Evaluation of In Situ Generated Valproyl 1- O-β-Acyl Glucuronide in Valproic Acid Toxicity in Sandwich-Cultured Rat Hepatocytes

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ABSTRACT

Acyl glucuronides are reactive electrophilic metabolites implicated in the toxicity of carboxylic acid drugs. Valproyl 1- O-β-acyl glucuronide (VPA-G), which is a major metabolite of valproic acid (VPA), has been linked to the development of oxidative stress in VPA-treated rats. However, relatively little is known about the toxicity of in situ generated VPA-G and its contribution to VPA hepatotoxicity. Therefore, we investigated the effects of modulating the in situ formation of VPA-G on lactate dehydrogenase (LDH) release (a marker of necrosis), BODIPY 558/568 C12 accumulation (a marker of steatosis), and cellular glutathione (GSH) content in VPA-treated sandwich-cultured rat hepatocytes. VPA increased LDH release and BODIPY 558/568 C12 accumulation, whereas it had little or no effect on total GSH content. Among the various uridine 5'-diphospho-glucuronosyltransferase inducers evaluated, β-naphthoflavone produced the greatest increase in VPA-G formation. This was accompanied by an attenuation of the increase in BODIPY 558/568 C12 accumulation, but did not affect the change in LDH release or total GSH content in VPA-treated hepatocytes. Inhibition of in situ formation of VPA-G by borneol was not accompanied by substantive changes in the effects of VPA on any of the toxicity markers. In a comparative study, in situ generated diclofenac glucuronide was not toxic to rat hepatocytes, as assessed using the same chemical modulators, thereby demonstrating the utility of the sandwich-cultured rat hepatocyte model. Overall, in situ generated VPA-G was not toxic to sandwich-cultured rat hepatocytes, suggesting that VPA glucuronidation per se is not expected to be a contributing mechanism for VPA hepatotoxicity.

Introduction

Drug-induced hepatotoxicity is a common cause of acute liver failure (Tijssen and Fontana, 2011). Evidence for drug-induced hepatotoxicity leads to attrition during drug development, refusal of drug approval, and black box warning or postmarketing withdrawal (Regev, 2013). Drug-induced hepatotoxicity can be intrinsic, the occurrence of which is relatively common, predictable, and dose-dependent (Russmann et al., 2009), or idiosyncratic, which occurs in a rare, unpredictable, and often dose-independent fashion in a few susceptible patients (Regev, 2013). Various mechanisms of drug-induced hepatotoxicity have been proposed, including formation of reactive electrophilic metabolites (Srivastava et al., 2010; Leung et al., 2012). One such class of reactive metabolites is the acyl glucuronides (Kalgutkar et al., 2005).

Acyl glucuronides, which are enzymatic products formed by glucuronidation of carboxylic acids (Stachulski et al., 2006), are capable of undergoing the following: 1) hydrolysis to parent aglycone mediated by β-glucuronidases, nonspecific esterases, hydroxyl ion, or serum albumin; 2) intramolecular acyl migration to form positional isomers that are resistant to β-glucuronidase-mediated hydrolysis; and 3) covalent binding to proteins via transacylation or glycation mechanisms (Regan et al., 2010). Acyl glucuronides, however, differ widely in their chemical reactivity, which is attributed to the chemical structure of the aglycone moiety (Stachulski et al., 2006). Postulated mechanisms for the toxicity of acyl glucuronides include the following: 1) a direct impairment of the function of a key protein that is covalently modified; 2) an indirect immune reaction to the antigenic drug-protein adducts; and 3) formation of more reactive acyl-glutathione thioester conjugates with intracellular glutathione (GSH), resulting in GSH depletion and possibly covalent binding to proteins (Shipkova et al., 2003; Skonberg et al., 2008). Although acyl glucuronides are hypothesized to be involved in various toxicities, including hepatotoxicity, of carboxylic acid-containing drugs (Regan et al., 2010; Boelsterli, 2011), it remains to be established whether there is a causal role for these reactive species.

Valproic acid (VPA) is a commonly used antiepileptic drug that is effective against various types of seizures and epileptic syndromes (Loscher, 2002). This drug undergoes microsomal glucuronidation, mitochondrial β-oxidation, and microsomal cytochrome P450-mediated oxidation (Abbott and Anari, 1999). Glucuronidation is a major metabolic pathway for VPA and contributes to the biotransformation of about 30–50% of the administered dose of VPA in humans (Silva et al., 2008). Valproyl 1- O-β-acetyl glucuronide (VPA-G) is one of the least reactive acyl glucuronides investigated to date (Stachulski et al., 2006). Yet, it undergoes intramolecular acyl migration to form positional isomers of VPA-G (Dickinson et al., 1984) and appears to be responsible, at least partly, for the formation of VPA-protein adducts in vitro in rat hepatocytes (Porubek et al., 1989). In rats (Tong et al., 2003) and pediatric patients (Michoulas et al., 2006), VPA increases the in vivo levels of 15-F2t-isoprostane, which is a marker of lipid peroxidation (Halliwell and
Sodium diclofenac (CAS 15307-79-6), sodium phenobarbital (CAS 57-30-7), attributed to the cytochrome P450–mediated oxidative metabolites of diclofenac (Tang, 2003). Although diclofenac undergoes glucuronidation to form an unstable acyl glucuronide, the hepatotoxicity of this drug has been discussed by Tang et al. (2003). At the present study was conducted to determine whether in situ generated VPA-G is toxic and whether it contributes to the toxicity of VPA in sandwich-cultured rat hepatocytes. It is now increasingly recognized that the sandwich-cultured hepatocyte model is appropriate for studying hepatic biotransformation and toxicity of drugs and other chemicals (Swift et al., 2010). Diclofenac is another well-known carboxylic acid drug that is associated with idiosyncratic hepatotoxicity (Tang, 2003). Although diclofenac undergoes glucuronidation to form an unstable acyl glucuronide, the hepatotoxicity of this drug has been attributed to the cytochrome P450–mediated oxidative metabolites of diclofenac (Tang, 2003). As a comparison, the present study also determined the effect of modulating the in situ formation of a more reactive acyl glucuronide, diclofenac 1-O-β-acetyl glucuronide (DFN-G), on the release of lactate dehydrogenase (LDH) in cultured hepatocytes treated with diclofenac. The findings of the present study provide insight to the question as to whether VPA-G plays a role in VPA hepatotoxicity.

Materials and Methods

Chemicals, Reagents, and Solvents. Sodium VPA (CAS 1069-66-5), sodium diclofenac (CAS 15307-79-6), sodium phenobarbital (CAS 57-30-7), β-naphthoflavone (CAS 6051-87-2), 3-methylcholanthrene (CAS 56-49-5), 1-sulfonaphthene (CAS 142825-10-3), quercetin (CAS 117-39-5), dexamethasone (CAS 50-02-2), clofibrate (CAS 637-07-0), pregnenolone 16α-carbinolone (PCN; CAS 1434-54-4), trans-stilbene oxide (CAS 1439-07-2), (−)-borneol (CAS 464-45-9), and dimethylsulfoxide (DMSO; CAS 67-68-5) were obtained from Invitrogen (Burlington, ON, Canada). Matrigel basement membrane matrix (C2), trans-C12 was used as an index of steatosis (Fujimura et al., 2009) and determined, as described previously (Tong et al., 2005b). In other experiments, at 48 hours after plating, cultured hepatocytes were pretreated with an inducer of uridine 5′-diphospho-glucuronyltransferase (UGT) or an inhibitor of glucuronidation and then treated with VPA, diclofenac, or vehicle, as described in each figure legend.

Quantification of VPA-G Concentration. At the end of the drug treatment period, culture supernatant was collected and hepatocytes were lysed with 2% Triton X-100 in PBS (pH 7.4) containing 20 mM EDTA. Each sample was transferred into a microfuge tube and stored at −80°C until analysis. VPA-G concentrations in culture supernatant and cell lysate were quantified using a validated ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) assay with [3H]VPA-G as the internal standard (Surendradoss et al., 2013). The UHPLC-MS/MS system consisted of an Agilent 1290 Infinity Binary Pump, a 1290 Infinity Autosampler, and a 1290 Infinity ThermoScientific Column Compartment (Agilent Technologies, Mississauga, Ontario, Canada), which was connected to an AB Sciex QTRAP 5500 hybrid linear ion-trap triple-quadrupole mass spectrometer equipped with a Turbo Spray source (AB Sciex, Concord, Ontario, Canada). The mass spectrometer was operated in negative ionization mode.

Quantification of DFN-G Concentration. As a precaution to maintain the stability of DFN-G, the culture supernatant samples and the stock solutions of calibration standards were thawed and maintained on wet ice during sample preparation and were acidic with 2 M acetic acid solution (4% v/v final concentration) to reduce intramolecular acyl migration and/or hydrolysis of DFN-G (Sparidans et al., 2008). To quantify DFN-G concentrations in culture supernatant from diclofenac-treated hepatocytes, 10 μl of sample and 10 μl of 50 μg/ml solution of [3H]DFN-G (internal standard) were added to 480 μl of assay diluent (85% of 2 mM ammonium acetate in water and 15% of 2 mM ammonium acetate in a 1:9 mixture of acetonitrile and water), vortex-mixed for 10 seconds, and centrifuged at 10,600g for 5 minutes at 4°C. A 15-μl volume was injected onto the UHPLC-MS/MS system. The calibration curve of DFN-G ranged from 2.1 to 2120 nM. The mobile phases, chromatographic gradient, and mass-spectrometric conditions were the same as those described previously for VPA-G assay (Surendradoss et al., 2013). The declustering potential and collision energy settings were −60 V and −12 V for DFN-G, and −40 V and −20 V for [3H]VPA-G, respectively. DFN-G was analyzed using the total ion current of the multiple reaction monitoring transition m/z 470.2→192.8 (Koga et al., 2011), with the internal standard [3H]VPA-G transition pairs being m/z 325.1→149.3 and 325.1→174.9.

LDH Assay. LDH release was used as a marker of cell necrosis (Jauregui et al., 1981). LDH activity in the culture supernatants and cell lysates was determined using the LDH Cytotoxicity Detection Kit (Roche Diagnostics). LDH released into the culture supernatant was quantified using the Cytotoxicity Detection Kit (Roche Diagnostics) expressed as a percentage of the total cellular LDH activity (i.e., sum of the LDH activity in the culture supernatant and cell lysate).

BODIPY 558/568 C12 Assay. Cellular accumulation of BODIPY 558/568 C12 was used as an index of steatosis (Fujimura et al., 2009) and determined, as described previously (Surendradoss et al., 2012). Fluorescence was measured at a λex of 484 nm and a λem of 618 nm in a Biotek Synergy Mx microplate reader (Biotek Instruments, Winooski, VT). Each blank well had culture medium containing BODIPY 558/568 C12 and a test compound, but without cells. BODIPY 558/568 C12 accumulation was expressed as fold increase in fluorescence in drug-treated wells over that in vehicle-treated control wells.

Total GSH Assay. Cellular content of total GSH was quantified using the Glutathione Assay Kit (Cayman Chemical), as described previously (Surendradoss et al., 2012). The rate of formation of the reaction product, 5-thio-2-nitrobenzoic acid, was determined spectrophotometrically in a kinetic mode at a wavelength of 405 nm in a Labsystems Multiskan Ascent multwell plate reader (Thermo Electron, Burlington, ON, Canada). The blank sample consisted of equal volumes of PBS (pH 7.4; supplemented with 1 mM EDTA) and metaphosphoric acid, but without cell homogenate.

Quantification of Oxidative Metabolites of VPA. Concentrations of the oxidative metabolites of VPA in culture supernatants from VPA-treated hepatocytes were quantified using a gas chromatography–mass spectrometry assay (Surendradoss et al., 2012). Determinations of 4′-OH-DFN and 5-OH-DFN. Concentrations of 4′-OH-DFN and 5-OH-DFN in the culture supernatants of diclofenac-treated rats.
hepatocytes were determined semiquantitatively using a liquid chromatography–
tandem mass spectrometry assay adapted from Spurians et al. (2008). Briefly, 50 μl
of the culture supernatant sample was added to 150 μl of assay diluent (60% of
solvent A, 8.5 mM ammonium acetate in water containing 0.0075% formic
acid, and 40% of solvent B, methanol), vortex-mixed for 10 seconds, and
centrifuged at 10,600g for 5 minutes at 4°C. A 5 μl volume of the supernatant
was injected into the UHPLC-MS/MS system. The calibration curves of 4’-OH-DFN
and 5-OH-DFN ranged from 0.03 to 16 μM. The mobile phases and the chromatographic gradient conditions used in this assay were the same as those described previously (Spurians et al., 2008). Analysis was performed
under multiple reaction monitoring mode on a QTRAP 5500 linear ion trap
mass spectrometer operated in positive electrospray ionization, using the
following instrument settings: curtain gas, 30 U; ion source gas 1, 60 U; ion
source gas 2, 40 U; collision-activated dissociation gas level, high; ion source
temperature, 400°C; ion spray voltage, 5500 V; collision cell exit potential,
18 V; entrance potential, 10 V; and dwell time, 150 ms. The 4’-OH-DFN and
5-OH-DFN were analyzed using the sum of the total ion currents of the multiple
reaction monitoring transitions m/z 312.0→230.9, m/z 312.0→266.0, and m/z
312.0→294.0. Whereas the declustering potential was 66 V, the collision
energy settings for the three MRM transitions were 27, 19, and 15 V for the
three transitions, respectively. Under the assay conditions employed, the retention times of 4’-OH-DFN and 5-OH-DFN were 4.39 and 5.03 minutes, respectively.

Statistical Analysis. Data were analyzed by one-way or two-way analysis of
variance, as appropriate, and when there were significant differences, the data
were further analyzed by the Student-Newman-Keuls multiple comparison test
(SigmaPlot for Windows, version 11.0; Systat Software, Chicago, IL). The
level of statistical significance was set a priori at P < 0.05.

Results

Concentration of In Situ Generated VPA-G in Culture Supernatant and Cell Lysate of Sandwich-Cultured Rat Hepatocytes Treated with VPA. As assessed in hepatocytes treated with VPA (1 mM), the concentration of VPA-G was 251 ± 12 and 6.8 ±
1.5 μM (mean ± S.E.M.; n = 4 rats per treatment group) in the culture supernatant and cell lysate, respectively. As more than 97% of the in situ generated VPA-G was localized in the culture supernatant, VPA-G concentration was quantified in culture supernatant in all the subsequent experiments, unless indicated otherwise.

Time Course and Concentration-Response Relationship in the In Situ Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA. As shown in Fig. 1A, the in situ concentration of VPA-G continued to increase in the culture supernatants in a linear fashion over the 1- to 24-hour post-treatment period. Concentration-response experiments indicated that the in situ formation of VPA-G increased from 20 to 348 μM in response to increases in VPA concentration from 0.03 to 3 mM, reached a peak at 3–10 mM VPA, and decreased at ≥20 mM VPA (Fig. 1B). Based on these results, VPA concentrations of 10 and 15 mM were chosen to investigate the toxicity of in situ generated VPA-G, as these concentrations of VPA resulted in maximal or near-maximal formation of VPA-G (Fig. 1B) and elicited a measurable response in the toxicity markers (Surendradoss et al., 2012).

Effect of Various Known UGT Inducers on In Situ Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA. Except for rat UGT2B1 (Pritchard et al., 1994), the identity of specific rat UGT enzymes catalyzing VPA glucuronidation is not known. Therefore, prior to investigating the toxicity of the in situ generated VPA-G in sandwich-cultured rat hepatocytes treated with VPA, initial experiments were performed to identify chemical modulators of VPA glucuronidation. Cultured hepatocytes were pretreated with a known UGT enzyme inducer, such as β-naphthoflavone (Viollon-Abadie et al., 2000), l-sulforaphane (Kohle and Bock, 2006), phenobarbital (Soars et al., 2004), 3-methylcholanthrene (Jennitz et al., 2000), quercetin (Soars et al., 2004), clofibrate (Jennitz et al., 2000), dexamethasone (Jennitz et al., 2000), PCN (Shelby and Klaassen, 2006), or trans-stilbene oxide (Shelby and Klaassen, 2006) once every 24 hours for 72 hours, followed by VPA treatment for the next 24 hours. As shown in Fig. 2A, β-naphthoflavone, l-sulforaphane, and phenobarbital increased the in situ formation of VPA-G by 2.3-, 1.7-, and 1.9-fold, respectively, whereas none of the other chemicals had an effect (data not shown). As a comparison, β-naphthoflavone, l-sulforaphane, and phenobarbital increased the in situ formation of DFN-G by 3.4-, 4.6-, and 2.9-fold, respectively (Fig. 2A). Among the UGT inducers investigated, β-naphthoflavone yielded the greatest increase in in situ VPA-G formation (Fig. 2A). As evident from the concentration-response data, pretreatment with β-naphthoflavone at 20 μM concentration produced the maximal increase in in situ concentrations of VPA-G in culture supernatants and cell lysates of VPA-treated cells (Table 1). Therefore, this concentration (20 μM) was used in subsequent modulation experiments involving β-naphthoflavone.

Effect of Increasing In Situ VPA-G Formation by β-Naphthoflavone on Markers of Toxicity in Sandwich-Cultured Rat Hepatocytes Treated with VPA. To investigate the effects of increasing the in situ formation of VPA-G on VPA toxicity in sandwich-cultured rat hepatocytes, we assessed LDH release (a marker of necrosis), BODIPY 558/568 C12 accumulation (marker of steatosis), and cellular content of total GSH, all of which are relevant to VPA hepatotoxicity (Jurima-Romet et al., 1996; Silva et al., 2008). Cultured hepatocytes were pretreated with β-naphthoflavone (20 μM) or DMSO (0.1% v/v; vehicle) once every 24 hours for 72 hours, followed by treatment with VPA (10 or 15 mM) or culture medium (vehicle) for the next 24 hours. At the end of the treatment period, LDH release, BODIPY 558/568 C12 accumulation, and cellular concentration of total GSH were measured. As shown in
Cultured Rat Hepatocytes Treated with VPA. Cultured hepatocytes were treated with VPA (10 or 15 mM), diclofenac (DFN; 400 μM), or DMSO (0.1% v/v; vehicle for DFN) for the next 24 hours. In a comparative experiment, β-naphthoflavone increased DFN-G concentrations by 3.4-fold (Fig. 2A), and this was accompanied by an attenuation of LDH release in diclofenac-treated hepatocytes (Fig. 2B). Pretreatment with β-naphthoflavone alone did not affect BODIPY 558/568 C12 accumulation, whereas 10 and 15 mM VPA increased BODIPY 558/568 C12 accumulation (Fig. 3A). This increase in BODIPY 558/568 C12 accumulation by VPA was attenuated by β-naphthoflavone pretreatment (Fig. 3A). In contrast to the increases observed for the LDH and BODIPY markers, VPA (10 and 15 mM) had no effect on total GSH content (Fig. 3B). As shown in Fig. 3B, β-naphthoflavone pretreatment alone led to an increase in the concentration of total GSH. However, treatment with 15 mM VPA, but not 10 mM VPA, decreased the concentration of total GSH (by ~25%) in the β-naphthoflavone–pretreated hepatocytes.

Effect of Borneol on In Situ Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA. Another approach to investigate the toxicity of in situ formed VPA-G is to determine the toxicological consequence of inhibiting its metabolic formation. Initial experiments were performed to determine the effect of borneol, which is a known inhibitor of glucuronidation (Watkins and Klaassen, 1983; Dong and Smith, 2009), on in situ formation of VPA-G in sandwich-cultured rat hepatocytes treated with VPA. Cultured hepatocytes were pretreated with borneol (0.25, 0.5, 0.75, or 1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 hour, followed by cotreatment with VPA (10 or 15 mM) or culture medium (vehicle) for the next 24 hours. Borneol, over the concentration range of 0.25–1 mM, decreased both extracellular and intracellular concentrations of VPA-G in VPA-treated hepatocytes (Table 1). As 1 mM borneol produced the greatest inhibition of in situ VPA-G formation (Table 1), this concentration was selected for the subsequent experiments. As shown in Fig. 4A, whereas 1 mM borneol decreased the extracellular concentration of VPA-G, the same concentration of borneol did not affect the in situ formation of DFN-G in hepatocytes treated with diclofenac for 24 hours (Fig. 4A).

Effect of Decreasing In Situ VPA-G Formation by Borneol on Markers of Toxicity in Sandwich-Cultured Rat Hepatocytes Treated with VPA. The next experiment was to investigate the effects of decreasing in situ formation of VPA-G by borneol on markers of toxicity in sandwich-cultured rat hepatocytes treated with VPA. Cultured hepatocytes were pretreated with borneol (1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 hour, followed by a 24-hour cotreatment with VPA (10 or 15 mM) or culture medium (vehicle). As shown in Fig. 4B, borneol alone did not affect LDH release, whereas 10 and 15 mM VPA treatment increased LDH release by 2- and 4.5-fold, respectively. In hepatocytes treated with 10 or 15 mM VPA, cotreatment with borneol (1 mM) decreased in situ concentration of VPA-G, but it had no effect on LDH release (Fig. 4B). By comparison, borneol (1 mM) attenuated LDH release in hepatocytes treated with 400 μM diclofenac (Fig. 4B), but this was not accompanied by a change in the in situ concentration of DFN-G (Fig. 4A). As shown in Fig. 5A, treatment of cultured hepatocytes with 10 and 15 mM VPA increased BODIPY

![Image](https://via.placeholder.com/150)

**Fig. 2.** Effect of UGT enzyme inducers on in situ formation of VPA-G and LDH release in sandwich-cultured rat hepatocytes treated with VPA. (A) Hepatocytes were cultured for 48 hours and then pretreated with β-naphthoflavone (BNF; 20 μM), t-sulfonamide (LSN; 5 μM), sodium phenobarbital (PB; 2 mM), or vehicle (0.1% DMSO for BNF and LSN; culture medium for PB) once every 24 hours for 72 hours. Subsequently, the hepatocytes were treated with VPA (10 mM), culture medium (vehicle for VPA), diclofenac (DFN; 400 μM), or DMSO (0.1% v/v; vehicle for DFN) for the next 24 hours. (B) Hepatocytes were pretreated with BNF and then treated with VPA (10 or 15 mM), diclofenac (400 μM), or their respective vehicle, as described above in (A). Data are expressed as mean ± S.E.M. for three rats. *Significantly different from the respective vehicle-pretreated control group, P < 0.05. †Significantly different from the respective culture medium-treated group, P < 0.05. ‡Significantly different from the DMSO + DMSO group, P < 0.05. *Significantly different from the BNF + DMSO and the DMSO + diclofenac groups, P < 0.05. VPA-G concentrations were 256 ± 45 and 213 ± 35 μM per 0.7 million cells in the culture medium- and DMSO-pretreated groups, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Pretreatment/Treatment</th>
<th>In Situ Concentration of VPA-G (fold increase over control)</th>
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<tbody>
<tr>
<td></td>
<td>Culture Supernatant</td>
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<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
<td>DMSO 0.1% v/v</td>
<td>1.0 ± 0.0</td>
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<tr>
<td>BNF 5 μM</td>
<td>1.7 ± 0.06*</td>
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<tr>
<td>BNF 10 μM</td>
<td>2.2 ± 0.23*</td>
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<tr>
<td>BNF 15 μM</td>
<td>2.2 ± 0.17*</td>
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<tr>
<td>BNF 20 μM</td>
<td>2.3 ± 0.20*</td>
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<tr>
<td>Experiment 2</td>
<td></td>
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<tr>
<td>DMSO 0.1% v/v</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Borneol 0.25 mM</td>
<td>0.90 ± 0.03*</td>
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<tr>
<td>Borneol 0.5 mM</td>
<td>0.83 ± 0.02*</td>
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<td>Borneol 0.75 mM</td>
<td>0.72 ± 0.04*</td>
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<tr>
<td>Borneol 1 mM</td>
<td>0.64 ± 0.04*</td>
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*Significantly different from the DMSO vehicle control group, P < 0.05.

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Dong and Smith, 2009), on in situ formation of VPA-G in sandwich-cultured rat hepatocytes (Fig. 2B). Pretreatment with β-naphthoflavone alone did not further increase the LDH release by VPA (Fig. 2B). In a comparative experiment, β-naphthoflavone increased DFN-G concentrations by 3.4-fold (Fig. 2A), and this was accompanied by an attenuation of LDH release in diclofenac-treated hepatocytes (Fig. 2B). Pretreatment with β-naphthoflavone alone did not affect BODIPY 558/568 C12 accumulation, whereas 10 and 15 mM VPA increased BODIPY 558/568 C12 accumulation (Fig. 3A). This increase in BODIPY 558/568 C12 accumulation by VPA was attenuated by β-naphthoflavone pretreatment (Fig. 3A). In contrast to the increases observed for the LDH and BODIPY markers, VPA (10 and 15 mM) had no effect on total GSH content (Fig. 3B). As shown in Fig. 3B, β-naphthoflavone pretreatment alone led to an increase in the concentration of total GSH. However, treatment with 15 mM VPA, but not 10 mM VPA, decreased the concentration of total GSH (by ~25%) in the β-naphthoflavone–pretreated hepatocytes.
558/568 C_{12} accumulation by 1.9- and 2.5-fold, respectively, and this increase was not affected by borneol. Treatment of cultured hepatocytes with 15 mM VPA decreased the total GSH content by 35%, which was further decreased by borneol (Fig. 5B). In contrast, neither 10 mM VPA nor cotreatment with borneol affected total GSH content (Fig. 5B).

**Time Course of the Effect of Borneol on the In Situ Formation of VPA-G and LDH Release in Sandwich-Cultured Rat Hepatocytes Treated with VPA.** A time course experiment was performed to further characterize the effect of borneol on in situ formation of VPA-G and LDH release in VPA-treated hepatocytes. VPA increased VPA-G concentrations in the culture supernatants over a 2- to 24-hour period, and this increase was attenuated by borneol at 4, 8, and 24 hours after VPA treatment (Fig. 6A). VPA increased LDH release, but this did not occur until 24 hours post-treatment, and the effect was further enhanced by borneol (Fig. 6B). By comparison, diclofenac increased DFN-G concentrations over the 2- to 24-hour time period (Fig. 6C), whereas borneol decreased the in situ formation of DFN-G only at 2 and 4 hours after drug treatment, but it had no effect at 8 hours, and in fact modestly increased DFN-G formation at 24 hours after drug treatment (Fig. 6C). Treatment with diclofenac resulted in an increase in LDH release at 8 and 24 hours post-treatment, and this increase was attenuated by cotreatment with borneol (Fig. 6D).

**Effect of β-Naphthoflavone and Borneol on In Situ Generated Oxidative Metabolites of VPA in Sandwich-Cultured Rat Hepatocytes Treated with VPA.** In addition to glucuronidation, VPA is also biotransformed by mitochondrial β-oxidation and cytochrome P450-mediated oxidation (Abbott and Anari, 1999). Therefore, we determined whether β-naphthoflavone and borneol are capable of modulating the oxidative biotransformation of VPA, as a means to gain insight into the observed lack of toxicity of VPA-G in cultured hepatocytes treated with VPA (e.g., Fig. 2B and 4B). As shown in Table 2, β-naphthoflavone increased the concentrations of 4-keto-VPA, 4-OH-VPA, and 3-OH-VPA (two- to threefold), but not the other oxidative metabolites of VPA in cultured hepatocytes treated with VPA. By comparison, borneol decreased the concentrations of only 4-OH-VPA (60%) and 5-OH-VPA (40%) (Table 3).

**Effect of β-Naphthoflavone and Borneol on In Situ Generated 4'-OH-DFN and 5-OH-DFN in Sandwich-Cultured Rat Hepatocytes Treated with Diclofenac.** Diclofenac undergoes biotransformation not only by glucuronidation, but also by cytochrome P450-mediated oxidation to produce 4'-OH-DFN and 5-OH-DFN, which undergo subsequent oxidation to form highly electrophilic benzoquinone imine intermediates (Tang, 2003). Therefore, we determined the effects of β-naphthoflavone and borneol on the in situ concentrations of 4'-OH-DFN and 5-OH-DFN in diclofenac-treated hepatocytes to rationalize the attenuation in LDH release by β-naphthoflavone (Fig. 2B) and borneol (Fig. 4B). Interestingly, β-naphthoflavone pretreatment differentially altered the concentrations of the two hydroxy metabolites of diclofenac; β-naphthoflavone increased the concentration of 4'-OH-DFN by over twofold, whereas it attenuated the concentration of 5-OH-DFN by 30% (Fig. 7). Borneol did not affect the concentration of 4'-OH-DFN, but it decreased the concentration of 5-OH-DFN by 80% (Fig. 7).
Discussion

Acyl glucuronides have been implicated in the hepatotoxicity of carboxylic acid drugs; however, there is no direct experimental evidence linking an acyl glucuronide to hepatotoxicity (Stachulski et al., 2013). VPA-G, which is an acyl glucuronide (Dickinson et al., 1984), has been linked to the formation of covalent VPA adducts with hepatocellular proteins (Porubek et al., 1989) and the development of oxidative stress in rats by VPA (Tong et al., 2005b). Yet, the hepatotoxic potential of VPA-G remains to be investigated. As shown in the present study, a major finding is that in situ generated VPA-G did not appear to be toxic to sandwich-cultured rat hepatocytes. This conclusion is based on the following experimental evidence: 1) an increase in the in situ formation of VPA-G by β-naphthoflavone was not accompanied by an increase in VPA toxicity (in fact, it led to an attenuation of BODIPY 558/568 C12 accumulation); and 2) inhibition of in situ VPA-G formation by borneol did not result in an attenuation...
of VPA toxicity. The lack of an effect of β-naphthoflavone on VPA toxicity cannot be due to the increased cellular GSH content by β-naphthoflavone because depletion of GSH has been reported as a consequence rather than a cause of VPA toxicity in cultured rat hepatocytes (Kiang et al., 2011). In support of our finding that in situ generated VPA-G is nontoxic to rat hepatocytes, previous studies have shown that VPA-G is one of the least reactive and the most stable acyl glucuronides (Stachulski et al., 2006). Furthermore, as demonstrated under the cell culture conditions employed in this study, acyl migration of VPA-G was not significant (Surendradoss et al., 2013), suggesting that VPA-G did not form more reactive positional isomers. Overall, VPA glucuronidation appears to be a typical detoxication pathway, as determined in the current study in sandwich-cultured rat hepatocytes.

Another novel finding of the present study was obtained from evaluating the effects of various known inducers of UGTs on increasing in situ formation of VPA-G in sandwich-cultured rat hepatocytes treated with VPA. Among the various inducers investigated in this study, only β-naphthoflavone, phenobarbital, and L-sulfonarphone, but not 3-methylcholanthrene, quercetin, clofibrate, dexamethasone, PCN, or trans-stilbene oxide, increased in situ formation of VPA-G in sandwich-cultured rat hepatocytes. The observed magnitude of the increase (~twofold) in VPA glucuronidation by β-naphthoflavone, phenobarbital, and L-sulfonarphone is consistent with the notion that the inducibility of UGT enzymes is less than that of cytochrome P450 enzymes (Soars et al., 2004). The increase in the extent of drug glucuronidation in response to prototypical UGT inducers is usually ~twofold (Lin and Wong, 2002; Soars et al., 2004). As shown in a previous ex vivo study (Shelby and Klaassen, 2006), β-naphthoflavone induces the hepatic gene expression of rat UGT1A3, UGT1A6, and UGT1A7, whereas PB induces UGT2B1. Therefore, these UGT enzymes are likely catalysts of VPA glucuronidation. In fact, rat recombinant UGT2B1 has been shown to glucuronidate VPA (Pritchard et al., 1994).

### Table 2

Effect of β-naphthoflavone on in situ formation of oxidative metabolites of valproic acid in sandwich-cultured rat hepatocytes treated with valproic acid

At 48 hours after plating, hepatocytes were pretreated with 20 μM β-naphthoflavone (BNF) or 0.1% dimethylsulfoxide (DMSO; vehicle) once every 24 hours for 72 hours and then treated with valproic acid (VPA; 10 or 15 mM) or culture medium (vehicle) for the next 24 hours. Culture supernatants were collected at the end of the 24-hour treatment period and subjected to gas chromatography-mass spectrometry assay for the quantification of the concentrations of oxidative metabolites of VPA. Data are expressed as mean ± S.E.M. for three rats.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>In Situ Concentration of VPA Metabolites (μM/0.7 × 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM VPA 0% DMSO 20 μM BNF 15 mM VPA 0% DMSO 20 μM BNF</td>
</tr>
<tr>
<td>4-ene-VPA</td>
<td>0.06 ± 0.01 0.09 ± 0.01 0.05 ± 0.01 0.08 ± 0.01</td>
</tr>
<tr>
<td>4-keto-VPA</td>
<td>0.15 ± 0.01 0.50 ± 0.06* 0.22 ± 0.02 0.58 ± 0.08*</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>2.5 ± 0.30 4.9 ± 0.54* 2.1 ± 0.25 5.1 ± 0.46*</td>
</tr>
<tr>
<td>5-OH-VPA</td>
<td>1.4 ± 0.27 1.2 ± 0.17* 1.3 ± 0.25 1.2 ± 0.24</td>
</tr>
<tr>
<td>(E)-2,4-diene-VPA</td>
<td>None detected None detected None detected None detected</td>
</tr>
<tr>
<td>(E,E)-2,3-diene-VPA</td>
<td>None detected None detected None detected None detected</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>1.2 ± 0.20 1.3 ± 0.14 1.0 ± 0.15 1.1 ± 0.13</td>
</tr>
<tr>
<td>(E)-2-ene-VPA</td>
<td>1.7 ± 0.13 1.5 ± 0.06 1.8 ± 0.21 1.8 ± 0.20</td>
</tr>
<tr>
<td>3-OH-VPA</td>
<td>5.7 ± 1.2 11 ± 4.2 5.5 ± 1.5 18 ± 5.0*</td>
</tr>
<tr>
<td>3-keto-VPA</td>
<td>2.0 ± 0.63 1.7 ± 0.32 2.1 ± 0.99 2.2 ± 0.79</td>
</tr>
</tbody>
</table>

*Significantly different from the DMSO-pretreated vehicle control group, P < 0.05.

### Table 3

Effect of borneol on in situ formation of oxidative metabolites of valproic acid in sandwich-cultured rat hepatocytes treated with valproic acid

At 120 hours after plating, hepatocytes were pretreated with 1 mM borneol or 0.1% dimethylsulfoxide (DMSO; vehicle) for 0.5 hour. Subsequently, hepatocytes were treated with valproic acid (VPA) (10 or 15 mM) or culture medium (vehicle) in the presence of 1 mM borneol or 0.1% DMSO for 24 hours. Culture supernatants were collected at the end of the 24-hour treatment period and subjected to gas chromatography-mass spectrometry assay for the quantification of the concentrations of oxidative metabolites of VPA. Data are expressed as mean ± S.E.M. for four rats.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>In Situ Concentration of VPA Metabolites (μM/0.7 × 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mM VPA 0% DMSO 1 mM Borneol 15 mM VPA 0% DMSO 1 mM Borneol</td>
</tr>
<tr>
<td>4-ene-VPA</td>
<td>0.08 ± 0.01 0.05 ± 0.01* 0.04 ± 0.01 0.04 ± 0.01</td>
</tr>
<tr>
<td>4-keto-VPA</td>
<td>0.09 ± 0.01 0.39 ± 0.02 0.13 ± 0.01 0.16 ± 0.02</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>3.4 ± 0.56 1.4 ± 0.45* 2.2 ± 0.22 1.9 ± 0.21</td>
</tr>
<tr>
<td>5-OH-VPA</td>
<td>1.6 ± 0.10 0.94 ± 0.05* 1.7 ± 0.23 1.3 ± 0.19</td>
</tr>
<tr>
<td>(E)-2,4-diene-VPA</td>
<td>None detected None detected None detected None detected</td>
</tr>
<tr>
<td>(E,E)-2,3-diene-VPA</td>
<td>None detected None detected None detected None detected</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>0.19 ± 0.02 0.19 ± 0.02 0.18 ± 0.01 0.20 ± 0.01</td>
</tr>
<tr>
<td>(E)-2-ene-VPA</td>
<td>1.5 ± 0.09 1.4 ± 0.20 1.6 ± 0.22 1.5 ± 0.14</td>
</tr>
<tr>
<td>3-OH-VPA</td>
<td>1.8 ± 0.16 1.2 ± 0.16 1.1 ± 0.13 1.0 ± 0.10</td>
</tr>
<tr>
<td>(E)-2-ene-VPA</td>
<td>7.1 ± 0.36 6.2 ± 1.5 11 ± 3.1 7.2 ± 1.1</td>
</tr>
<tr>
<td>3-keto-VPA</td>
<td>1.4 ± 0.33 1.1 ± 0.35 2.8 ± 0.75 2.1 ± 0.97</td>
</tr>
</tbody>
</table>

*Significantly different from the DMSO-pretreated vehicle control group, P < 0.05.
As a comparison with the results obtained with VPA-G, the present study also investigated the effects of modulating in situ formation of DFN-G on hepatocyte toxicity of diclofenac. The increase in VPA-G formation by β-naphthoflavone did not affect LDH release in VPA-treated sandwich-cultured rat hepatocytes, whereas the increase in DFN-G formation by β-naphthoflavone resulted in a decrement of LDH release in diclofenac-treated hepatocytes. The reason for the differential effect of β-naphthoflavone on the toxicity of VPA and diclofenac is not known, but it may involve distinct modulation of other biotransformation pathways of these two drugs. Other than glucuronidation, pretreatment of rat hepatocytes with β-naphthoflavone also increased cytochrome P450-mediated biotransformation of VPA, as shown in the present study. However, the in situ formation of these VPA oxidative metabolites has been reported not to influence VPA toxicity in sandwich-cultured rat hepatocytes (Kiang et al., 2010; Surendradoss et al., 2012). In the case of diclofenac, the hepatocyte toxicity of this drug has been attributed to the formation of cytochrome P450-mediated oxidative metabolites of diclofenac (Kretz-Rommel and Boelsterli, 1993). In the present study, β-naphthoflavone attenuated the hepatocyte toxicity of diclofenac, even though it increased in situ formation of 4′-OH-DFN. The reason for the attenuation of diclofenac toxicity is not known, but it may relate to the increase in cellular GSH content by β-naphthoflavone. GSH is involved in the conjugation and detoxification of cytochrome P450-mediated reactive metabolites of diclofenac (Tang et al., 1999). Overall, the increase in the in situ formation of two acyl glucuronides (VPA-G and DFN-G), which differ greatly (~150-fold) in chemical stability (Stachulski et al., 2006), did not enhance the hepatocyte toxicity of the parent drugs VPA and diclofenac.

As shown in the present study, cotreatment of sandwich-cultured rat hepatocytes with borneol and VPA decreased VPA-G formation over a period of 4–24 hours after VPA treatment, but this was not associated with a decrease in LDH release over the same time period. By comparison, cotreatment of hepatocytes with borneol and diclofenac was seen to decrease DFN-G levels for only up to 4 hours after diclofenac treatment. The apparent recovery of the in situ levels of DFN-G over the 4–24 hour period was accompanied by a marked decrease in the extent of LDH release in the 8- and 24-hour time points after the cotreatment of hepatocytes with borneol and diclofenac. A possible explanation for the observed decrease in LDH release by borneol in diclofenac-treated hepatocytes is the substantive attenuation of the in situ levels of the oxidative metabolite 5-OH-DFN. Previously, borneol was reported to increase rather than decrease LDH release in diclofenac-treated rat hepatocytes (Kretz-Rommel and Boelsterli, 1993). The reason for the differences in the effect of borneol on LDH release in diclofenac-treated hepatocytes shown in the present study and in the earlier study is not known; however, it may relate to the differences in the duration of borneol treatment (24 hours in the present study and 2 hours in the study by Kretz-Rommel and Boelsterli, 1993).

Borneol inhibits the glucuronidation of drugs and other chemicals by depleting the hepatocellular levels of the cofactor uridine 5′-diphospho-glucuronic acid and inhibiting the catalytic activity of specific UGT enzymes that catalyze glucuronidation (Watkins and Klaassen, 1982). As evident by our time course data, the inhibitory effect of borneol on in situ VPA-G formation was apparent from 4 to 24 hours after VPA treatment, whereas its inhibition of in situ DFN-G formation no longer occurred at 8 hours after diclofenac treatment. The differences in the temporal profile on the effect of borneol on in situ formation of VPA-G and DFN-G suggest that borneol does not inhibit the glucuronidation of VPA and diclofenac by the same mechanism.

Sandwich-cultured hepatocytes have been used to study the toxicity of acyl glucuronides (Dong and Smith, 2009). However, a limitation of this model is the lack of enterohepatic recycling. As evident from a preliminary experiment, hydrolysis of VPA-G was not apparent in our sandwich-cultured rat hepatocyte model. Pretreatment or cotreatment with an inhibitor of VPA-G hydrolysis (Suzuki et al., 2010), 3-sacccharalactone or a carbapenem (meropenem or imipenem), did not enhance in situ concentrations of VPA-G, as assessed at 24 hours after treatment with VPA (data not shown). However, VPA-G is subject to efficient hydrolysis and enterohepatic recycling in rats in vivo (Dickinson et al., 1979; Liu and Smith, 2006). Thus, the conjugation-deconjugation cycling may increase the hepatocellular exposure to VPA-G in vivo. It remains to be investigated whether such an increase in exposure to VPA-G in vivo contributes to the hepatotoxicity of VPA.

In conclusion, similar to the findings on DFN-G in the same cell culture model, in situ generated VPA-G was not toxic to sandwich-cultured rat hepatocytes, as evident from the experiments using chemical modulators of VPA glucuronidation. The findings of this study, together with the available information regarding the chemical reactivity of VPA-G (Stachulski et al., 2006), lead us to conclude that in situ generated VPA-G does not contribute to the hepatocyte toxicity of VPA. As reported previously, the in situ formation of other reactive metabolites of VPA, such as 4-ene-VPA (Kiang et al., 2010) and (E)-2,4-diene-VPA (Surendradoss et al., 2012), also does not play a role in VPA toxicity. Valproyl-S-acyl CoA is another metabolite of VPA formed by the β-oxidation pathway (Silva et al., 2008). It has been proposed that bioactivation of carboxylic acids may also involve acyl-CoA thioesters and acyl-glutathione thioesters, which are even more reactive than acyl glucuronides (Skonberg et al., 2008). The toxicological significance of acyl-CoA thioester metabolites has been relatively less studied in comparison with acyl glucuronides, although the intracellular localization of acyl-CoA thioesters makes them more probable mediators of hepatotoxicity associated with carboxylic acid drugs (Darnell and Weidolf, 2013). In a previous study, it was speculated that the formation of valproyl-S-acyl CoA thioester may contribute to the hepatotoxicity of VPA (Grillo et al., 2001). It would be of interest in the future to investigate the role of valproyl-S-acyl-CoA and other downstream β-oxidation metabolites.
of VPA in the hepatotoxicity of VPA using the sandwich-cultured hepatocyte model.

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Authorship Contributions

Participated in research design: Surendradoss, Chang, Abbott.

Conducted experiments: Surendradoss.

Performed data analysis: Surendradoss.

Wrote or contributed to the writing of the manuscript: Surendradoss, Chang, Abbott.

References


