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 is intrinsic, the occurrence of which is relatively common, predictable, and dose-dependent (Rusmann 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...
Whitman, 2004). The increase in plasma and hepatic levels of 15-F$_2$-isoprostane in rats administered VPA was accompanied by an increase in the levels of VPA-G (Tong et al., 2005b). In another in vivo study in rats, hepatic and urinary concentrations of VPA-G did not correlate with serum levels of α-glutathione-S-transferase (Lee et al., 2009), which is a marker of hepatotoxicity (Bailey et al., 2012).

The therapeutic use of VPA by humans is associated with a rare, but potentially fatal, idiosyncratic hepatotoxicity (Nanau and Neuman, 2013). Although the mechanism of VPA hepatotoxicity is not understood, VPA-G has been proposed to play a role (Tong et al., 2005b). However, there is no direct evidence as to whether in situ generated VPA-G is hepatotoxic. Therefore, to increase our understanding of the toxicological significance of VPA-G, 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). Dichlofenac is another well-known carboxylic acid drug that is associated with idiosyncratic hepatotoxicity (Tang, 2003). Although dichlofenac undergoes glucuronidation to form an unstable acyl glucuronide, the hepatotoxicity of this drug has been attributed to the cytochrome P450 (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$_6$]VPA-G as the internal standard (Suredradoss 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 acidified 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 dichlofenac-treated hepatocytes, 10 μl of sample and 10 μl of 50 μg/ml solution of [3H$_6$]VPA-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 9:1 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 (Suredradoss 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$_6$]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$_6$]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 CytoTox-96 Non-Radioactive Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN), and the Glutathione Assay Kit was from Cayman Chemical (Ann Arbor, MI). Williams’ medium E, liver perfusion medium, hepatocyte wash medium, heat-inactivated fetal bovine serum, phosphate-buffered saline (PBS; pH 7.4), 10% Hank’s balanced salt solution, 10% Dulbecco’s PBS, penicillin-streptomycin, 1-glutamine, and BODIPY 558/568 C12 were obtained from Invitrogen (Burlington, ON, Canada). Matrigel basement membrane matrix was obtained from BD Biosciences (Mississauga, ON, Canada). Percoll was purchased from GE Healthcare (Baie d’Urfe, QC, Canada). Ammonium acetate, ethyl acetate, diethyl ether, acetonitrile, methanol, n-hexane, glacial acetic acid, and sodium hydroxide were obtained from Fisher Scientific (Ottawa, ON, Canada).

**Animals.** Adult male Sprague-Dawley rats (175–200 g) were obtained from Charles River Laboratories (Sennville, QC, Canada) and were housed and cared for in our animal care facility, as described previously (Surendradoss et al., 2012). All animal experiments were approved by the University of British Columbia Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

**Isolation, Culture, and Treatment of Rat Hepatocytes.** Rat hepatocytes were isolated by a two-step collagenase perfusion method (Seglen, 1993), as described previously (Surendradoss et al., 2012). The isolated hepatocytes were treated with plating medium (vehicle for VPA, diclofenac, or DMSO (vehicle for dichlofenac) for the next 24 hours at the concentrations indicated in each figure legend. In other experiments, at 48 hours after plating, cultured hepatocytes were pretreated with an inducer of uridine 5′-diphospho-glucuronosyltransferase (UDT) or an inhibitor of glucuronidation and then treated with VPA, diclofenac, or vehicle, as described in each figure legend.

**VPA culture medium (vehicle for VPA), diclofenac, or DMSO (vehicle for dichlofenac) for the next 24 hours at the concentrations indicated in each figure legend. In other experiments, at 48 hours after plating, cultured hepatocytes were pretreated with an inducer of uridine 5′-diphospho-glucuronosyltransferase (UDT) or an inhibitor of glucuronidation and then treated with VPA, diclofenac, or vehicle, as described in each figure legend.
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 on 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
(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 Hepato
cytes 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 Hepato
cytes 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-sulfuraphane (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-sulfuraphane, 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-sulfuraphane, 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
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-Romert 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

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Fig. 1. Time course and concentration-response relationship in the in situ formation of VPA-G in sandwich-cultured rat hepatocytes treated with VPA. (A) Hepatocytes were cultured for 120 hours and then treated with VPA (1 mM) or culture medium (vehicle). At 1, 2, 4, 8, 12, and 24 hours after drug treatment, an aliquot of culture supernatant was collected and VPA-G concentration was quantified by UHPLC-MS/MS. (B) Hepatocytes were cultured for 120 hours and then treated with VPA (0.03–100 mM) or culture medium (vehicle) for 24 hours. Data are expressed as mean ± S.E.M. for three or four rats.
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), l-sulforaphane (LSFN; 5 μM), sodium phenobarbital (PB; 2 mM), or vehicle (0.1% DMSO for BNF and L-SFN; 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 culture medium-treated group, P < 0.05. †Significantly different from the respective culture medium-treated group, P < 0.05. ‡Significantly different from the respective culture medium-treated group, P < 0.05. 

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). As shown in Fig. 3A, pretreatment with borneol (1 mM) decreased in situ concentration of VPA-G, but it 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 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

<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>DMSO 15 μM</td>
<td>2.2 ± 0.23*</td>
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<tr>
<td>BNF 20 μM</td>
<td>2.3 ± 0.17*</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.
DFN-G concentration was 20\% different from the DMSO + culture medium group or borneol + culture medium group, and then pretreated with borneol (1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 hour. Subsequently, the hepatocytes were treated with VPA (10 or 15 mM) or culture medium (vehicle) for the next 24 hours. Data are expressed as mean ± S.E.M. for three to five rats. *Significantly different from the DMSO + culture medium group, \( P < 0.05 \); †significantly different from the corresponding DMSO + culture medium or DMSO + VPA groups, \( P < 0.05 \); ‡significantly different from the BNF + culture medium and DMSO + 15 mM VPA groups, \( P < 0.05 \). The total GSH content was 7.4 ± 1.6 \( \mu \)M per 0.7 million cells in the DMSO + culture medium group.

**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 \( \beta \)-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 \( \beta \)-oxidation and cytochrome P450-mediated oxidation (Abbott and Anari, 1999). Therefore, we determined whether \( \beta \)-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, \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-naphthoflavone (Fig. 2B) and borneol (Fig. 4B). Interestingly, \( \beta \)-naphthoflavone pretreatment differentially altered the concentrations of the two hydroxy metabolites of diclofenac; \( \beta \)-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).

**Fig. 4.** Effect of borneol on in situ formation of VPA-G and LDH release in sandwich-cultured rat hepatocytes treated with VPA. Hepatocytes were cultured for 120 hours and then pretreated with borneol (1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 hour. Subsequently, the hepatocytes were treated with 10 nM VPA (A and B), 15 mM VPA (B), culture medium (vehicle for VPA; A and B), 400 \( \mu \)M diclofenac (DFN; A and B), or DMSO (0.1% v/v; vehicle for DFN; A and B) for the next 24 hours in the presence of borneol (1 mM) or DMSO (0.1% v/v). Data are expressed as mean ± S.E.M. for three rats. *Significantly different from the DMSO + VPA group, \( P < 0.05 \); †significantly different from the DMSO + culture medium or borneol + culture medium groups, \( P < 0.05 \); ‡significantly different from the DMSO + DMSO and diclofenac groups, \( P < 0.05 \); §significantly different from the DMSO + culture medium group or borneol + culture medium group, \( P < 0.05 \); ‡significantly different from the DMSO + DMSO group, \( P < 0.05 \); ‡significantly different from the DMSO + DMSO + diclofenac groups, \( P < 0.05 \). VPA-G concentration was 283 ± 41 \( \mu \)M per 0.7 million cells in the DMSO + VPA group. DFN-G concentration was 20 ± 1.8 \( \mu \)M per 0.7 million cells in the DMSO + diclofenac group.
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 detoxification 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-sulfonate, 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-sulfonate 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

<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</td>
</tr>
<tr>
<td></td>
<td>0.1% DMSO</td>
</tr>
<tr>
<td></td>
<td>0.1% DMSO</td>
</tr>
<tr>
<td>4-ene-VPA</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>4-keto-VPA</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>2.5 ± 0.30</td>
</tr>
<tr>
<td>5-OH-VPA</td>
<td>1.4 ± 0.27</td>
</tr>
<tr>
<td>(E)-2,4-diene-VPA</td>
<td>None detected</td>
</tr>
<tr>
<td>(E,E)-2,3'-diene-VPA</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>1.7 ± 0.27</td>
</tr>
<tr>
<td>3-keto-VPA</td>
<td>1.2 ± 0.20</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>1.7 ± 0.13</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>5.7 ± 1.2</td>
</tr>
<tr>
<td>3-keto-VPA</td>
<td>2.0 ± 0.63</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

<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</td>
</tr>
<tr>
<td></td>
<td>0.1% DMSO</td>
</tr>
<tr>
<td></td>
<td>0.1% DMSO</td>
</tr>
<tr>
<td>4-ene-VPA</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>4-keto-VPA</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>4-OH-VPA</td>
<td>3.4 ± 0.56</td>
</tr>
<tr>
<td>5-OH-VPA</td>
<td>1.6 ± 0.10</td>
</tr>
<tr>
<td>(E)-2,4-diene-VPA</td>
<td>None detected</td>
</tr>
<tr>
<td>(E,E)-2,3'-diene-VPA</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>1.5 ± 0.09</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>1.3 ± 0.13</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>1.8 ± 0.16</td>
</tr>
<tr>
<td>3-ene-VPA</td>
<td>7.1 ± 0.36</td>
</tr>
<tr>
<td>3-keto-VPA</td>
<td>1.4 ± 0.33</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 DFN-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 an attenuation 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- to 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), D-saccharolactone 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, Chang, Abbott.

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

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