DMD #32938

Identification of valproic acid glucuronide hydrolase as a key enzyme for the interaction of valproic acid with carbapenem antibiotics

Eiko Suzuki, Naotoshi Yamamura, Yuji Ogura, Daisuke Nakai, Kazuishi Kubota, Nobuhiro Kobayashi, Shin-ichi Miura and Osamu Okazaki.

Drug Metabolism & Pharmacokinetics Research Laboratories (E.S., N.Y., D.N., N.K. and O.O.) and Translational Medicine & Clinical Pharmacology Department (Y.O., K.K. and S.M.), Daiichi Sankyo Co., Ltd., Tokyo, Japan

1

DMD #32938

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Address correspondence to:

Eiko Suzuki

Drug Metabolism & Pharmacokinetics Research Laboratories, Daiichi Sankyo Co., Ltd., 1-2-

58, Hiromachi, Shinagawa-Ku, Tokyo 140-8710, Japan

Tel: +81-3-3492-3131

Fax: +81-3-5436-8567

E-mail: tsukada.eiko.x7@daiichisankyo.co.jp

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Abbreviations: VPA, valproic acid; PAPM, panipenem; VPA-G, VPA glucuronide; APEH,

acylpeptide hydrolase; DFP, diisopropyl fluorophosphate; DTNB, 5,5'-dithiobis(2-

nitrobenzoic acid); PCMB, p-chloromercuribenzoic acid sodium salt; DRPM, doripenem;

BNPP, bis-p-nitrophenyl phosphate

Abstract

Plasma levels of valproic acid (VPA) are decreased by the concomitant use with carbapenem antibiotics, such as panipenem (PAPM). One of the plausible mechanisms of this interaction is the inhibition of VPA glucuronide (VPA-G) hydrolysis by carbapenems in the liver. In order to elucidate this interaction mechanism, we purified VPA-G hydrolase from human liver cytosol, in which the hydrolytic activity was mainly located. After a chromatographical purification, the VPA-G hydrolase was identified as acylpeptide hydrolase (APEH). APEH depleted cytosol, prepared by an immunodepletion method, completely lacked the hydrolytic activity. These results demonstrate that APEH is a single enzyme involved in PAPM-sensitive VPA-G hydrolysis in cytosol. In addition, the hydrolytic activity of recombinant human APEH was inhibited by PAPM and the inhibition profile by typical esterase inhibitors (DFP, DTNB, PCMB and D-saccharic acid 1,4-lactone) was similar to that of human liver cytosol. Cytosolic VPA-G hydrolase activity was slightly inhibited by cholinesterase and carboxylesterase inhibitors. β-Glucuronidase activity remained in APEH depleted cytosol, whereas the VPA-G hydrolase activity was completely abolished. Thus, either cholinesterase, carboxylesterase or β-glucuronidase in cytosol would hardly be involved in VPA-G hydrolysis. Collectively, APEH plays a major role in the PAPM-sensitive VPA-G hydrolysis in the liver. These findings suggest that APEH could be a key enzyme for the drug interaction of VPA with carbapenems via VPA-G hydrolysis.

Introduction

Valproic acid (VPA) is widely used as an antiepileptic drug with a narrow therapeutic

window (50 - 100 µg/mL) (Rimmer and Richens, 1985). The plasma levels of VPA decrease

below the window while patients are receiving carbapenem antibiotics, resulting in the

recurrence of epileptic seizures (Nagai et al., 1997; De Turck et al., 1998). In 1996, the

prohibition of the concomitant use of these drugs was added to the Information on Adverse

Reactions to Drugs from the Ministry of Welfare in Japan, whereas in other countries a

careful concomitant use is allowed and clinical interactions have still been reported (Fudio et

al., 2006; Lunde et al., 2007; Gu and Huang, 2009).

The mechanism of this interaction has been studied by a number of researchers (Mori et al.,

2007). Some mechanisms related to absorption, distribution and metabolism of VPA have

been proposed (Kojima et al., 1998; Yamamura et al., 1999; Torii et al., 2001; Nakajima et

al., 2004; Omoda et al., 2005). Among them, metabolism in the liver has been considered to

be the most important for interaction, based on the following findings. VPA is mainly

metabolized to the acylglucuronide (VPA-G) in the liver and subsequently excreted into bile

in rats. In hepatectomized rats, the interaction of VPA with carbapenems was not observed

(Yamamura et al., 1999). After treatment with panipenem (PAPM) to rats, the biliary

excretion of VPA-G increased and the biliary excretion clearance did not change, suggesting

that the formation of VPA-G was apparently accelerated. Although UDP

glucuronosyltransferases were not induced or activated by PAPM in rat liver microsomes,

UDPGA level was increased by 1.7 times, consistent with 1.8 times increase of the apparent

glucuronidation clearance of VPA (Yamamura et al., 2000). However, VPA-G formation in

monkey liver slice was increased 10 to 20 times by doripenem (DRPM), while the UDPGA

level in the slice was increased only 1.4 times (Nakajima et al., 2004), suggesting that other

processes contribute to the apparent acceleration in VPA-G formation. As one of the

processes, it is considered that the inhibition of the hydrolysis of VPA-G to VPA apparently

accelerates the VPA-G formation. The hydrolysis of VPA-G was observed in rat and monkey

liver homogenates (Nakajima et al., 2004). The hydrolysis in rat liver cytosol was strongly

inhibited by DRPM (Nakajima et al., 2004). After intravenous administration of VPA-G to

rats, the exposure of VPA in plasma was decreased by carbapenems, demonstrating that VPA-

G hydrolysis and the inhibition by carbapenems also occur *in vivo* (Nakajima et al., 2004).

Collectively, VPA-G hydrolase(s) seems to be a key enzyme for the interaction of VPA with

carbapenems.

In this study, we purified a human liver VPA-G hydrolase from cytosol, in which the

hydrolytic activity was mainly located. Then, we estimated the contribution of the enzyme to

VPA-G hydrolysis to judge if the enzyme is a key enzyme for the interaction of VPA with

carbapenems via VPA-G hydrolysis.

Material and Methods

Materials

VPA-G was synthesized enzymatically in Chemtech Labs, Inc. (Tokyo, Japan). PAPM was

synthesized in Sankyo Co., Ltd. (Tokyo, Japan). VPA sodium salt and

ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Sigma Chemical

Co. (St. Louis, MO). Diisopropyl fluorophosphate (DFP), eserine, *p*-nitroaniline, *p*-

nitrophenyl β-glucuronide (pNPG) and *p*-nitrophenol were purchased from Wako Pure

Chemicals (Osaka, Japan). Bis-*p*-nitrophenyl phosphate (BNPP) and 5,5'-dithiobis(2-

nitrobenzoic acid) (DTNB) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). *p*-

Chloromercuribenzoic acid sodium salt (PCMB) and D-saccharic acid 1,4-lactone

monohydrate (saccharolactone) were purchased from Research Organics (Cleveland, OH) and

MP Biomedicals, Inc. (Solon, OH), respectively. Acetyl-alanine *p*-nitroanilide (AANA) was

purchased from Bachem AG (Bubendorf, Switzerland). The other reagents were all

commercially available and of guaranteed grade. Pooled human liver subcellular fractions

and human liver tissue used for the purification of VPA-G hydrolase were obtained from

XenoTech, LLC. (Lenexa, KS) and Human & Animal Bridging Research Organization

(Chiba, Japan). Bovine liver β-glucuronidase was purchased from ProZyme, Inc. (San

Leandro, CA).

VPA-G hydrolysis and inhibition

Either human liver subcellular fraction (cytosol, microsomes, mitochondria and lysosomes; 1

mg/mL each) or recombinant human APEH (0.1 mg/mL) was preincubated in 50 mM Tris-

HCl buffer (pH 7.4) at 37°C. Then, VPA-G (30 μM) was added to make a final volume of

0.05 - 0.1 mL and further incubation was performed at 37°C. The reaction was stopped by

DMD #32938

adding the same volume of acetonitrile containing chlorzoxazone (0.5 - 5 µM, internal

standard (IS)) as the reaction mixture and was centrifuged. An aliquot of 10 µL of each

supernatant was injected into an LC/MS system. Human liver lysosomes were also incubated

in 50 mM sodium acetate buffer (pH 5). In the inhibition studies, either PAPM, β-

glucuronidase inhibitor (saccharolactone) or esterase inhibitor (DFP, eserine, BNPP, DTNB,

EDTA, and PCMB) was added to the reaction mixture before preincubation. In the β-

glucuronidase study, bovine liver β-glucuronidase (200 Fishman unit/mL) was preincubated

in 50 mM sodium acetate buffer (pH 5) and further incubation was performed after the

addition of VPA-G.

Measurement of VPA concentration

The concentrations of VPA in the incubation samples were analyzed using an LC/MS system

consisting of an LC-10A (Shimadzu Corporation, Kyoto, Japan) coupled to an API3000

(Applied Biosystems/MDS SCIEX; Foster City, CA). Chromatographic separation was

achieved on a Capcell Pak C18 MG column (5 µm, 2.0 × 150 mm; Shiseido Co., Ltd., Tokyo,

Japan) at a column temperature of 40°C. A mixture of acetonitrile/water (5/95, v/v)

containing 5 mM ammonium acetate (mobile phase A) and acetonitrile/water (95/5, v/v)

containing 5 mM ammonium acetate (mobile phase B) were used at a flow rate of 0.3

mL/min. The following gradient elution program was used: a linear increase of mobile phase

B from 40% to 60% for 4 min followed by elution with 60% mobile phase B for 0.45 min.

The eluent from the column was introduced directly to the API3000 using the electrospray

ionization interface in negative ion mode. The ions of *m/z* 143 for VPA and *m/z* 168 for IS

were monitored using the selected ion monitoring mode. For both VPA and IS, the

declustering potential, curtain gas, ion spray voltage and ion source temperature were -31 V, 6

psi, -3,500 V and 550°C, respectively.

LDH activity in human liver subcellular fractions

The LDH activity in human liver subcellular fractions was measured using an LDH assay kit

(L-type Wako LDH, Wako Pure Chemicals) according to the manufacturer's instructions.

Purification of VPA-G hydrolase from human liver cytosol

Human liver tissue was homogenized in a buffer (250 mM sucrose, 3 mM Tris and 1 mM

EDTA (pH 7.4)) and the cytosol fraction was prepared by a conventional method. Briefly, the

cytosol was fractionated by ammonium sulfate and 20 to 70% (w/v) precipitate was obtained.

The precipitate was dissolved in 20 mM HEPES (pH 7) and dialyzed against the same buffer.

The dialysate was applied to a HiPrep 16/10 Q FF column (20 mL, GE Healthcare, Uppsala,

Sweden) equilibrated with 20 mM HEPES (pH 7) and then a linear gradient of 0 to 0.5 M

NaCl was applied to elute the enzyme. The active fractions were combined and dialyzed

against 10 mM potassium phosphate buffer (pH 6.8) containing 0.3 mM $CaCl_2$. The dialysate

was applied to a Bio-Scale CHT2-I column (2 mL, Bio-Rad Laboratories, Hercules, CA) and

then a linear gradient of 0 to 50% potassium phosphate buffer (500 mM, pH 6.8) containing

0.01 mM $CaCl_2$ was applied to elute the enzyme. After each active fraction was analyzed by

SDS-PAGE, a 75-kDa protein band visualized by silver staining (EzStain Silver, ATTO

Corporation, Tokyo, Japan) was excised and digested with trypsin within the gel. The

resulting peptides were extracted and sequenced by LC/MS/MS, which consisted of a DiNa

nano-flow liquid chromatography system (KYA Tech, Tokyo, Japan) and an LTQ Orbitrap

(Thermo Fisher Scientific, Waltham, MA). The MS/MS spectra data were searched against a

composite target/decoy IPI human database (version 3.72; 172784 forward and reversed

protein sequences) using Mascot 2.2 (Matrix Science, London, UK). Parent mass and

MS/MS tolerances were set at 50 ppm and 0.8 Da, respectively. We required strict enzyme

specificity and allowed for up to two missed cleavage sites. Carbamidomethylation of
cysteine was set as fix modification, and oxidation of methionine and N-acetylation of protein
were searched as variable modifications. The search results were filtered and summarized
using in-house developed software. In the software, the estimated false discovery rate of all
peptide identifications was fixed at less than 1% by automatically filtering on mass error and
peptide score of all forward and reverse peptide identifications.

Preparation of recombinant human APEH with FLAG tag

The expression vector, pcDNA-NFLAG-GW, was prepared by Daiichi-Sankyo Co., Ltd.
Ultimate ORF Clone (IOH3679, Matching nucleotide accession number: NM_001640.3,
Invitrogen Corporation, Carlsbad, CA), which has an open reading frame of human APEH,
was recombined with pcDNA-NFLAG-GW by a Gateway LR reaction. This vector was
transfected into FreeStyle 293F cells (Invitrogen) using 293fectin (Invitrogen) according to
the manufacturer's instructions. The transfected cells were cultured for 96 h and the harvest
was centrifuged. Recombinant APEH in the supernatant was applied to anti-FLAG M2
affinity gel (Sigma) and was eluted with FLAG peptide (Sigma). The eluate was purified
using the same procedure as described above.

Antiserum and immunodepletion

A rabbit was immunized 8 times over a period of 3 months by subcutaneous injection of a
mixture of recombinant APEH (100 μg) and oil adjuvant (Adjuvant complete fleund (1st
immunization only) or Adjuvant incomplete fleund, Difco Laboratories Inc, Detroit, MI). In 7
days after the final immunization, whole blood was collected and anti-APEH antiserum was
obtained by centrifugation. Aliquots of 100 μL of Protein G Sepharose 4 Fast Flow beads
(GE Healthcare) were washed with 500 μL of TNN.15 buffer (20 mM Tris-HCl (pH 7.4), 150

DMD #32938

mM NaCl and 0.1% NP-40) and centrifuged. After discarding the supernatant, the precipitate

was suspended in 400 µL of the same buffer. A 50-µL aliquot of the antiserum was applied to

the prepared beads and was gently mixed overnight at 4°C to retain the enzyme on the beads.

The antiserum binding beads were washed with 500 µL of TNN.15 buffer 3 times, followed

by a wash with the same volume of TNN.1 (20 mM Tris-HCl (pH 7.4), 100 mM NaCl and

0.1% NP-40). The beads were suspended with 500 µL of TNN.1. Aliquots of 250 µL of the

antiserum binding bead suspension were centrifuged and the supernatant was discarded. To

the beads, 40 µL of human liver cytosol and 360 µL of TNN.1 buffer were added and the

suspension was mixed gently for 2 h at 4°C. After centrifugation, the supernatant was treated

with 250 µL of the bead suspension again. The resulting supernatant obtained after

centrifugation was used as APEH depleted cytosol and Western blot analysis was performed

to check the depletion of APEH. The various hydrolytic activity in the cytosol was also

measured. As a negative control, cytosol was also applied to beads treated with either

preimmune serum or TNN.15 buffer.

Western blotting

APEH depleted cytosol (10 µg) was separated by SDS-PAGE and was transferred

electrophoretically onto a polyvinylidene difluoride membrane (Immun-Blot PVDF

membrane, 0.2 µm, Bio-Rad). APEH protein was detected with anti-APEH antiserum as a

primary antibody and ECL anti-rabbit IgG horseradish peroxidase-linked, from donkey (GE

Healthcare, 1:1,000,000 dilution) as a secondary antibody. The immunoblots were visualized

by chemiluminescence with an ECL Advance Western blotting detection kit (GE Healthcare).

APEH activity

DMD #32938

APEH activity in immunodepleted cytosol (0.1 mg/mL) was assayed as described below.

Each cytosol was incubated with AANA as a substrate in 100 mM Tris-HCl buffer (pH 7.4) at

37°C. The formation of *p*-nitroaniline was measured spectrophotometrically by monitoring

the increase in absorbance at 405 nm.

β-Glucuronidase activity

β-Glucuronidase activity in immunodepleted cytosol (0.1 mg/mL) was measured as described

previously with a little modification (Akao et al., 1987). Each cytosol was incubated with

pNPG (5 mM) as a substrate in 100 mM sodium acetate buffer (pH 5) or 100 mM Tris-HCl

buffer (pH 7.4) at 37°C. The formation of *p*-nitrophenol was measured

spectrophotometrically by monitoring the increase in absorbance at 405 nm.

11

DMD #32938

Results

VPA-G hydrolysis in human liver subcellular fraction and inhibition by PAPM

VPA-G hydrolase activity in human liver cytosol, microsomes, mitochondria and lysosomes at pH 7.4 are shown in Figure 2. All the subcellular fractions showed hydrolase activity and cytosol had the highest activity. Lysosomes also showed the VPA-G hydrolase activity at pH 5. PAPM (0.03 mM) inhibited the VPA-G hydrolase activity in all the subcellular fractions, except for lysosomes at pH 5. The subcellular distribution of LDH activity, a marker enzyme of cytosol, was almost consistent with that of the VPA-G hydrolase activity (Figure 3).

VPA-G hydrolysis by β-glucuronidase

Because VPA-G hydrolysis is a deconjugation of glucuronide, we examined the possibility of the hydrolysis by β-glucuronidase in cytosol. Saccharolactone, known as a β-glucuronidase inhibitor, decreased the hydrolytic activity in cytosol at pH 7.4 by 53.8% at 5 mM and abolished the activity at 25 mM (Table 1). However, purified β-glucuronidase hardly hydrolyzed VPA-G at the same pH (0.710 pmol/Fishman unit/h). Since β-glucuronidase is mainly located in lysosomes, the activity of this enzyme at lysosomal pH was also tested. This enzyme showed greater activity at pH 5 (110 pmol/Fishman unit/h) than at pH 7.4. However, the hydrolase activity at pH 5 was not inhibited by PAPM at 0.3 mM (10 times higher than the inhibitory concentration in cytosol, Table 2).

Inhibition of VPA-G hydrolase activity by typical esterase inhibitors

Because VPA-G hydrolysis is a cleavage reaction of the carboxylester bond, we examined the possibility of the hydrolysis by typical esterases in cytosol. As shown in Table 1, DFP, a strong serine esterase inhibitor, completely inhibited the hydrolytic reaction at more than 0.1

12

DMD #32938

mM. Eserine and BNPP, a specific inhibitor for cholinesterase and carboxylesterase, showed

little or no inhibition of the hydrolysis, respectively. DTNB and PCMB, known as inhibitors

of esterase containing SH-groups, completely inhibited the hydrolysis at 5 and 1 mM,

respectively. The inhibitory effect of EDTA, a chelate agent, was very weak.

Purification of VPA-G hydrolase from human liver cytosol

We tried to purify VPA-G hydrolase(s) from human liver cytosol by anion exchange

chromatography and hydroxyapatite affinity chromatography. The active fractions were

subjected to SDS-PAGE and a thick band was found at 75 kDa (Figure 4). The band was

identified as APEH (IPI accession number: IPI00337741) with a sequence coverage of 71%

by LC/MS/MS (Table 3).

Inhibitory effect of PAPM and esterase inhibitors on the VPA-G hydrolytic activity of
recombinant APEH

The construct of human APEH (derived from IOH3679, Matching nucleotide accession

number: NM_001640.3, Invitrogen) with FLAG tag was transfected in FreeStyle 293F cells

and the resulting protein was purified using the affinity tag and two-step column

chromatography. Then the inhibitory effect of PAPM and esterase inhibitors on the VPA-G

hydrolytic activity of the recombinant human APEH was examined. As shown in Table 1,

PAPM significantly inhibited the hydrolase activity of the recombinant APEH at 0.03 mM.

The effect of DFP, DTNB, PCMB and saccharolactone on the recombinant APEH was similar

to human liver cytosol as well.

Immunodepletion of APEH by rabbit anti-human APEH antiserum

13

To examine the contribution ratio of APEH to VPA-G hydrolysis in human liver cytosol,

rabbit anti-human APEH antibody was raised by an injection of recombinant human APEH.

The resultant antiserum showed a binding ability to APEH protein but it could not inhibit the

APEH activity (data not shown), therefore, we used an immunodepletion method. Human

liver cytosol was treated with the antiserum to deplete APEH. Western blot analysis revealed

that APEH was successfully depleted in the resulting cytosol (Figure 5a). The APEH

depleted cytosol completely lacked the VPA-G hydrolase activity, whereas cytosol treated

with preimmune serum kept 97% of the control activity (Figure 5b). Similarly, AANA, a

typical substrate of APEH, was hardly hydrolyzed in APEH depleted cytosol (Figure 5b). In

contrast, cytosolic β-glucuronidase activity, measured as pNPG activity, was not affected by

depletion of APEH at pH 5 (Figure 5b). Neither cytosol treated with anti-APEH antiserum,

preimmune serum, nor buffer showed β-glucuronidase activity at pH 7.4.

Discussion

We examined the localization of VPA-G hydrolase in the liver to determine a target fraction

for the hydrolase identification (Figure 2). The highest hydrolase activity was found in

cytosol at neutral pH. The other fractions, such as microsomes, mitochondria and lysosomes

also showed the hydrolase activity. However, the activity of these fractions was almost in

parallel with the contaminated fraction of cytosol. Lysosomes showed the hydrolase activity

at acidic pH. This lysosomal activity was not changed by PAPM, whereas the cytosolic

activity at neutral pH was inhibited. Thus, VPA-G hydrolase, subjected to inhibition by

PAPM, is mainly located in cytosol.

From human liver cytosol, we purified a single enzyme with a molecular weight of 75 kDa as

a VPA-G hydrolase (Figure 4). This enzyme was identified as APEH, a serine peptidase.

Then we prepared the recombinant human APEH and found that the inhibition profiles of the

recombinant enzyme by PAPM and enzyme inhibitors (DFP, DTNB, PCMB and

saccharolactone) was similar to that of human liver cytosol (Table 1). APEH depleted cytosol

prepared by anti-APEH antiserum completely lacked the VPA-G hydrolytic activity (Figure

5b). These results demonstrate that APEH is a single enzyme involved in PAPM-sensitive

VPA-G hydrolysis in human liver cytosol.

We examined VPA-G hydrolysis by other cytosolic enzymes (Table 1). Cholinesterase

inhibitor (eserine) and carboxylesterase inhibitor (BNPP) slightly inhibited the VPA-G

hydrolase activity. EDTA showed a weak inhibitory effect on the hydrolytic activity.

Therefore, the contribution of cholinesterase, carboxylesterase and metalloenzyme to VPA-G

hydrolysis was minor in cytosol. β-Glucuronidase is known as a lysosomal enzyme, but the

localization in cytosol has been suggested (Beem et al., 1987). Purified bovine liver β-

glucuronidase hardly hydrolyzed VPA-G at cytosolic pH (Table 2). In addition, APEH

15

depleted cytosol lacked VPA-G hydrolase activity but showed β-glucuronidase activity as same as the control (Figure 5b). These results suggest that cytosolic β-glucuronidase does not contribute to VPA-G hydrolysis.

On the other hand, Nakamura et al. (2008) concluded that VPA-G is hydrolyzed by β-glucuronidase other than serine esterase(s) in cytosol because the VPA-G hydrolase activity was inhibited by saccharolactone but not by phenylmethylsulfonyl fluoride, a serine esterase inhibitor. However, this inhibition profile is consistent with that of APEH (Raphel et al., 1999; this study). Therefore, the previous findings also suggested that cytosolic VPA-G hydrolase is APEH.

APEH (EC 3.4.19.1) is known as a cytosolic serine peptidase, which consists of four identical monomers with a molecular weight of 75 kDa. It catalyzes the hydrolysis of N-acylated peptide to an acylamino acid and a peptide with a free N-terminus (Perrier et al., 2005). The physiological role of APEH is considered as a regulation of the turnover and function of proteins because it has been reported that the acylation of proteins is related to the stability, function and interaction with other proteins. Other than N-acylated peptides, APEH also hydrolyzes simple esters such as *p*-nitrophenyl esters (Scaloni et al., 1994). As far as we know, there are no articles that report the involvement of APEH in drug metabolism.

APEH has been found in various tissues including the kidney, intestine and erythrocytes as well as the liver (Giardina et al., 1999). Therefore, we can not rule out the possibility that APEH in these tissues contributes to the interaction of VPA with carbapenems. However, we have considered the major contribution of the liver to this interaction based on the following published data. In rats, the liver is the most important tissue because interaction did not occur after hepatectomy (Yamamura et al., 1999), although it has not been clarified whether hepatic hydrolysis was mainly responsible for the interaction. Nakajima et al. (2004) reported that urinary excretion of VPA-G after intravenous administration of VPA-G in rats failed to

16

increase by concomitant administration of DRPM. No significant change of VPA-G in

plasma and urine after the treatment of VPA with carbapenems was observed in monkeys

(Nakajima et al., 2004), suggesting that the biliary excretion of VPA-G would increase by the

inhibition of hepatic APEH. The above results indicate that the main tissue of hydrolysis of

VPA-G is the liver. In rabbits, the urinary excretion of VPA-G was increased from 72% to

88% by the treatment of VPA-G with meropenem, although the plasma level of VPA-G only

changed slightly (Yokogawa et al., 2001). In this case, renal APEH seems to contribute to the

interaction via VPA-G hydrolysis, however, the extent of the impact is unknown because the

change in biliary excretion has not been examined. In the case of humans, information on

VPA-G disposition in plasma, urine and bile in patients who are concomitantly received VPA

and carbapenems is not available. The VPA glucuronidation activity in the human liver and

kidney microsomes are very similar (Soars et al., 2001). Moreover, we observed that the

VPA-G hydrolase activities in both tissues are also comparable (data not shown). However,

the total activity in the liver is greater than that in the kidney because of the difference of the

blood flow and total protein between both tissues. Taken together, the APEH in the liver is

considered to be the most important for the interaction via VPA-G hydrolysis in humans as

well.

The inhibition of VPA-G hydrolysis by carbapenems is the most plausible mechanism for the

interaction in human as well as in animals. Significant increase of the formation of VPA-G

by PAPM was observed in human liver slice (Yamamura et al., 2001). Likewise, DRPM

accelerated the VPA-G formation in rat and monkey liver slice and inhibited VPA-G

hydrolysis in rat and monkey liver homogenate (Nakajima et al., 2004). No induction or

activation of UDP glucuronosyltransferases by PAPM was observed in rats (Yamamura et al.,

2000). Moreover, we found that the VPA-G hydrolytic activity was highly located in cytosol

in human liver and was inhibited by PAPM, consistent with the fact that DRPM-sensitive

17

VPA-G hydrolytic activity was abundant in rat liver cytosol (Nakajima et al., 2004). These

findings suggest that the inhibition of VPA-G hydrolysis in the liver is observed as the

acceleration of VPA-G formation, leading to the rapid decrease of VPA in plasma. In

contrast, the other 3 proposed mechanisms, namely, 1) interaction at intestinal absorption

process, 2) interaction at enterohepatic circulation process, and 3) interaction at blood cell

distribution process (Mori et al., 2007), are less likely in human than the interaction at VPA-G

hydrolysis process. Torii et al. (2001) reported that the Cmax and AUC of VPA was

decreased by 50-60% after oral administration of VPA with treatment of imipenem and

PAPM to rats, suggesting that the intestinal absorption of VPA might be inhibited by

carbapenems. However, the plasma level of VPA was also decreased at later time points even

after intravenous administration of VPA with the carbapenems. In a case report of a Chinese

patient who received 800 mg of VPA (p.o., q.d.), the plasma concentration of VPA was

decreased from 39 µg/mL to 10 µg/mL after treatment with meropenem treatment (1 g, i.v.

b.i.d.) and it was not improved by the change of the dose regimens of VPA (400 mg i.v. and

1200 mg p.o. q.d.) and meropenem (0.5 g i.v. b.i.d.) (Gu and Huang, 2009). These findings

indicate that the interaction of VPA and carbapenems occur after intravenous administration

of VPA as well as after the oral administration and the inhibition of intestinal absorption of

VPA would not be significant in rat and human. Kojima et al. (1998) reported that the

decrease of VPA in plasma was not observed after intravenous administration of VPA and

PAPM to bile duct-cannulated rats, suggesting that carbapenems excreted into bile may

suppress the enterohepatic recirculation of VPA by killing intestinal flora, which deconjugate

VPA-G in gut. However, this result has been controversial because the other report showed

that the interaction occurred in bile duct-cannulated rats (Yamamura et al., 1999). In addition,

the biliary concentrations of carbapenems in human are less than that of other β-lactam

antibiotics, which have been reported not to interact with VPA (Thomas et al., 1981;

DMD #32938

Shiramatsu et al., 1988; Morimoto et al., 1991; Granai et al., 1992; De Turck et al., 1998; Yamagata et al., 1998), supporting that the interaction at enterohepatic circulation process would be improbable in human. As to the blood cell distribution process, Omoda et al. (2005) reported that the blood levels of VPA was not changed when the plasma level was decreased after intravenous administration of VPA with carbapenems in rats. However, both the blood and plasma levels of VPA were decreased in patients (Omoda et al., 2005), therefore, this mechanism does not contribute to the interaction in human. Thus, the inhibition of VPA-G hydrolysis in the liver is the most plausible mechanism of the interaction of VPA with carbapenems in human at present.

In conclusion, VPA-G hydrolase activity is mainly located in cytosol in human liver. In addition, VPA-G hydrolysis in cytosol is inhibited by PAPM and caused by APEH only, suggesting that APEH could play an important role for the drug interaction of VPA and carbapenems via VPA-G hydrolysis.

DMD #32938

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20

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Figure legend

Figure 1 Chemical structures of VPA, VPA-G and PAPM.

Figure 2 VPA-G hydrolase activity in human liver subcellular fractions. Subcellular

fractions without indication of pH were tested at pH 7.4 (N=2).

Figure 3 VPA-G hydrolase activity and LDH activity in human liver subcellular fractions at

pH 7.4. Each activity is shown as % of cytosol (N=2).

Figure 4 SDS-PAGE of VPA-G hydrolase purified from human liver. Lane 1, molecular

mass marker; lane 2, purified VPA-G hydrolase. The gel was revealed by silver staining.

Figure 5 APEH depletion (a) and various hydrolytic activity (b) in human liver cytosol after

treatment with preimmune serum and anti-human APEH antiserum. Immunodepleted

samples prepared in duplicate. One sample each was subjected to Western blot analysis.

Hydrolytic activity is shown as the mean value or the value of the pooled sample. The cytosol

treated with buffer is shown as the control.

25

DMD #32938

Table 1 Residual VPA-G hydrolase activity in human liver cytosol (HLC) and recombinant human APEH (rhAPEH) at pH 7.4 after treatment with various inhibitors (N=2). N.D., not determined.

Typical target	Inhibitors	Conc. (mM)	Residual activity (%Control)	
			HLC	rhAPEH
β-Glucuronidase	Saccharolactone	5 25	53.8 0.0	79.6 17.4
Serine esterase	DFP	0.1 1	0.0 0.0	18.0 18.0
Cholinesterase	Eserine	0.1 1	88.1 102.5	N.D. N.D.
Carboxylesterase	BNPP	0.1 1	110.2 85.6	N.D. N.D.
Esterase with SH-group	DTNB	1 5	19.4 0.0	29.6 19.2
	PCMB	1	0.0	19.5
Metalloenzyme	EDTA	1 5	108.9 82.4	N.D. N.D.
Carbapenem-sensitive VPA-G hydrolase	PAPM	0.03	14.0	23.1

DMD #32938

Table 2 Residual VPA-G hydrolase activity of purified bovine liver β-glucuronidase at pH 5

after treatment with inhibitors (N=2).

Inhibitors	Conc. (mM)	Residual activity (%Control)
Saccharolactone	5	0.0
	25	0.0
PAPM	0.03	101.9
	0.3	101.0

Table 3 Correspondence of tryptic peptides of a 75 kDa protein purified from human liver to human APEH (IPI accession number:
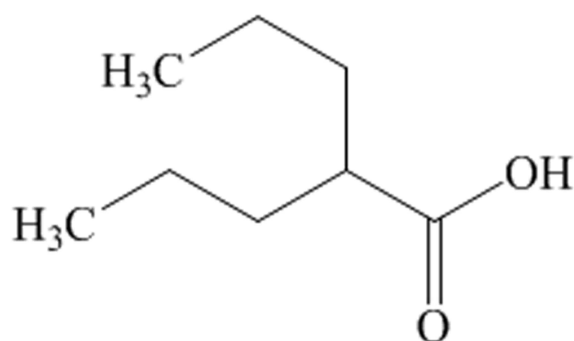
IPI00337741). Sequence coverage is 71%.

Peptide Sequence	Localization of peptide in APEH sequence	Mascot Score	Peptide Sequence	Localization of peptide in APEH sequence	Mascot Score
ERQVLLSEPEEAAALYR	2-18	79	SALYYVDLIGGK	280-291	87
QVLLSEPEEAAALYR	4-18	71	CELLSDDSLAVSSPR	292-306	118
QPALSAACLGPEVTTQYGGQYR	23-44	83	IVYLQYPSLIPHHQCSQLCLYDWYTK	314-339	65
TVHTEWTQR	45-53	45	VTSVVVDVVPR	340-350	64
TVHTEWTQRDLER	45-57	20	QLGENFSGIYCSLLPLGCWSADSQR	351-375	71
QYLVFHDGDSVVFAGPAGNSVETR	66-89	114	VVFDSAQR	376-383	55
GELLSRESPSGTMK	90-103	49	SRQDLFAVDTQVGTVTSLTAGGSGGSWK	384-411	120
ESPSGTMKAVLR	96-107	54	QDLFAVDTQVGTVTSLTAGGSGGSWK	386-411	143
KAGGTGPGEEKQFLEVWEK	108-126	32	LLTIDQDLMVAQFSTPSLPPTLK [a]	412-434	98
QFLEVWEKNRK	119-129	45	VGFLPSAGKEQSVLWVSLEEAEPIPDIHWGIR	435-466	77
KLKSFNLSALEK	129-140	49	VGFLPSAGK	435-443	48
LKSFNLSALEK	130-140	53	EQSVLWVSLEEAEPIPDIHWGIR	444-466	85
SFNLSALEK	132-140	53	VLQPPPEQENVQYAGLDFEAILLQPGSPPDK	467-497	51
HGPVYEDDCFGCLSWSHSETHLLYVAEK	141-168	87	TQVPMVVMPHGGPHSSFVTAWMLFPAMLCK	498-527	53
AESFFQTKALDVSASDDEIAR	173-193	52	MGFAVLLVNYR [a]	528-538	77
AESFFQTK	173-180	49	GSTGFGQDSILSLPGNVGHQDVK	539-561	75
ALDVSASDDEIAR	181-193	83	TPLLLMLGQEDR [a]	665-676	112
LKKPDQAIKGDQFVFYEDWGENMVSK	194-219	87	TPLLLMLGQEDRR	665-677	49
LKKPDQAIK	194-202	40	RVPFKQGMEYYR	677-688	29
GDQFVFYEDWGENMVSK [a]	203-219	99	STHALSEVEVESDSFMNAVLWLR	705-727	115
RSALYYVDLIGGK	279-291	70			

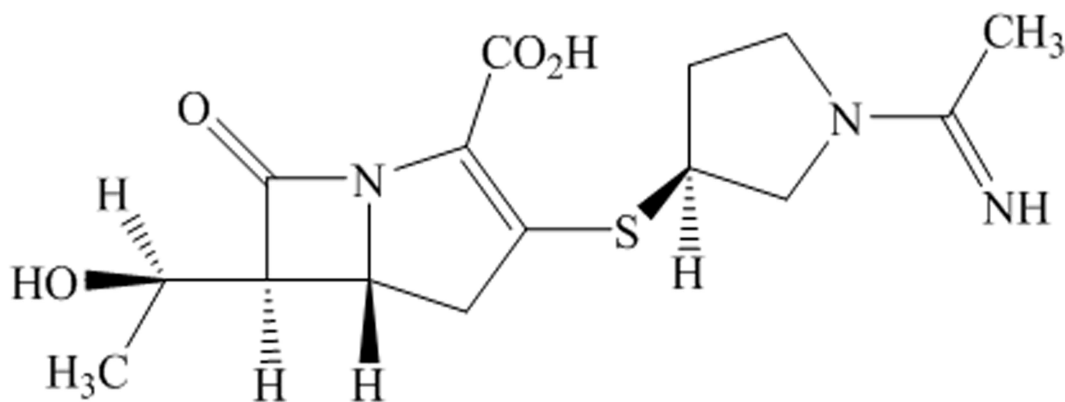[a] Methionine in the peptides is identified as the oxidized form.

Figure 1

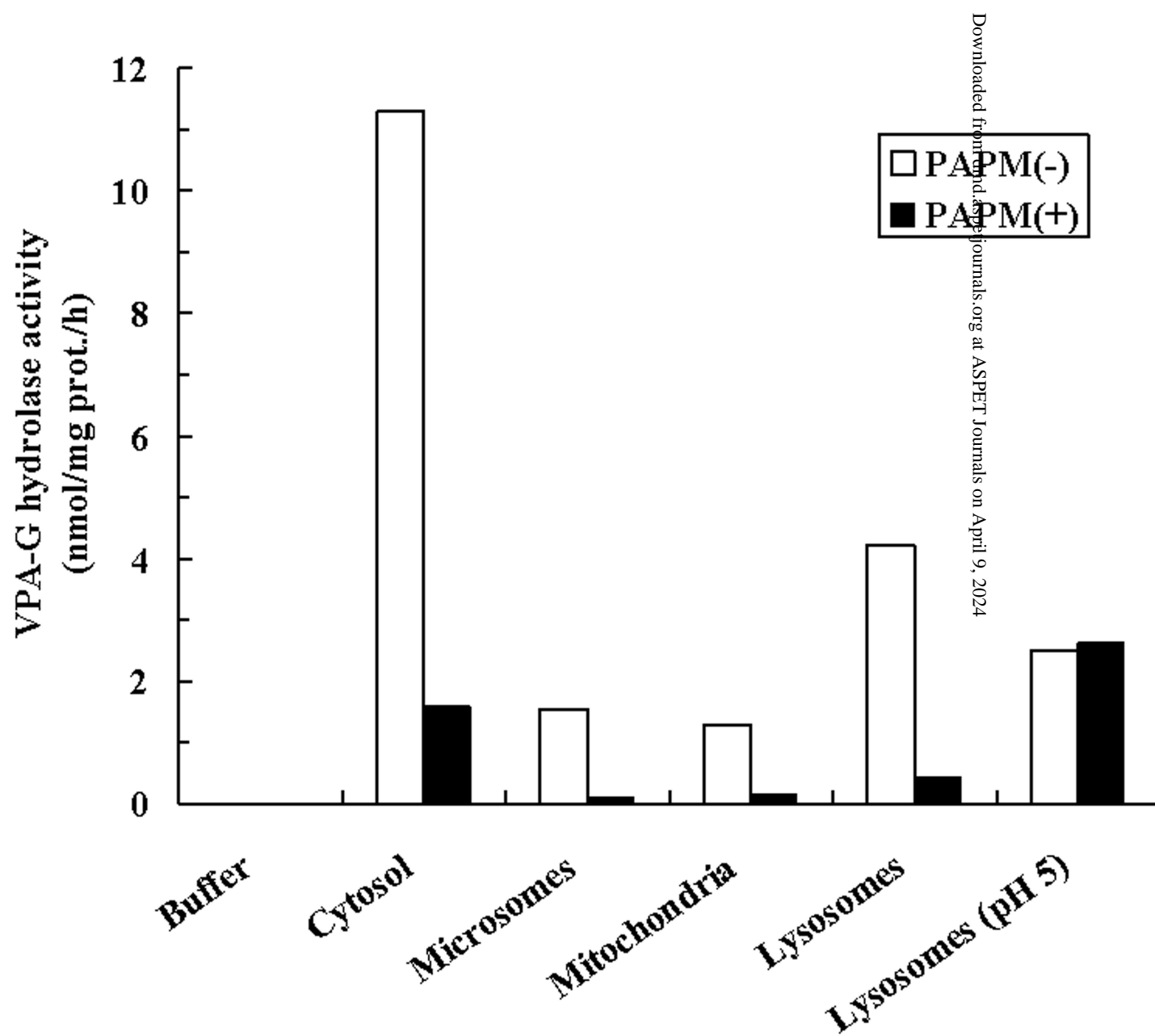Valproic acid
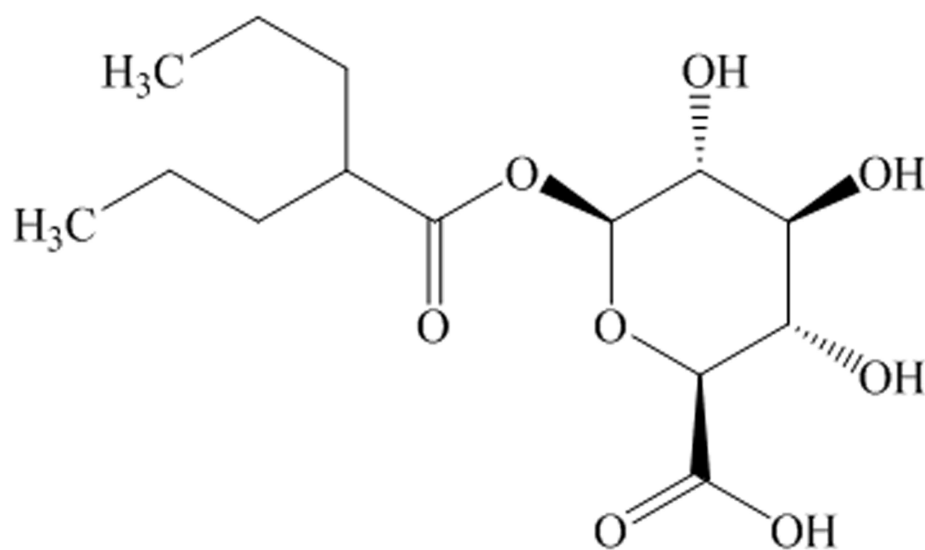
Valproic acid glucuronide
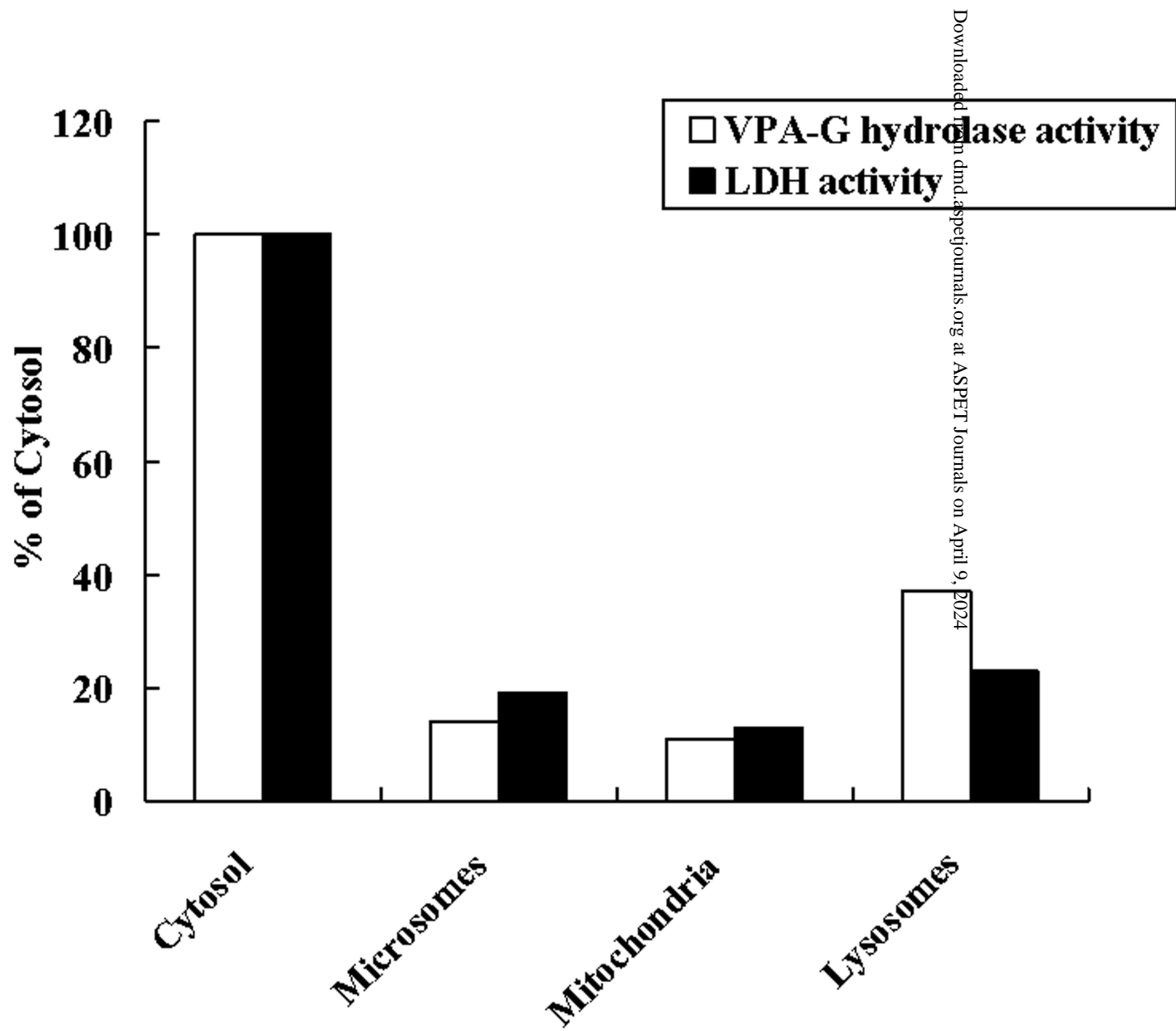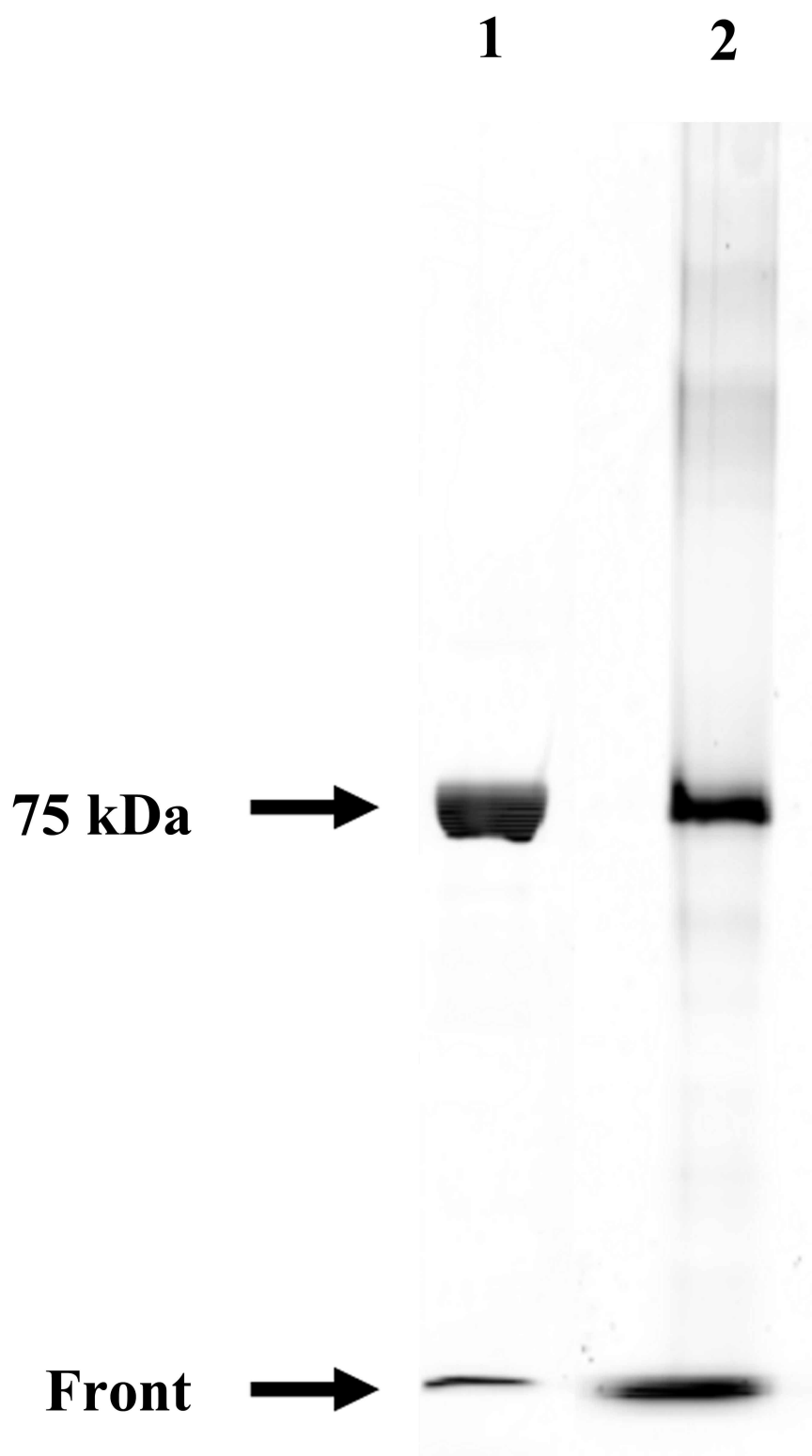
Panipenem

Figure 2

Figure 3

Figure 4

1   **2**

75 kDa ➡

Front ➡

Figure 5

a)

b)