} = 1 - \left( \frac{E_0}{E_{ss}} - E_{ss}\right) \exp(-k_{deg}t)
\]  

(5)

**Determination of the amount of active VPAGase**

Without irreversible inhibitors, the change in enzyme amount can be accounted for by Eq. 3. In contrast, in the presence of irreversible inhibitors, the change in enzyme amount can be described by Eq. 6.

\[
\frac{dE}{dt} = v_{syn} - (k_{obs} + k_{deg}) \times E
\]

(6)

The time profile of enzyme amount after CBPM administration to human can be calculated from Eq. 6 with kinetic parameters for inactivation of human enzyme \((K_{\text{inact}}\) and \(K_{\text{app}}')\) and rat \(k_{deg}\). After intravenous infusion of CBPM for \(Time_{\text{inf}}\) hr, the relative enzyme amount can be given by Eq. 7. In this estimation, CBPM concentration \((I(t))\) in blood is assumed to be given by a one-compartment model, and \(I(t)\) was defined by dividing the time during CBPM infusion \((0 < t < Time_{\text{inf}})\) and time
after termination of CBPM infusion \((Time_{inf} < t)\). \(Dose, K_p, MW, k_{el} \) and \(V_d\) represent the dose of CBPM, the liver-to-blood concentration ratio, the molecular weight, the elimination rate constant and the distribution volume of each CBPM, respectively.

\[
\frac{dE(t)}{dt} = k_{deg} \cdot E_{ss} \cdot \left( \frac{k_{muc} \cdot K_p I(t)}{K_{app} + K_p I(t)} + k_{deg} \right) \times E(t) \tag{7}
\]

where,

i) \(0 < t < Time_{inf}\)

\[
I(t) = \frac{Dose}{MW \cdot Time_{inf} \cdot k_{el} \cdot V_d} \left\{1 - \exp(-k_{el} t)\right\}
\]

ii) \(Time_{inf} < t\)

\[
I(t) = \frac{Dose}{MW \cdot Time_{inf} \cdot k_{el} \cdot V_d} \left\{1 - \exp(-k_{el} \cdot Time_{inf})\right\} \cdot \exp\left\{-k_{el} (t - Time_{inf})\right\}
\]

In the calculation, all parameters except for \(K_p\) were obtained from the package inserts of each CBPM and summarized in Table 3. The \(K_p\) value was changed from 0.01 to 1. Simulations were performed by using Scientist program (Micromath, MO). Repeated administration condition (12 hr intervals) was examined for some CBPMs to predict the prolonged effect.
Results

**Substrate specificity and inhibitor sensitivity of VPA-G de-conjugation activity**

In human and rat liver cytosol, the extent of VPA-G de-conjugation activity was similar. These activities were inhibited by MEPM (Fig. 2A and 2B) as previously reported (Nakajima et al., 2004; Nakamura et al., 2008). 4-MUG and MFA-G were used as authentic substrates to examine whether MEPM inhibits the de-conjugation of other glucuronides. Although these glucuronides were de-conjugated in liver cytosol, no inhibition by MEPM was observed (Fig. 2C - 2F). Consequently, inhibition by MEPM may be selective for the VPA-G de-conjugation reaction.

Since glucuronides are de-conjugated by β-glucuronidase expressed ubiquitously in the body and enterobacteria in general, the involvement of β-glucuronidase in VPA-G de-conjugation, which was inhibited by MEPM, was examined using SL, a well established β-glucuronidase inhibitor. It was found that 5 mM SL inhibited de-conjugation of 4-MUG completely and MFA-G by 75% in both human and rat liver cytosol, but only partially inhibited de-conjugation of VPA-G in human and rat liver cytosol by 30% and 25%, respectively (Fig. 2). These results suggest the involvement of an unidentified enzyme other than β-glucuronidase for the de-conjugation of VPA-G. Hereinafter, this unidentified enzyme is referred to as VPAGase.

Although we tried to determine $K_m$ and $v_{max}$ values of VPAGase, it was found that the VPA-G de-conjugation reaction was already saturated at the concentration range from 12.5 to 100 μM. Unfortunately, experiment using a lower concentration of VPA-G was difficult, because of the poor sensitivity of VPA detection under the present experimental conditions. Assuming that the VPAGase activity was saturated under these experimental conditions, the $v_{max}$ values of VPAGase for VPA-G de-conjugation in human and rat liver cytosol were determined to be 0.15 and 0.10 nmol/min/mg protein,
Inhibition mode of CBPM against VPAGase

Although the half-life of CBPM in humans is approximately 1 hr, the decrease in serum VPA concentration continues for several days after stopping CBPM administration (Haroutiunian et al., 2009). It is difficult to explain such a prolonged effect of CBPM by the time lag to reach another steady-state of VPA, since the half life of VPA is as short as 10 hr (Addison et al., 2000). Taking these facts into consideration, it is possible that the prolongation of this interaction may be caused by an irreversible process.

As shown in Fig. 3, pre-incubation time- and CBPM concentration-dependent inactivation of de-conjugation activity for VPA-G was observed in human and rat liver cytosol for all the CBPMs examined (MEPM, PAPM, DRPM and BIPM). Parameters for irreversible inactivation ($k_{\text{inact}}$ and $K_{\text{app}}'$) calculated from the results shown in Fig. 3 are summarized in Table 2. Since saturation of $k_{\text{obs}}$ was not observed for BIPM up to 256 μM (Fig. 4), the inhibition potential was calculated from the relationship between $k_{\text{obs}}$ and BIPM concentration. Comparison of the inhibition potentials ($k_{\text{inact}}/K_{\text{app}}'$) among CBPM in human liver cytosol revealed that PAPM had the strongest inhibition potential followed by MEPM, DRPM and BIPM (Table 2). There was little species difference in these parameters between humans and rats.

Calculation of $k_{\text{deg}}$ of VPAGase in rats

In addition to the parameters for the irreversible inactivation by CBPM, degradation rate constant of enzyme itself is required for the purpose of predicting the in vivo effect of CBPM on the disposition of VPA. Assuming that velocity of enzyme
synthesis is constant, the $k_{deg}$ was calculated from the recovery profile of VPA-G de-conjugation activity in rat liver cytosol, prepared at different time points after intravenous bolus administration of MEPM. Soon after MEPM administration, VPA-G de-conjugation activity was significantly decreased and the relative activities at 3, 12, 24, 48, and 72 hr after MEPM administration were 19, 34, 44, 61 and 72 % of the control, respectively (Fig. 5). Finally, the $k_{deg}$ of rat VPAGase was calculated to be $0.39 /\text{day}$ and $E_0 / E_{ss}$, the relative activity soon after MEPM administration, was 0.16.

**Prediction of the amount of active VPAGase**

Finally, we simulated the hepatic VPAGase activity in human liver after CBPM administration (500 mg/body) using the pharmacokinetic parameters of CBPM (Table 3), the parameters for irreversible inactivation obtained for human liver cytosol (Table 2), and the $k_{deg}$ obtained in rats (Fig. 5). Since there is little information about the $K_p$ values of CBPM, these values were changed from 0.01 to 1. When the $K_p$ value was set at 1 or 0.1, complete inactivation of VPAGase was predicted within less than 2 hr after administration of all the CBPMs examined (Fig. 6). Complete and rapid inactivation was predicted for PAPM when the $K_p$ value was set at 0.01. Although significant activity still remained at 2 hr after a single administration of MEPM, DRPM and BIPM (20, 6 and 44 %, respectively), almost complete inactivation is expected after repeated administration of these CBPMs even under such stringent condition ($K_p = 0.01$). (Fig. 7).
Discussion

Although inhibition of VPA-G de-conjugation has been proposed as one of the mechanisms of the drug-drug interaction between VPA and CBPM, the identity and characteristics of the de-conjugation activity have not been fully established. In the present study, we demonstrated that the de-conjugation of VPA is mediated by enzyme(s) other than β-glucuronidase. This enzyme selectively accepts VPA-G as a substrate, and is also selectively inhibited by CBPM. Moreover, we demonstrated that CBPM extensively inactivates this putative enzyme (VPAGase) in an irreversible manner.

Concerning the characterization of the putative VPAGase, we found that the activity to de-conjugate VPA-G in liver cytosol was only minimally inhibited by 5 mM of SL, a β-glucuronidase inhibitor (Fig. 2). This was in marked contrast to a previous report in which Nakamura et al. (2008) showed complete inhibition of VPA-G de-conjugation by 10 mM SL in human liver cytosol. One of the possible reasons to account for such a discrepancy may be the difference in the experimental conditions. Nakamura et al. (2008) used water as an incubation medium for the inhibition study with SL, whereas we used buffered solution (pH 7.4) throughout the experiments. Since SL is an acidic compound, the pH of the incubation medium may be decreased by the addition 10 mM SL to water and this may result in non-specific inhibition (Oleson and Court, 2008). In accordance with this hypothesis, we found the complete inhibition of VPA-G de-conjugation activity by 10 mM SL in water and confirmed that the pH of the incubation medium was as low as pH = 2 under this condition. Moreover, VPA-G de-conjugation activity was also completely inhibited by acidifying the incubation solution to pH 2 in the absence of SL. It can also be concluded that the putative enzyme is pH-sensitive but insensitive to SL. Concerning the subcellular
localization of VPA-G de-conjugation activity, it is reported that the activity is higher in the liver cytosol fraction than in the liver microsome fraction (Nakajima et al., 2004; Nakamura et al., 2008) where β-glucuronidase is enriched (Medda and Swank, 1985; Shipley et al., 1993). Considering the inhibitor sensitivity and subcellular localization of the activity, it is not likely that β-glucuronidase is the predominant enzyme involved in the de-conjugation of VPA-G.

VPAGase may be relatively selective for VPA-G, since it is not involved in the de-conjugation of other glucuronide conjugates, including 4-MUG and MFA-G (Fig. 2). From a structural point of view, glucuronide conjugates can be classified by conjugated functional groups; if carboxyl, hydroxyl and amino groups are conjugated, they are classified to acyl-, O-, and N-glucuronides, respectively. Based on this classification, both VPA-G and MFA-G are acyl-glucuronides. If VPAGase generally recognizes this bond moiety, inhibition by CBPM would be also expected for MFA-G. However, this was not the case (Fig. 2E and 2F). This result indicates that VPAGase does not necessarily accept acyl-glucuronide in general but is selective for VPA-G. Moreover, 4-MUG, a typical O-glucuronide, is not recognized as a good substrate for VPAGase (Fig. 2C and 2D). These results suggest that not only glucuronide or the bond moieties, but also the structure of the parent compound (VPA), may be important for substrate recognition by VPAGase. Although our in vitro results showing that VPAGase is relatively selective for VPA-G are consistent with the clinical observations that such an interaction has been only reported for VPA, we cannot exclude the effect of CBPM on other drug conjugates. Since therapeutic dose monitoring is performed for a limited number of drugs, it is possible that there are drugs whose serum concentrations are altered by CBPM that have not been detected yet.

In addition, we have demonstrated that VPAGase is inactivated by all the
CBPMs examined (PAPM, MEPM, DRPM and BIPM) in an irreversible manner. Calculated $k_{inact}$ values were approximately 10 to 100 times higher than the values reported for CYP inactivation via the mechanism-based inhibition (Chiba et al., 1995). This inactivation is not merely an artifact obtained from in vitro experiments, since CBPM-sensitive de-conjugation activity in rat liver cytosol was also reduced to the same extent after in vivo administration of CBPM (Fig. 5). Consistent with the irreversible inactivation of VPAGase by MEPM, the recovery of the de-conjugation activity for VPA was slow enough ($t_{1/2} = 1.8$ day) compared to the relatively rapid elimination of MEPM ($t_{1/2} = 1$ hr in humans). If inhibition of VPAGase by CBPM is the mechanism responsible for the drug-drug interaction between VPA and CBPM, VPAGase should be significantly inactivated by CBPM in clinical situations. According to the time profiles for the recovery of CBPM-sensitive VPA-G de-conjugation activity in rat liver cytosol, the $k_{deg}$ of VPAGase in rat was calculated to be 0.39 /day, and half-life of VPAGase was approximately 2 days. Such slow turn-over rate of VPAGase is also consistent with the observations in humans, where the interaction between VPA and CBPM continues for a week after the final administration of CBPM.

Finally, we performed simulation analyses to examine the time profiles of the active amount of VPAGase after CBPM administration in clinical situations. According to the standard regimen of CBPM in Japan, 0.5 g CBPM is intravenously administered for 1 hr, and repeated for 2 times a day. Since the tissue concentration of CBPM in humans is mostly unknown, the $K_p$ value for the liver was changed from 0.01 to 1 according to the information from mouse experiments; the $K_p$ values for DRPM and imipenem (IPM) were more than 0.1 at 5 min after administration (Horiuchi et al., 2006). When the $K_p$ value was set at 0.1 or 1, VPAGase would be almost completely
inactivated after a single administration of CBPM, irrespective of difference in parameters among these four CBPMs. Even if the $K_p$ value was set to 0.01, almost complete inactivation was expected after repeated administration of these CBPMs. The limitation of this simulation is that the $k_{deg}$ values of VPAGase determined in rats are used for the prediction of drug-drug interactions in humans. Such substitution is not always suitable; indeed, the $k_{deg}$ values of the most CYP enzymes in humans are smaller than those in rats (Venkatakrishnan et al., 2007). However, the results of our simulation indicated that, even if $k_{deg}$ values of VPAGase in humans are also smaller than those in rats, complete inactivation may still be observed as shown in Figs. 6 and 7. The simulated results are consistent with the fact that the serum VPA is decreased rapidly after initiation of CBPM therapy. In spite of the lack in clinical evidence for DRPM, and BIPM, co-administration of these CBPMs with VPA are all contraindicated in Japan. From our simulation, it is supported that DRPM and BIPM may be as potent as other CBPM and may cause a severe interaction with VPA.

In conclusion, we have demonstrated for the first time that the predominant de-conjugation activities for VPA in human and rat liver cytosol are extensively inactivated by CBPM in an irreversible manner. Such inactivation may take place under clinical situations based on our simulation analyses.
References


Footnotes

Pharmacokinetic parameters of VPA (DEPAKENE®), PAPM (CARBENIN®), MEPM (MEROPEN®), DRPM (FINIBAX®) and BIPM (Omegacin®) were obtained from the Japanese Pharmaceuticals and Medical Devices Agency website’s section on package inserts (http://www.info.pmda.go.jp/psearch/html/menu_tenpu_base.html).
Figure Legends

Figure 1 Chemical structures of CBPMs.

Figure 2 Inhibitor sensitivity and substrate sensitivity of VPA-G de-conjugation activity. Glucuronide de-conjugation activities in human (A, C, E) and rat (B, D, F) liver cytosol were examined in the presence of 50 μM MEPM or 5 mM SL. VPA-G (A, B), 4-MUG (C, D) and MFA-G (E, F) were used as substrates. Each value represents mean ± S.D. of triplicate experiments. *p<0.05; **p<0.01, significantly different from control. N.D.; not detected.

Figure 3 Pre-incubation time and CBPM concentration-dependent inactivation of VPA-G de-conjugation activity. VPA-G de-conjugation activities in human (A, C, E, G) and rat (B, D, F, H) liver cytosol were inhibited by CBPM (Panels A and B, PAPM; Panels C and D, MEPM; Panels E and F, DRPM, and Panels G and H, BIPM). Each value represents mean ± S.D. of triplicate experiments.

Figure 4 Concentration-dependent inactivation of VPA-G de-conjugation activity by CBPM. Each $k_{obs}$ value was calculated from the data shown in Fig. 3. The $k_{obs}$ values were plotted against the CBPM concentration for human (A) and rat (B) liver cytosol. Fitted curves are shown as solid lines. Each value represents mean ± S.D. of triplicate experiments.

Figure 5 Recovery of VPA-G de-conjugation activity after MEPM administration to rats. After intravenous bolus administration of MEPM (100 mg/kg), liver cytosol was prepared at indicated times and VPA-G de-conjugation activity was examined.
The control value corresponds to the activity of untreated rat liver cytosol. Fitted curve is shown by solid line. Each value represents the average of duplicate experiments.

Figure 6  Simulated time profiles of CBPM concentration and VPAGase remaining activity after administration of CBPM. Five hundred milligrams of each CBPM (Panel A, PAPM; Panel B, MEPM; Panel C, DRPM; and Panel D, BIPM) were assumed to be administered to humans by intravenous infusion for 1 hr. Time profiles for the remaining VPAGase activity in the liver (dotted line) and those for serum concentrations of CBPM (solid line) were simulated. The $K_p$ values were set at 1, 0.1, and 0.01 for each CBPM.

Figure 7  Simulated time profiles of CBPM concentration and VPAGase remaining activity after repeated administration of CBPM. Five hundred milligrams of each CBPM (Panel A, MEPM; Panel B, DRPM; and Panel C, BIPM) were assumed to be repeatedly administered to humans by intravenous infusion at 12 hr intervals. Time profiles for remaining VPAGase activity in the liver (dotted line) and those for serum concentrations of CBPM (solid line) were simulated. The $K_p$ values were set at 0.01 for each CBPM.
Table 1

Conditions involving the CBPM concentrations and pre-incubation times.

<table>
<thead>
<tr>
<th></th>
<th>Concentration µM</th>
<th>Pre-incubation Time min</th>
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<td>PAPM</td>
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<td>0, 3, 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0, 1.5, 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0, 1.5, 3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0, 1, 2</td>
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<tr>
<td></td>
<td>8</td>
<td>0, 0.5, 1</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>0, 0.25, 0.5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0, 0.25, 0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0, 3, 5</td>
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<tr>
<td></td>
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<tr>
<td>MEPM</td>
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</tr>
<tr>
<td></td>
<td>32</td>
<td>0, 0.5, 1</td>
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<tr>
<td></td>
<td>64</td>
<td>0, 0.5, 1</td>
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<tr>
<td></td>
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<td>0, 0.25, 0.5</td>
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<tr>
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<td></td>
<td>8</td>
<td>0, 3, 5</td>
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<tr>
<td>DRPM</td>
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<td></td>
<td>16</td>
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<td>BIPM</td>
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<td>64</td>
<td>0, 1, 2</td>
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Table 2

Calculated parameters for irreversible inactivation.

<table>
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<tr>
<th></th>
<th>Human Liver Cytosol</th>
<th>Rat Liver Cytosol</th>
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<tr>
<td></td>
<td>( k_{\text{inact}} )</td>
<td>( K'_{\text{app}} )</td>
</tr>
<tr>
<td></td>
<td>/min</td>
<td>( \mu M )</td>
</tr>
<tr>
<td>PAPM</td>
<td>9.66</td>
<td>51</td>
</tr>
<tr>
<td>MEPM</td>
<td>7.34</td>
<td>166</td>
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<tr>
<td>DRPM</td>
<td>6.92</td>
<td>142</td>
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<tr>
<td>BIPM</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

The \( k_{\text{inact}} \) and \( K'_{\text{app}} \) values for each CBPM were obtained by non-linear curve fitting of Fig. 3 into equation (1).
Table 3

Parameters for simulation of CBPM concentration and VPAGase remaining activity after CBPM administration.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Timeinf (hr)</th>
<th>Dose (mg)</th>
<th>MW (g/mol)</th>
<th>k_d (1/hr)</th>
<th>V_d (L/body)</th>
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<tr>
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<td>500</td>
<td>339</td>
<td>1.07</td>
<td>20.1</td>
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<tr>
<td>MEPM</td>
<td>1</td>
<td>500</td>
<td>438</td>
<td>0.684</td>
<td>22.0</td>
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<tr>
<td>DRPM</td>
<td>1</td>
<td>500</td>
<td>350</td>
<td>1.01</td>
<td>16.4</td>
</tr>
<tr>
<td>BIPM</td>
<td>1</td>
<td>500</td>
<td>439</td>
<td>0.81</td>
<td>12.4</td>
</tr>
</tbody>
</table>
Figure 1

Figure 1: Structures of PAPM, MEPM, DRPM, and BIPM.
Figure 2

Human Liver Cytosol

De-conjugation Activity [% of control]

Control MEPM SL

Rat Liver Cytosol

De-conjugation Activity [% of control]

Control MEPM SL

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Figure 3

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Figure 4

A  Human Liver Cytosol

B  Rat Liver Cytosol
Figure 5

This article has not been copyedited and formatted. The final version may differ from this version.

VPA-G De-conjugation Activity [% of control]

Time After MEPM Administration [hr]
Figure 6
Figure 7

A

Relative VPAGase Amount

Time [hr]

B

Relative VPAGase Amount

Time [hr]

C

Relative VPAGase Amount

Time [hr]