VALPROIC ACID BIOTRANSFORMATION AND TOXICITY IN SANDWICH-CULTURED RAT HEPATOCYTES

by

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Abstract

Valproic acid (VPA) is a widely prescribed broad-spectrum antiepileptic drug. Clinical use of VPA is associated with a rare, but possibly fatal, idiosyncratic hepatotoxicity. The mechanism of VPA hepatotoxicity is not known, but it may involve reactive metabolites of VPA. Using a sandwich-cultured rat hepatocyte model, the present work investigated the toxicity of two specific VPA metabolites, (*E*)-2,4-diene-VPA and valproyl-1-*O*-β acyl glucuronide (VPA-G), and their role in the hepatocyte toxicity of VPA. The overall experimental strategy was to modulate the *in situ* formation of these two metabolites and determine the consequences on VPA toxicity in sandwich-cultured rat hepatocytes. VPA toxicity was assessed by markers such as 2′,7′-dichlorofluorescein formation (oxidative stress), BODIPY 558/568 C_{12} accumulation (steatosis), lactate dehydrogenase release (necrosis), and cellular content of total glutathione (antioxidant status). (*E*)-2-ene-VPA, which is a β-oxidation metabolite of VPA, was also included in the experiments concerning (*E*)-2,4-diene-VPA, as it formed relatively large amounts of (*E*)-2,4-diene-VPA. Based on the modulatory experiments with phenobarbital and 1-aminobenzotriazole, *in situ* generated (*E*)-2,4-diene-VPA did not appear to contribute to VPA toxicity in sandwich-cultured rat hepatocytes. However, the results from (*E*)-2-ene-VPA experiments indicated the toxic potential of *in situ* generated (*E*)-2,4-diene-VPA in sandwich-cultured rat hepatocytes, when generated at high concentrations.

As part of this study, a sensitive and rapid ultra-high performance liquid chromatography – tandem mass spectrometry method for the quantification of VPA-G in hepatocyte culture medium was developed, validated, and applied successfully to quantify *in situ* concentrations of VPA-G. From a comprehensive screening of several known inducers of uridine 5'-diphospho-

glucuronosyltransferase enzymes, this study identified β-naphthoflavone, L-sulforaphane, and phenobarbital to be effective in increasing the *in situ* formation of VPA-G from VPA in sandwich-cultured rat hepatocytes. According to the findings with β-naphthoflavone and borneol, *in situ* generated VPA-G did not appear to be toxic to sandwich-cultured rat hepatocytes and was unlikely to contribute to the hepatocyte toxicity of VPA. Overall, the results of the present study add value to the existing knowledge on the role of reactive metabolites of VPA in VPA hepatotoxicity. Future studies should investigate the role of VPA-CoA thioester formation on VPA toxicity in sandwich-cultured rat hepatocytes.

Preface

Chapter 2. A version of Chapter 2 has been published. Surendradoss, J, Chang, TKH, and Abbott, FS (2012) Assessment of the role of *in situ* generated (*E*)-2,4-diene-valproic acid in the toxicity of valproic acid and (*E*)-2-ene-valproic acid in sandwich-cultured rat hepatocytes. *Toxicol Appl Pharmacol* 264: 413-422. With guidance from Dr. Chang and Dr. Abbott, I was responsible for the design and conduct of the experiments, data collection, analysis and interpretation of the results, and writing the manuscript. The metabolites were synthesized and purified by Dr. Stoyan Karagiozov.

Chapter 3. A version of Chapter 3 has been published. Surendradoss, J, Szeitz, A, Teng, XW, Chang, TK, and Abbott, FS (2013) A rapid and sensitive assay to quantify valproyl 1-*O*-acyl glucuronide in supernatants of sandwich-cultured rat hepatocytes using ultra-high performance liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 932: 40-49. This method is an improvement of the UPLC-MS/MS method previously developed in the laboratory by Dr. Xiaowei Teng. Dr. Teng also contributed to the design of the method validation study and Andras Szeitz helped with the UHPLC-MS/MS method development. With guidance from Dr. Chang and Dr. Abbott, I was responsible for the design and conduct of the experiments, data collection, analysis and interpretation of the results, and writing the manuscript. Dr. Teng and Andras Szeitz also reviewed and provided suggestions to the manuscript.

Chapter 4. With guidance from Dr. Chang and Dr. Abbott, I was responsible for the design and conduct of the experiments, data collection, analysis, and interpretation of the results, and writing this chapter. Chapter 4 will be further revised and submitted for publication.

Animal Care Ethics. All animal experiments mentioned in Chapters 2, 3, and 4 of this thesis were approved by The University of British Columbia Animal Care Committee under the Animal Care Certificate Nos. A08-0347 and A12-0302. These experiments were conducted in accordance to the guidelines of Canadian Council on Animal Care.

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List of Abbreviations

1-ABT	1-aminobenzotriazole
4'-OH-DFN	4'-hydroxy diclofenac
5-OH-DFN	5-hydroxy diclofenac
APAP	acetaminophen
BROD	7-benzyloxyresorufin *O*-dealkylation
BSO	DL-buthionine-[S,R]-sulfoximine
CPT 1A	carnitine palmitoyl-transferase 1A
DCF	2′,7′-dichlorofluorescein
DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
DEM	diethyl maleate
DFN	diclofenac
DFN-G	diclofenac acyl-β-D-glucuronide
DMSO	dimethylsulfoxide
GABA	γ-aminobutyric acid
GC-MS	gas chromatography–mass spectrometry
GSH	glutathione
HPLC	high performance liquid chromatography
HQC	high quality control sample
IS	internal standard
LDH	lactate dehydrogenase
LQC	low quality control sample

MQC	middle quality control sample
MRM	multiple reaction monitoring
NAC	*N*-acetylcysteine
PB	phenobarbital
ROS	reactive oxygen species
SIM	selected ion monitoring
TIC	total ion current
UDP-GA	uridine 5'-diphospho-glucuronic acid
UGT	uridine 5'-diphospho-glucuronosyltransferase
UHPLC-MS/MS	ultra-high performance liquid chromatography – tandem mass spectrometry
VPA	valproic acid
VPA-G	valproyl 1-*O*-β acyl glucuronide

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Dedicated to My Family

Chapter 1: Introduction

1.1 Drug-Induced Hepatotoxicity

Drug-induced hepatotoxicity refers to the occurrence of liver injury accompanied by an impairment of liver function, resulting from exposure to drugs or herbal medicines and is one of the major causes for acute liver failures in the United States (Navarro and Senior, 2006; Bjornsson, 2010). Evidence for drug-induced hepatotoxicity leads to attrition of drugs during late-stage clinical development, refusal of drug approvals, and post-marketing withdrawals or black-box warnings of drugs that cause huge financial losses to pharmaceutical companies (Davern, 2012; Kaplowitz, 2005; Regev, 2013). There are no reliable biomarkers for drug-induced hepatotoxicity, but clinical manifestations include fatigue, anorexia, nausea, vomiting, right upper quadrant abdominal discomfort, and dark urine (Davern, 2012). Presence of these symptoms together with biochemical evidence of liver injury (elevation of serum transaminases) and impaired liver function (high serum levels of total or conjugated bilirubin, low serum albumin, and prolonged prothrombin time) are suggestive of drug-induced hepatotoxicity, after all other probable causes are ruled out (Navarro and Senior, 2006). The prognosis of drug-induced hepatotoxicity depends on the severity and extent of liver damage. Whereas the majority of patients undergoing drug-induced hepatotoxicity recover following discontinuation of the hepatotoxic drug, severe cases of drug-induced hepatotoxicity may lead to liver failure resulting in fatality or necessitating an urgent liver transplantation in those patients (Bjornsson, 2010). Treatment options are limited to cessation of the perpetrator drug and general palliative therapy, except in cases involving acetaminophen and valproic acid, where administration of *N*-

acetylcysteine and L-carnitine, respectively, are suggested to be beneficial in improving the clinical outcome (Senior, 2007).

Based on the pattern of liver injury, drug-induced hepatotoxicity can be classified as hepatocellular (e.g., acetaminophen, valproic acid), cholestatic (e.g., amoxicillin-clavulanic acid, phenothiazines), or mixed type (e.g., amitryptiline, sulfonamides) liver injuries (Navarro and Senior, 2006). From a mechanistic perspective, drug-induced hepatotoxicity is classified into two major types: intrinsic and idiosyncratic hepatotoxicity (Russmann et al., 2009).

1.1.1 Intrinsic Hepatotoxicity

Intrinsic hepatotoxicity, which is also known as dose-dependent hepatotoxicity, is predictable and occurs in most patients when a hepatotoxic drug is taken at doses exceeding a threshold dose (Russmann et al., 2009). This type of hepatotoxicity exhibits a consistent period of latency following drug exposure (Roth and Ganey, 2010). Drugs producing dose-dependent hepatotoxicity can be detected during preclinical animal toxicity studies (Regev, 2013). A classic example of an intrinsic hepatotoxin is acetaminophen (APAP), which is responsible for more than half of the acute liver failure cases in the United States. APAP hepatotoxicity results from metabolic activation of APAP to an electrophilic metabolite, *N*-acetylbenzoquinoneimine (NAPQI) which leads to subsequent depletion of glutathione (GSH), covalent protein binding, and oxidative stress (Russmann et al., 2009).

1.1.2 Idiosyncratic Hepatotoxicity

Idiosyncratic hepatotoxicity is rare, unpredictable, not dose-dependent, and occurs only in a few susceptible individuals (Regev, 2013). Unlike intrinsic hepatotoxicity, the latency

period is highly variable and can range from a few days to a year (Navarro and Senior, 2006). Although idiosyncratic hepatotoxicity is considered to be dose-independent, the incidence appears to be higher for drugs that are administered at doses ≥ 50 mg/day, when compared to drugs with daily doses of ≤ 10 mg/day or 10-50 mg/day (Lammert et al., 2008). In addition to drug-related causes, a convergence of genetic and environmental factors predispose a susceptible individual to develop idiosyncratic hepatotoxicity to a particular drug (Kaplowitz, 2005). Preclinical and clinical studies are usually not helpful in detecting drugs that are capable of producing idiosyncratic hepatotoxicity. With a reported incidence of ≤ 1 in 10,000 drug-induced idiosyncratic hepatotoxicities being encountered, mostly during post-marketing use of drugs, presents a formidable challenge to physicians, pharmaceutical companies, and regulatory agencies (Navarro and Senior, 2006; Regev, 2013).

Idiosyncratic hepatotoxicity can be either allergic or non-allergic. Allergic idiosyncratic hepatotoxicity is usually delayed, occurs shortly after repeat exposure to the hepatotoxic drug, and is accompanied by typical signs of an adaptive immune response such as fever, rash, eosinophilia, and the presence of autoantibodies (Russmann et al., 2009). Phenytoin, nitrofurantoin, and halothane are some of the drugs that cause allergic idiosyncratic hepatotoxicity (Navarro and Senior, 2006). Non-allergic idiosyncratic hepatotoxicity is characterized by a sudden and unpredictable onset of toxicity after a long latent period of several months and the lack of hypersensitivity-like symptoms. Examples of drugs associated with this type of hepatotoxicity include bromfenac, troglitazone, and valproic acid (Kaplowitz, 2005).

1.2 Valproic Acid

Valproic acid (2-propylpentanoic acid; VPA; Fig. 1.1) is an antiepileptic drug with a broad spectrum of activity. Following the serendipitous discovery of its anticonvulsant activity by Pierre Eymard in 1963, VPA has been used in antiepileptic therapy since 1967 in France (Simon and Penry, 1975) and since 1978 in the U.S.A. (Koch-Weser and Browne, 1980). Chemically, VPA is a simple eight carbon branched-chain fatty acid (Fig. 1), which makes it unique from all the other anticonvulsant drugs (Bialer, 2012; Loscher and Vetter, 1985).

1.2.1 Mechanism of Action

VPA exerts its anticonvulsant effects by multiple mechanisms, all of which are not fully understood. VPA has been shown to enhance the inhibitory neurotransmission mediated by γ-aminobutyric acid (GABA) in specific regions of the brain which are concerned with the development of seizures (Loscher and Vetter, 1985). An attenuation of GABA degradation resulting from the inhibition of the enzymes GABA transaminase and succinate semialdehyde dehydrogenase, as well as an increase in GABA biosynthesis owing to the activation of the enzyme glutamic acid decarboxylase have been suggested to be responsible for the increase in GABA levels and GABAergic neurotransmission caused by VPA (Loscher, 1981a; Loscher, 1981b). Other effects of VPA include inhibition of the synaptic release of γ-hydroxybutyric acid (Vayer et al., 1988) and attenuation of sodium channel conductance (McLean and Macdonald, 1986). However, the relevance and relative contribution of these effects to the anticonvulsant effects of VPA have not been established.

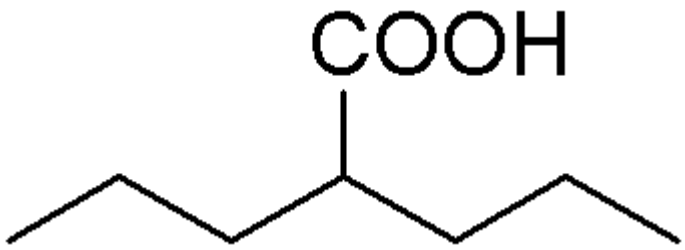

Figure 1.1 Chemical Structure of Valproic Acid.

1.2.2 Therapeutic Indications

VPA has a broad spectrum of activity against several types of seizures, and is used in the management of generalized tonic clonic seizures, myoclonic seizures, absence seizures, partial seizures, and status epilepticus (Chateauvieux et al., 2010; Guerrini, 2006; Johannessen and Johannessen, 2003). Besides epilepsy, VPA is also useful in several neuro-psychiatric indications, such as bipolar disorder, migraine, and anxiety (Ghodke-Puranik et al., 2013). The discovery of inhibition of histone deacetylases by VPA has opened the potential for its use in anticancer therapy (Gurvich et al., 2004), and is currently being evaluated for its effects on hematological and solid tumors (Tan et al., 2010).

1.2.3 Biotransformation

VPA undergoes extensive hepatic biotransformation in humans, with only ~3% of the administered dose excreted unchanged as parent drug in urine (Gugler et al., 1977; Silva et al., 2008). The major pathways of VPA metabolism include β-oxidation, cytochrome P450-mediated oxidation, and glucuronidation.

1.2.3.1 β-Oxidation

VPA, being a medium-chain fatty acid, undergoes mitochondrial β-oxidation resulting in the formation of typical β-oxidation metabolites, such as (*E*)-2-ene-VPA, 3-OH-VPA, and 3-keto-VPA in rats (Matsumoto et al., 1976; Li et al., 1991) and humans (Kuhara et al., 1978). Previous studies on the effects of fasting and glucose infusion on VPA metabolism in both rats (Koch et al., 1989a) and humans (Koch et al., 1989b) not only support the role of fatty acid β-oxidation in VPA metabolism, but also suggest a competition between VPA and endogenous

fatty acids for the enzymes and co-factors involved in β-oxidation (Baillie and Levy, 1991). Other metabolites that are formed in this pathway include non-classical β-oxidation metabolites, such as 3-ene-VPA and its secondary metabolite (*E*,*E*)-2,3'-diene-VPA (Bjorge and Baillie, 1991). The chemical structures of the β-oxidation metabolites of VPA are shown in Fig. 1.2. β-oxidation of 4-ene-VPA, which is a cytochrome P450-mediated metabolite of VPA (Rettie et al., 1987), results in the formation of another secondary metabolite of VPA namely (*E*)-2,4-diene-VPA (Fig. 1.3) (Rettenmeier et al., 1985). The β-oxidation metabolite 3-keto-VPA-CoA is reported to be resistant to thiolytic cleavage by 3-keto-acyl-CoA thiolase (Li et al., 1991), whereas another *in vitro* study has shown that VPA could undergo one full cycle of β-oxidation, resulting in the thiolytic cleavage of 3-keto-VPA-CoA to form propionyl-CoA and pentanoyl-CoA (Silva et al., 2002). β-oxidation was reported to account for the metabolism of over 35% of the administered dose of VPA in patients on monotherapy with VPA (Levy et al., 1990). Although peroxisomes are capable of catalyzing the β-oxidation of VPA-CoA (Vamecq et al., 1993), mitochondrial enzymes appear to be predominantly responsible for the metabolism of VPA through β-oxidation (Bjorge and Baillie, 1991; Draye and Vamecq, 1987).

1.2.3.2 Cytochrome P450-Mediated Oxidation

Cytochrome P450 enzymes catalyze the metabolism of VPA *via* ω-, ω-1-, and ω-2-hydroxylation pathways, leading to the formation of 5-OH-VPA, 4-OH-VPA, and 3-OH-VPA, respectively (Prickett and Baillie, 1984). Thus, the metabolite 3-OH-VPA can originate from both β-oxidation and cytochrome P450-mediated oxidation of VPA (Prickett and Baillie, 1984; Rettenmeier et al., 1987). Cytochrome P450 enzymes are responsible for the direct desaturation of VPA to form 4-ene-VPA, which is also derived from a common intermediate in ω- or ω-1-

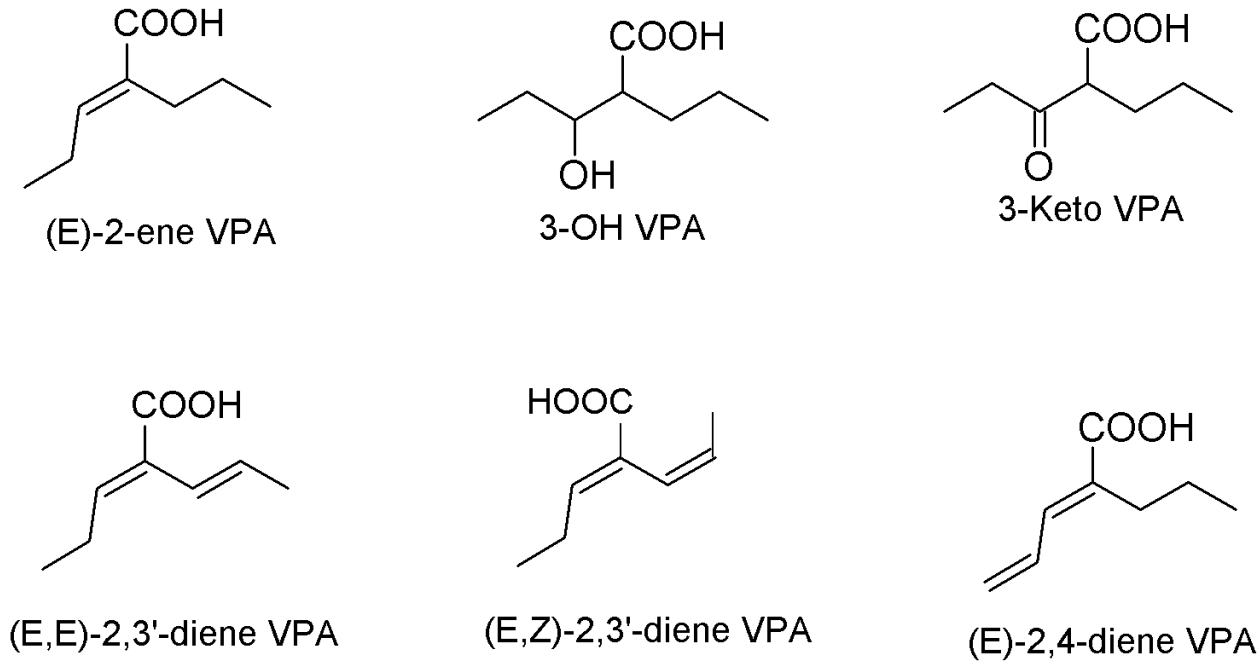

Figure 1.2 Chemical Structures of β-Oxidation Metabolites of VPA (Abbott and Anari, 1999).

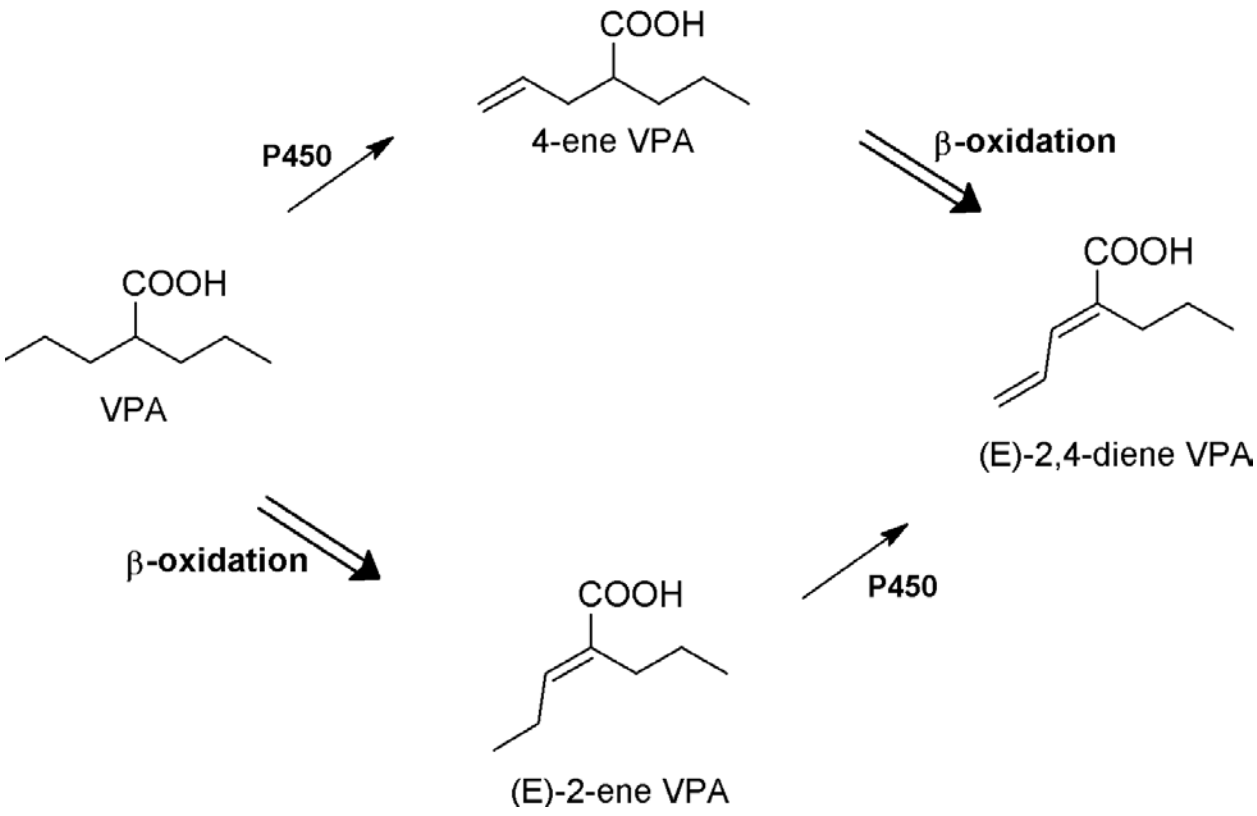

Figure 1.3 Metabolic Pathways Involved in the Formation of (*E*)-2,4-Diene-VPA (Abbott and Anari, 1999).

hydroxylation pathways (Rettie et al., 1987). Subsequent oxidation of 4-OH-VPA leads to the formation of 4-keto-VPA and a dicarboxylic acid metabolite, 2-propyl succinic acid (2-PSA), whereas 5-OH-VPA undergoes further oxidation to form another dicarboxylic acid, 2-propyl glutaric acid (2-PGA) (Granneman et al., 1984). The chemical structures of the cytochrome P450-mediated metabolites of VPA are shown in Fig. 1.4. As mentioned earlier, β-oxidation of 4-ene-VPA generates the reactive metabolite (*E*)-2,4-diene-VPA (Granneman et al., 1984; Rettenmeier et al., 1985), which can also be formed by cytochrome P450-mediated oxidation of the β-oxidation metabolite (*E*)-2-ene-VPA (Fig. 1.3) (Fabre et al., 1992). Although cytochrome P450-mediated oxidation contributes less than 10% of the total metabolism of VPA in patients under VPA monotherapy (Levy et al., 1990), this pathway is of toxicological interest as the formation of 4-ene-VPA and (*E*)-2,4-diene-VPA has been implicated in VPA hepatotoxicity. Human cytochromes P450 2A6, 2B6, and 2C9 have been shown to catalyze the formation of 4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA in human liver microsomes (Kiang et al., 2006). Whereas rat cytochrome P450 2B enzymes have been reported to mediate the formation of 4-ene-VPA (Rettie et al., 1995), the identity of various rat cytochrome P450 enzymes involved in the oxidation of VPA remains unknown.

1.2.3.3 Glucuronidation

VPA undergoes conjugation with uridine 5'-diphospho glucuronic acid (UDP-GA) in the presence of uridine 5'-diphospho-glucuronosyltransferases (UGTs) to form valproyl 1-*O*-β acyl glucuronide (VPA-G; Fig. 1.5) (Ethell et al., 2003; Kuhara et al., 1978). Glucuronidation is a major metabolic pathway for VPA, with ~ 40% of the administered dose of VPA subject to glucuronidation in patients undergoing monotherapy (Levy et al., 1990). VPA-G, which is

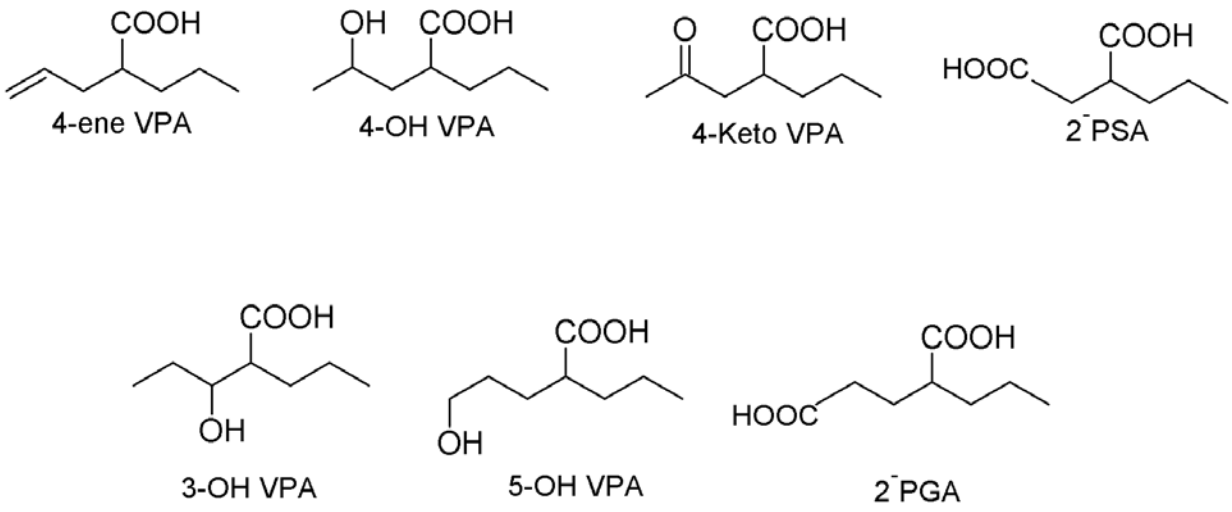

Figure 1.4 Chemical Structures of Cytochrome P450-Mediated Metabolites of VPA (Abbott and Anari, 1999).

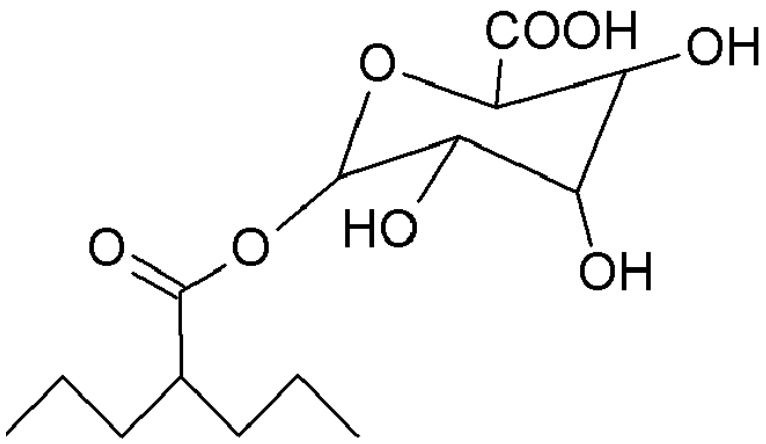

Figure 1.5 Chemical Structure of VPA-G (Abbott and Anari, 1999).

considered to be one of the least reactive and highly stable acyl glucuronides (Stachulski et al., 2006), has been shown to undergo intra-molecular rearrangement to form positional isomers that are resistant to β-glucuronidase-mediated hydrolysis (Dickinson et al., 1984). Studies with human recombinant UGTs have identified the UGT enzymes, such as UGT1A3, UGT1A4, UGT1A6, UGT1A8, UGT1A9, UGT1A10, and UGT2B7 to be capable of glucuronidating VPA, with recombinant UGT2B7 having the highest intrinsic clearance (Argikar and Remmel, 2009; Ethell et al., 2003). However, the identity of rat UGT enzymes involved in VPA glucuronidation has not been established.

1.2.4 VPA Hepatotoxicity

Therapeutic use of VPA is associated with two types of hepatotoxicity, namely dose-dependent intrinsic hepatotoxicity and idiosyncratic hepatotoxicity (Silva et al., 2008). Dose-dependent hepatotoxicity of VPA is usually characterized by a mild and brief increase in the serum levels of transaminase enzymes, and is reversible following a dose reduction (Silva et al., 2008; Willmore, 1991). On the other hand, VPA therapy sometimes results in a rare idiosyncratic hepatotoxicity, which is frequently irreversible and fatal, and is characterized by microvesicular steatosis with or without hepatic necrosis (Bryant, III and Dreifuss, 1996; Dreifuss et al., 1987; Silva et al., 2008; Zimmerman and Ishak, 1982). Retrospective analyses of VPA-associated hepatic fatalities in humans have revealed that the hepatotoxicity was idiosyncratic and not dose-related, with the susceptibility being greater in patients treated with VPA as part of multi-drug anticonvulsant therapy, mostly with phenytoin and phenobarbital (Dreifuss et al., 1987; Zimmerman and Ishak, 1982). Children in the 0 – 2 years of age group, who were subjected to polytherapy with VPA, were at the greatest risk for VPA hepatotoxicity

(Dreifuss et al., 1987; Zimmerman and Ishak, 1982). The estimated incidence of fatal VPA hepatotoxicity in this group was the highest (1/500), followed by young children (0 – 2 years of age) receiving VPA monotherapy (1/7000), older (age > 2 years) children receiving polytherapy (1/12000), and older children receiving monotherapy (1/40000) (Bryant, III and Dreifuss, 1996; Dreifuss et al., 1987; Zimmerman and Ishak, 1982). In addition to young age and polytherapy with cytochrome P450-inducing anticonvulsants, other risk factors included mental retardation, developmental delays, presence of other neurological diseases and metabolic disorders (Dreifuss et al., 1987; Zimmerman and Ishak, 1982). Fatal hepatotoxicity has also been reported in patients receiving VPA, including adults on monotherapy, who did not have any recognized risk factors (Bauer, 2005; Koenig et al., 2006). This, together with the expanding therapeutic indications for VPA, emphasizes the need for a better understanding of the mechanisms of VPA hepatotoxicity.

In the majority of cases, the onset of hepatotoxicity is within the first six months of VPA therapy and is often characterized by symptoms like nausea, vomiting, anorexia, lethargy, jaundice, generalized edema, ascites, hemorrhage and an exacerbation of seizures (Zimmerman, 1991; Zimmerman and Ishak, 1982). Absence of fever, rash, and eosinophilia suggest that there is no involvement of immune-mediated hypersensitivity reaction (Riela, 1991). Biochemical analyses revealed increases in serum levels of transaminases, ammonia, bilirubin, and also hypoglycemia. Though sometimes useful, these biochemical alterations were not always suggestive of the underlying VPA-induced hepatotoxicity (Dreifuss et al., 1987). Histologically, most cases of VPA hepatotoxicity are characterized by the presence of microvesicular steatosis in liver, and are often accompanied by necrosis (Dreifuss et al., 1987; Zimmerman and Ishak, 1982). The exact mechanism of VPA-associated idiosyncratic hepatotoxicity is unknown.

However, various mechanisms have been proposed to explain this phenomenon, which include the formation of reactive metabolites of VPA, interference with mitochondrial β-oxidation of fatty acids, and development of oxidative stress (Chang and Abbott, 2006). The major focus of this dissertation was to investigate the toxicity of *in situ* generated reactive metabolites of VPA, (*E*)-2,4-diene-VPA and VPA-G in sandwich-cultured rat hepatocytes, and to assess their role in the concentration-dependent toxicity of VPA in sandwich-cultured rat hepatocytes.

1.2.5 Proposed Mechanisms of VPA Hepatotoxicity

1.2.5.1 Formation of Reactive Metabolites of VPA

Retrospective analysis of VPA-associated fatal liver injuries suggest that polytherapy with cytochrome P450 enzyme-inducing anticonvulsants increase the risk of VPA hepatotoxicity by 14-fold over monotherapy with VPA among children in the age group of 0 – 2 years (Zimmerman and Ishak, 1982). This led to the proposition that cytochrome P450-mediated formation of reactive metabolites such as 4-ene-VPA could be responsible for VPA-associated hepatotoxicity (Rettie et al., 1987). Synthetic 4-ene-VPA is toxic to cultured rat hepatocytes (Kingsley et al., 1983) and produces hepatic steatosis in rats (Kesterson et al., 1984). Although the studies mentioned above indicate the toxic potential of synthetic 4-ene-VPA, recent rodent studies have questioned the role of *in situ* generated 4-ene-VPA in VPA hepatotoxicity. Inhibition of the metabolic formation of 4-ene-VPA by the use of 1-aminobenzotriazole (1-ABT), which is a mechanism-based inactivator of cytochrome P450 enzymes (Ortiz de Montellano and Mathews, 1981), does not attenuate VPA-associated oxidative stress in rats (Tong et al., 2003). Similarly, pretreatment of sandwich-cultured rat hepatocytes with 1-ABT attenuates the *in situ* formation of 4-ene-VPA, but does not influence the toxicity of VPA as

determined by the markers of cell viability, oxidative stress, necrosis, and cellular content of total GSH (Kiang et al., 2010; Kiang et al., 2011). Nevertheless, studies with the fluorinated analogue of VPA, α-fluoro-VPA, appear to suggest a role for the reactive metabolites of VPA in VPA hepatotoxicity. α-Fluoro-VPA, which is resistant to metabolism by β-oxidation (Grillo et al., 2001) and glucuronidation (Tang and Abbott, 1997), is either non-toxic or less toxic to sandwich-cultured rat hepatocytes as assessed by the markers of cell viability, oxidative stress, necrosis, and cellular content of total GSH (Kiang et al., 2010; Kiang et al., 2011).

4-Ene-VPA has been shown to undergo further metabolism by β-oxidation to form a reactive metabolite, (*E*)-2,4-diene-VPA (Granneman et al., 1984; Rettenmeier et al., 1985). The fluorinated analogue of 4-ene-VPA, α-fluoro-4-ene-VPA, which is resistant to mitochondrial β-oxidation, does not produce steatosis in rats and is less able to deplete hepatic content of total GSH (Tang et al., 1995). Furthermore, the detection of glutathione- and *N*-acetylcysteine (NAC)-conjugates of (*E*)-2,4-diene-VPA in the bile and urine samples, respectively, of rats administered 4-ene-VPA or (*E*)-2,4-diene-VPA implied a role for (*E*)-2,4-diene-VPA in VPA hepatotoxicity (Kassahun et al., 1991). NAC conjugates of (*E*)-2,4-diene-VPA are also found in the urine samples of patients treated with VPA (Gopaul et al., 2000; Kassahun et al., 1991), with the levels of a NAC conjugate reported to be 3-4 times greater in patients with VPA-associated hepatic failure than patients with no hepatotoxicity (Kassahun et al., 1991). Another study reported an increased urinary excretion of NAC conjugates of (*E*)-2,4-diene-VPA in VPA patients aged $\leq$ 7.5 years or undergoing polytherapy with cytochrome P450-enzyme inducing anticonvulsants, suggesting an increased systemic exposure to this metabolite in patients with known risk factors of VPA hepatotoxicity (Gopaul et al., 2003). GSH conjugation of (*E*)-2,4-diene-VPA has also been reported *in vitro* in a reaction catalyzed by rat GST enzymes, which

happened more readily when this metabolite was present in an activated ester-form e.g., (*E*)-2,4-diene-VPA-CoA thioester, (*E*)-2,4-diene-VPA-*N*-acetylcysteamine thioester, or (*E*)-2,4-diene-VPA-glucuronide (Tang and Abbott, 1996; Tang et al., 1996). Synthetic (*E*)-2,4-diene-VPA was also reported to be more potent than VPA in its toxicity to cultured rat hepatocytes (Kiang et al., 2010; Kiang et al., 2011) and in producing hepatic steatosis in rats (Kesterson et al., 1984). Although the above findings indicate the reactive nature of (*E*)-2,4-diene-VPA and the toxic potential of synthetic (*E*)-2,4-diene-VPA, it is still not known whether *in situ* generated (*E*)-2,4-diene-VPA is toxic to hepatocytes and if this metabolite plays a causal role in VPA hepatotoxicity.

Glucuronidation of VPA results in the formation of an acyl glucuronide, VPA-G. Acyl glucuronides, in general, are reactive electrophilic metabolites (Boelsterli and Ramirez-Alcantara, 2011), and are implicated in the toxicities associated with carboxylic acid drugs, although there is no evidence for the causal association (Boelsterli, 2011). Despite being classified as one of the least reactive and most stable acyl glucuronides (Skonberg et al., 2008), a previous study has reported that VPA-G undergoes pH-, temperature-, and time-dependent intra-molecular rearrangement to form 2-, 3-, or 4-*O* positional isomers, which were resistant to β-glucuronidase-mediated hydrolysis (Dickinson et al., 1984). Incubation of VPA with isolated rat hepatocytes was reported to cause covalent binding of VPA to hepatocellular proteins, which is attenuated, at least partly, by the presence of borneol (Porubek et al., 1989), which is a known inhibitor of glucuronidation (Watkins and Klaassen, 1983). Furthermore, the increases in the plasma and hepatic concentrations of 15-F_{2t}-isoprostane, which is an *in vivo* marker for lipid peroxidation, in rats treated with VPA was linked to VPA-G formation (Tong et al., 2005c).

Thus, it is possible that VPA-G may play a role in VPA-associated hepatotoxicity and this proposition has not been investigated in an experimental model.

1.2.5.2 Interference with β-Oxidation of Fatty Acids

The presence of microvesicular steatosis in the livers of patients who underwent VPA hepatotoxicity and the reported similarity of VPA hepatotoxicity to Reye's syndrome suggest an inhibition of mitochondrial β-oxidation of endogenous fatty acids by VPA (Fromenty and Pessayre, 1995). Multiple mechanisms are suggested to be responsible for VPA-associated impairment of mitochondrial β-oxidation (Silva et al., 2008). Besides the consumption of free CoASH during β-oxidation of VPA and its metabolites (Becker and Harris, 1983; Ponchaut et al., 1992; Thurston and Hauhart, 1993; Li et al., 1991), branched chain CoA thioesters of VPA and its metabolites were reported to be relatively less susceptible to hydrolysis, thereby resulting in accumulation of VPA-CoA and metabolite-CoA thioesters, and subsequent depletion of the cellular CoA pool (Moore et al., 1988; Silva et al., 2002; Li et al., 1991). Studies have suggested that VPA-associated hypocarnitinemia (Lheureux and Hantson, 2009; Nakajima et al., 2011; Melegh et al., 1987) and the reported inhibition of human hepatic carnitine palmitoyl-transferase 1A (CPT 1A) enzyme by VPA-CoA (Aires et al., 2010) could affect the transport of long chain fatty acids into mitochondria via 'carnitine shuttle', leading to impaired β-oxidation and accumulation of fatty acids. Accordingly, administration of L-carnitine was associated with a protective effect against VPA-associated toxicity in rat hepatocytes (Takeuchi et al., 1988) and carnitine deficiency in rats (Nishida et al., 1987; Sugimoto et al., 1987). Similarly, intravenous administration of L-carnitine has been reported to increase the chances of survival in VPA hepatotoxicity (De Vivo et al., 1998; Lheureux et al., 2005); however the therapeutic use of L-

carnitine has not been evaluated in controlled clinical trials (Lheureux and Hantson, 2009). Besides CoA depletion and carnitine deficiency, VPA and/or its metabolites have been reported to cause direct inhibition of enzymes involved in mitochondrial β-oxidation. VPA-CoA, but not VPA, was reported to inhibit the activities of human short-chain acyl-CoA dehydrogenase and medium-chain acyl-CoA dehydrogenase *in vitro* (Ito et al., 1990). The observed resistance of 3-keto-VPA-CoA to thiolytic cleavage by 3-keto-acyl-CoA thiolase led to a postulation by Li et al. (1991) that 3-keto-VPA-CoA could be an inhibitor of the β-oxidation enzymes, 3-keto-acyl-CoA thiolase and 3-hydroxy-acyl-CoA dehydrogenase. The hypoglycemic agent 4-pentenoic acid undergoes β-oxidation to form a highly reactive metabolite, 3-keto-4-pentenoic acid, which is a potent inhibitor of the terminal β-oxidation enzyme 3-keto-acyl-CoA thiolase (Schulz, 1983). Based on the structural similarity of 4-ene-VPA to that of 4-pentenoic acid, it was proposed that the β-oxidation metabolite of 4-ene-VPA, namely 3-keto-4-ene-VPA could be an inhibitor of the β-oxidation enzyme 3-keto-acyl-CoA thiolase, and be responsible for the toxicity of VPA and its metabolites (Baillie, 1988), although these propositions have yet to be experimentally verified.

1.2.5.3 Development of Oxidative Stress

Oxidative stress refers to the state wherein the formation of reactive oxygen species (ROS) exceeds the ability of the cellular antioxidant defense mechanism to remove the ROS (Chen et al., 2013). Oxidative stress is now recognized as one of the mechanisms leading to idiosyncratic drug-induced liver injury (Amacher, 2012). Various studies have postulated that oxidative stress could play a contributory role in VPA-associated hepatotoxicity (Graf et al., 1998; Klee et al., 2000; Tabatabaei et al., 1999). Whereas few rodent and human studies have reported a lack of oxidative stress following VPA administration (Seckin et al., 1999; Ono et al.,

2000; Aycicek and Iscan, 2007), there are many studies that have shown that VPA causes an elevation of oxidative stress biomarkers in rats (Tong et al., 2003; Tong et al., 2005b; Natarajan et al., 2006; Jafarian et al., 2013) as well as humans (Michoulas et al., 2006; Varoglu et al., 2010). Few studies have suggested hydrogen peroxide (H_2O_2) to be the ROS involved in VPA-associated oxidative stress (Graf et al., 1998; Natarajan et al., 2006; Olson et al., 1986; Tabatabaei et al., 1999); however, there has been no systematic investigation of the involvement of various ROS in the oxidative stress caused by VPA. Following administration of VPA to rats, development of oxidative stress precedes the onset of hepatotoxicity, hepatic steatosis, and necrosis (Tong et al., 2005a); but, there is no evidence of a causative role for oxidative stress in VPA hepatotoxicity. VPA and/or its metabolites have been shown to cause depletion of hepatic GSH in rats (Tang et al., 1995) and cultured rat hepatocytes (Jurima-Romet et al., 1996; Kiang et al., 2011), and furthermore, prior depletion of GSH by DL-buthionine-[S,R]-sulfoximine (BSO) and diethyl maleate (DEM) increases the toxicity of VPA (Tong et al., 2005b) or its metabolites (Jurima-Romet et al., 1996) in hepatocytes. However, recent time course experiments have indicated that GSH depletion is not responsible for the onset of oxidative stress in sandwich-cultured rat hepatocytes treated with VPA (Kiang et al., 2011).

Studies in cultured rat hepatocytes have been performed to investigate whether VPA causes oxidative stress, as determined by the assessment of various markers. Formation of 2′,7′-dichlorofluorescein (DCF) from 2′,7′-dichlorodihydrofluorescein diacetate (DCF DA) is a commonly used marker for the assessment of intracellular oxidative stress (Kalyanaraman et al., 2012). Intracellular hydrolysis of DCFDA yields 2′,7′-dichlorofluorescin (DCFH), which subsequently gets oxidized to form a highly fluorescent product DCF (Bartosz, 2006). DCF assay is one of the most widely used markers for oxidative stress, despite several limitations of

this assay which include the requirement of a catalyst to react with ROS, its lack of specificity for H_2O_2, self-generation of H_2O_2 during the oxidation of DCFH, and its susceptibility to autooxidation (Winterbourn, 2014). Treatment of rat hepatocytes with VPA results in concentration- and time-dependent increases in DCF formation (Kiang et al., 2010; Tong et al., 2005b), and this was consistent with the increase in the concentration of 15-F_{2t}-isoprostanes (Tong et al., 2005b), which is a marker for lipid peroxidation (Halliwell and Whiteman, 2004). Depletion of cellular GSH by BSO and DEM further enhanced VPA-mediated oxidative stress in rat hepatocytes, as evident from DCF formation and the concentration of 15-F_{2t}-isoprostanes (Tong et al., 2005b). The experimental evidence described above suggests the development of oxidative stress following VPA treatment *in vitro* and *in vivo*; however, whether VPA-associated oxidative stress has any role in the hepatotoxicity of VPA is still not known.

1.2.6 Sandwich-Cultured Rat Hepatocytes

Primary cultures of hepatocytes are effective *in vitro* models for the investigation of metabolism and toxicity of xenobiotics (Wang et al., 2002). However, hepatocytes cultured in monolayers undergo rapid de-differentiation and lose the expression of drug metabolizing enzymes, hepatobiliary transporter proteins, and other liver-specific functions (Olsavsky Goyak et al., 2010). Sandwich-cultured rat hepatocytes, which refer to the culture of primary rat hepatocytes between two layers of extracellular matrix, overcomes these limitations and helps to maintain the hepatocytes in a differentiated state and retain hepatocyte-specific functions for up to a few weeks (Dunn et al., 1991; De Bruyn et al., 2013). In addition to promoting longevity, hepatocytes cultured in a sandwich-configuration are known to exhibit polarized cell architecture (sinusoidal and canalicular surfaces), site-specific expression of specific transporter proteins, and

also form functional biliary canalicular networks (Swift et al., 2010). Various factors such as the culture configuration, extracellular matrix, cell density, culture medium composition, presence of serum and other supplements have been shown to influence the morphology and function of hepatocytes (Gross-Steinmeyer et al., 2005; Kern et al., 1997; LeCluyse et al., 1999; Mingoia et al., 2007; Richert et al., 2002; Turncliff et al., 2006; Tuschl and Mueller, 2006). Thus, a potential limitation of the sandwich-cultured rat hepatocyte model is that the culture conditions employed could significantly impact the results obtained, necessitating a careful choice of the experimental conditions for this model. This limitation should be considered while comparing the results from different studies involving sandwich-cultured rat hepatocytes. The present study employed rat hepatocytes cultured in a Matrigel-Matrigel sandwich configuration in the presence of Williams' medium E supplemented with insulin and dexamethasone. Rat hepatocytes cultured in Matrigel-Matrigel sandwich configuration have been shown to appear rounded, compact, and form spheroidal aggregates (Moghe et al., 1996). Culturing in Williams' medium E was reported to facilitate the formation of biliary canalicular networks (Turncliff et al., 2006) and yield the highest degree of cytochrome P450 2B induction (LeCluyse et al., 1999) in rat hepatocytes when compared to other media formulations. Prolonged cell survival and function, increased formation of canalicular networks, enhanced expression of cytochrome P450 enzymes and hepatocellular uptake/efflux transporters, and better induction of cytochrome P450 enzymes are some of the advantages of supplementing the hepatocyte culture medium with insulin and dexamethasone (De Bruyn et al., 2013).

1.2.6.1 Use of Sandwich-Cultured Rat Hepatocytes to Study Drug-Induced Hepatotoxicity

The sandwich-cultured rat hepatocyte model is an useful tool for the mechanistic investigation of drug-induced hepatotoxicity, because of its general ability to maintain liver specific functions, including drug metabolism and transport (Swift et al., 2010). However, the culture time-dependent expression and function of individual drug metabolizing enzymes and transporter proteins are highly dependent on the exact culture conditions and vary widely between different studies (De Bruyn et al., 2013). Sandwich-cultured rat hepatocytes were shown to exhibit greater sensitivity to the metabolism-mediated toxicity of acetaminophen, diclofenac, and cyclophosphamide, when compared to 3T3, HeLa, and HepG2 cell lines (Wang et al., 2002). Contrarily, Farkas and Tannenbaum (2005) reported a lack of sensitivity of sandwich-cultured rat hepatocytes to the metabolism-mediated toxicity of acetaminophen, possibly due to the loss of cytochrome P450 2E1 enzymes in culture. Recently, sandwich-cultured rat hepatocytes were successfully employed to study the role of *in situ* generated acyl glucuronides in the covalent protein binding and toxicity of non-steroidal anti-inflammatory drugs, such as benoxaprofen, flunoxaprofen, and ibuprofen (Dong and Smith, 2009). Sandwich-cultured rat hepatocytes have also been used to investigate the role of hepatobiliary transport in the hepatotoxicity of various drugs (Lee et al., 2008; Chatterjee et al., 2014; Marion et al., 2011).

1.2.6.2 VPA Toxicity in Sandwich-Cultured Rat Hepatocytes

Treatment of sandwich-cultured rat hepatocytes with VPA resulted in a concentration-dependent decrease in markers of cell viability (formazan formation) and cellular antioxidant status (total GSH content), and an increase in markers of oxidative stress (DCF formation), cell

necrosis (LDH release), and steatosis (BODIPY 558/568 C_{12} accumulation) (Kiang et al., 2010; Kiang et al., 2011; Fujimura et al., 2009). Kiang et al. (2010; 2011) showed that the cytochrome P450-mediated formation of 4-ene-VPA, 4-OH-VPA, 3-OH-VPA, and 5-OH-VPA did not contribute to VPA-associated oxidative stress, necrosis, and GSH depletion in sandwich-cultured rat hepatocytes (Kiang et al., 2010; Kiang et al., 2011). The fluorinated analogue of VPA, α-fluoro-VPA, which is less able to form glucuronide conjugates, CoA thioesters, and (*E*)-2,4-diene-type reactive metabolite, was reported to be non-toxic to sandwich-cultured rat hepatocytes (Kiang et al., 2010) and did not deplete cellular content of total GSH (Kiang et al., 2011). The authors had suggested that mitochondrial β-oxidation and/or glucuronidation could be responsible for the toxicity of VPA in sandwich-cultured rat hepatocytes (Kiang et al., 2010; Kiang et al., 2011). Furthermore, the aforementioned studies were not able to investigate the role of (*E*)-2,4-diene-VPA in VPA toxicity as this metabolite was not detected in their culture conditions. Although the profile of VPA metabolites that are formed *in situ* in sandwich-cultured rat hepatocytes treated with VPA (Kiang et al., 2010) appear to be broadly similar to the metabolite profiles reported in patients undergoing monotherapy with VPA (Anderson et al., 1992; Levy et al., 1990), there are certain differences with respect to individual metabolites. Whereas (*E*)-2,4-diene-VPA was not detectable in sandwich-cultured rat hepatocytes treated with VPA (Kiang et al., 2010), this metabolite was quantified in the plasma (Levy et al., 1990) and urine samples (Anderson et al., 1992; Levy et al., 1990) of patients undergoing VPA monotherapy. Therefore, sandwich-cultured rat hepatocyte model would require pretreatment with a cytochrome P450 inducer, such as phenobarbital, to be useful in evaluating the role of (*E*)-2,4-diene-VPA in VPA toxicity.

1.3 Research Rationale and Hypothesis

VPA is known to cause a rare, but possibly fatal, idiosyncratic hepatotoxicity (Nanau and Neuman, 2013), the mechanism of which is still unknown. 4-Ene-VPA and (*E*)-2,4-diene-VPA, which are the reactive metabolites of VPA, were believed to be responsible for VPA hepatotoxicity (Kesterson et al., 1984). Recent rodent studies have shown that the cytochrome P450-mediated formation of 4-ene-VPA had no role in VPA-associated oxidative stress in rats (Tong et al., 2003) and toxicity in sandwich-cultured rat hepatocytes (Kiang et al., 2010). Although previous studies have shown that *in situ* formed (*E*)-2,4-diene-VPA is highly reactive (Gopaul et al., 2000) and that the synthetic form of (*E*)-2,4-diene-VPA is more hepatotoxic than VPA and 4-ene-VPA (Kesterson et al., 1984; Kiang et al., 2010), it is still not known whether *in situ* generated (*E*)-2,4-diene-VPA is toxic and whether it contributes to VPA hepatotoxicity. In order to make progress towards addressing the aforementioned knowledge gaps, there is a need for further studies to investigate the toxicity of *in situ* generated (*E*)-2,4-diene-VPA in an experimental model and whether modulating the *in situ* formation of this metabolite affects the toxicity of VPA.

VPA undergoes glucuronidation to form an acyl glucuronide, VPA-G (Argikar and Remmel, 2009; Abbott and Anari, 1999). Acyl glucuronides are highly reactive electrophiles, which are unstable and undergo spontaneous hydrolysis and/or intra-molecular acyl migration to form positional isomers (Skonberg et al., 2008). Acyl glucuronides and their positional isomers, which are known to undergo covalent binding with proteins and other nucleophiles through transacylation or glycation mechanisms, have been implicated in the toxicities of several drugs (Regan et al., 2010). Although VPA-G is very stable and less reactive (Stachulski et al., 2006), previous studies have shown that VPA-G undergoes intramolecular acyl migration to form

positional isomers of VPA-G (Dickinson et al., 1984) and covalent protein binding (Cannell et al., 2002; Porubek et al., 1989). A recent study in rats appeared to suggest that VPA-associated oxidative stress, as determined by the increases in plasma and hepatic concentrations of 15-F_{2t}-isoprostanes (Tong et al., 2003), is linked to VPA-G formation (Tong et al., 2005c). Despite its proposed role in VPA-associated oxidative stress, the toxicity of *in situ* generated VPA-G has not been studied yet and its role in VPA hepatotoxicity is also not known. In a recent study (Dong and Smith, 2009), sandwich-cultured rat hepatocyte model was used to assess the exposure and covalent protein binding of acyl glucuronides and the results were found to be consistent with those observed in sandwich-cultured human hepatocytes. Therefore, it would be worthwhile to investigate the toxicity of *in situ* generated VPA-G in sandwich-cultured rat hepatocytes and its contribution, if any, to the toxicity of VPA observed in this model.

Investigating the toxicity of *in situ* generated VPA-G in sandwich-cultured rat hepatocytes requires an accurate and precise quantification of VPA-G, in order to determine if the formation of VPA-G has any effect on the response of toxicity markers. Only few studies have employed a direct quantification of VPA-G, whose lower limits of quantification were 0.125 and 0.5 µg/mL VPA-G (Tong et al., 2005c; Wong et al., 2007). Furthermore, these assays require a large sample volume (0.2 – 0.5 mL). Considering the relatively low levels of metabolite formation in cell culture studies, there is a need for the development of a sensitive method for the accurate quantification of VPA-G in small volumes of sample.

In the present work, the hypothesis is that (*E*)-2,4-diene-VPA and VPA-G generated *in situ* from VPA are toxic to sandwich-cultured rat hepatocytes and contribute to the toxicity of VPA in this experimental model.

1.4 Research Objectives

The research objectives for the individual thesis chapters are as follows:

Chapter 2:

i. To characterize the concentration-response effect of VPA, (*E*)-2-ene-VPA, and (*E*)-2,4-diene-VPA on markers of oxidative stress, steatosis, and necrosis in sandwich-cultured rat hepatocytes.

ii. To determine the effects of modulating the *in situ* formation of (*E*)-2,4-diene-VPA on markers of oxidative stress, steatosis, and necrosis, and cellular total glutathione (GSH) levels in sandwich-cultured rat hepatocytes treated with VPA or (*E*)-2-ene-VPA.

Chapter 3:

i. To develop and validate an ultra-high performance liquid chromatography – tandem mass spectrometry method for the quantification of VPA-glucuronide in culture supernatants of sandwich-cultured rat hepatocytes.

Chapter 4:

i. To identify appropriate experimental conditions to investigate the role of *in situ* generated VPA-G in VPA-associated toxicity using the sandwich-cultured rat hepatocyte model.

ii. To investigate the effects of modulating *in situ* formation of VPA-G on VPA-associated toxicity in sandwich-cultured rat hepatocytes.

Chapter 2: Assessment of the Role of *In Situ* Generated (*E*)-2,4-Diene-Valproic Acid in the Toxicity of Valproic Acid and (*E*)-2-Ene-Valproic Acid in Sandwich-Cultured Rat Hepatocytes

2.1 Introduction

Valproic acid (VPA, Fig. 2.1) is an efficacious broad-spectrum antiepileptic drug (Owens, 2010) that has several other indications, including bipolar disorder and migraine (Rosenberg, 2007). VPA therapy, although well-tolerated in most patients, is associated with a rare, but potentially fatal, idiosyncratic hepatotoxicity (Gerstner et al., 2008) characterized by the presence of microvesicular steatosis (Zimmerman and Ishak, 1982). The mechanism of VPA hepatotoxicity is not yet known; however, formation of reactive metabolites and development of oxidative stress have been proposed to explain the toxicity (Chang and Abbott, 2006).

VPA undergoes extensive biotransformation by multiple pathways; namely, glucuronidation, β-oxidation, and cytochrome P450-mediated oxidation (Silva et al., 2008). As illustrated in Fig. 2.1, cytochrome P450 enzymes desaturate VPA to form 4-ene-VPA, which upon subsequent mitochondrial β-oxidation, yields *(E)*-2,4-diene-VPA (Abbott and Anari, 1999). *(E)*-2,4-diene-VPA can also be formed by cytochrome P450-mediated terminal desaturation of *(E)*-2-ene-VPA (Fabre et al., 1992; Kassahun and Baillie, 1993), which is a mitochondrial β-oxidation metabolite of VPA (Fig. 2.1). *(E)*-2,4-diene-VPA has been linked to the hepatotoxicity of VPA based on the following experimental findings: 1) products of glutathione (GSH) conjugates of *(E)*-2,4-diene-VPA are present in the bile and urine samples of rats treated with

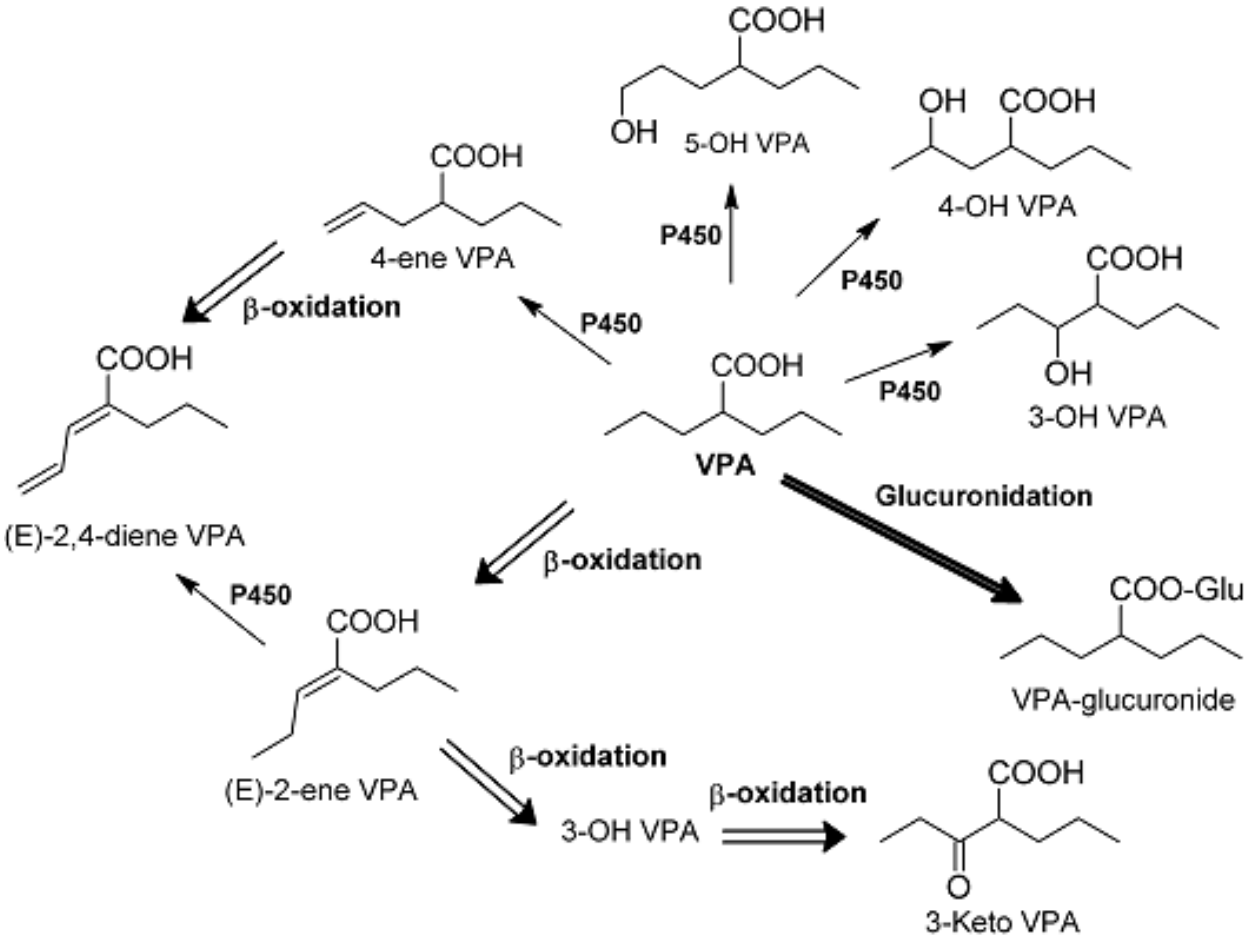

Figure 2.1 Simplified Schematic Representation of the Major Biotransformation Pathways of VPA (as modified from Chang and Abbott (2006)).

4-ene-VPA or (*E*)-2,4-diene-VPA (Kassahun et al., 1991) and in urine samples of patients treated with VPA (Gopaul et al., 2000; Kassahun et al., 1991); and 2) administration of large dosages/concentrations of synthetic (*E*)-2,4-diene-VPA leads to hepatic microvesicular steatosis in rats (Kesterson et al., 1984) and cytotoxicity in sandwich-cultured rat hepatocytes (Kiang et al., 2010). The mechanisms proposed for the cytotoxicity of (*E*)-2,4-diene-VPA include depletion of the mitochondrial GSH pool (Kassahun et al., 1991; Tang et al., 1995) and inhibition of mitochondrial β-oxidation (Kesterson et al., 1984; Ponchaut et al., 1992). A recent study showed that the *in situ* generated 4-ene-VPA, 4-OH-VPA, 3-OH-VPA, and 5-OH-VPA metabolites are not responsible for the toxicity of VPA in sandwich-cultured rat hepatocytes (Kiang et al., 2010). However, it is still not known whether the *in situ* generated (*E*)-2,4-diene-VPA contributes to VPA toxicity in hepatocytes.

As a means to enhance the understanding of the role of reactive metabolites in the pathogenesis of VPA hepatotoxicity, the present study was undertaken to investigate the toxicity of *in situ* generated (*E*)-2,4-diene-VPA in sandwich-cultured rat hepatocytes. The experimental strategies of this study involved the use of: 1) (*E*)-2-ene-VPA, which is a mitochondrial β-oxidation metabolite of VPA (Fig. 2.1), because it undergoes direct cytochrome P450-mediated desaturation to form (*E*)-2,4-diene-VPA (Fabre et al., 1992; Kassahun and Baillie, 1993), yielding 50-fold greater levels of (*E*)-2,4-diene-VPA than from an equivalent dose of VPA (Lin et al., 1991); 2) phenobarbital (PB), which is an inducer of cytochromes P450 (Waxman, 1999), to increase the enzymatic conversion of VPA or (*E*)-2-ene-VPA to form the (*E*)-2,4-diene-VPA reactive metabolite; and 3) 1-aminobenzotriazole (1-ABT), which is an inhibitor of cytochromes P450 (Ortiz de Montellano and Mathews, 1981), to decrease the enzymatic formation of (*E*)-2,4-diene-VPA from VPA or (*E*)-2-ene-VPA. The overall aim was to determine the effects of

modulating the *in situ* formation of (*E*)-2,4-diene-VPA on markers of oxidative stress, steatosis, necrosis, and total GSH levels in sandwich-cultured rat hepatocytes treated with VPA or (*E*)-2-ene-VPA. The results are discussed in the context of whether *in situ* generated (*E*)-2,4-diene-VPA is a contributory mechanism in the hepatotoxicity of VPA.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

Sodium VPA, sodium PB, 1-ABT, acetaminophen, Williams' medium E, insulin, dexamethasone, collagenase (type IV), trypsin inhibitor (type II-S), Triton X-100, dimethyl sulfoxide (DMSO), trypan blue, and 2′,7′-dichlorodihydrofluorescin diacetate (DCF-DA) were obtained from Sigma-Aldrich (St. Louis, MO). Lactate Dehydrogenase (LDH) Cytotoxicity Detection Kit and β-glucuronidase/arylsulfatase enzyme mixture were obtained from Roche diagnostics (Indianapolis, IN). The Glutathione Assay Kit was bought from the Cayman Chemical Co. (Ann Arbor, MI). Liver perfusion medium, hepatocyte wash medium, heat-inactivated fetal bovine serum, 10× Hank's balanced salt solution, 10× Dulbecco's phosphate buffered saline, penicillin-streptomycin, L-glutamine, and BODIPY 558/568 C_{12} 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). Ethyl acetate, methanol, *n*-hexanes, and sodium hydroxide were obtained from Fisher Scientific (Ottawa, ON, Canada).

2.2.2 Synthesis of VPA Metabolites

(E)-2-ene-VPA and *(E)*-2,4-diene-VPA were synthesized as described previously (Kassahun et al., 1991; Zheng, 1993). The purity was >99% as determined by liquid chromatography and mass spectrometry.

2.2.3 Animals

Adult male Sprague-Dawley rats (225-250 g) were obtained from the Centre for Disease Modeling, University of British Columbia, and were housed in the University of British Columbia Animal Resource Unit facility on a 12 h light/12 h dark cycle at standard temperature (22^{o}C) and humidity. The rats were fed *ad libitum* with food (Labdiet 5001 rodent diet, PMI Feeds Inc., Richmond, IN, USA) and water. 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.

2.2.4 Isolation, Culture, and Treatment of Rat Hepatocytes

Rat hepatocytes were isolated by a two-step collagenase perfusion method (Seglen, 1993), as described previously (Tong et al., 2005c). Isolated cells were suspended in supplemented Williams' medium E containing heat-inactivated fetal bovine serum (10% v/v), insulin (1 µM), dexamethasone (100 nM), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cell viability, as determined by trypan blue exclusion, was ≥ 85%. Culture plates (12-well) were coated with Matrigel (40 µl per well) at about 1 h prior to plating of cells at a density of 0.56 x 10^6 cells per well in a volume of 0.8 ml. Cells were allowed to attach for 4 h. Subsequently, the medium containing unattached cells was aspirated and fresh

serum-free, supplemented Williams' medium E (0.8 ml) containing Matrigel (2.5% v/v) was added. Thereafter, culture medium was replaced every 24 h with 1 ml of supplemented Williams' medium E. After a total culture period of 48 h, the sandwich-cultured hepatocytes were subjected to pretreatment with either PB (100 µM final concentration) or culture medium (vehicle) for a further 72 h, with medium freshly replaced every 24 h. At the end of 72 h of pretreatment, the hepatocytes were treated with VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, culture medium (vehicle for VPA and its metabolites), acetaminophen (positive control), or DMSO (vehicle for acetaminophen) for the next 24 h at the concentrations indicated in each figure legend. In experiments involving 1-ABT, hepatocytes were pretreated with 100 µM PB or culture medium every 24 h for 72 h, and this was followed by either 0.5 mM 1-ABT or culture medium (vehicle) for 30 min. Subsequently, the cells were treated with VPA, (*E*)-2-ene-VPA, culture medium (vehicle for VPA and (*E*)-2-ene-VPA), acetaminophen (10 mM), or DMSO (vehicle for acetaminophen; 0.5% v/v) and either 0.5 mM 1-ABT or culture medium (vehicle) for 24 h. Each experiment had two wells per treatment group. A total of three to five rats were used in each experiment.

2.2.5 Dichlorofluorescein (DCF) Assay

Oxidative stress was assessed by measuring the conversion of DCF-DA to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Bartosz, 2006). At the end of the 24 h drug treatment period, hepatocytes were washed with prewarmed phosphate-buffered saline and incubated for 120 min with 1 ml of supplemented Williams' medium E containing 5 µM DCF-DA. Fluorescence was measured at a λ_{ex} of 485 nm and λ_{em} of 527 nm in a Biotek Synergy Mx microplate reader (Biotek Instruments, Winooski, VT). The blank wells contained culture

medium, but without a drug or cells. Fluorescence in each drug-treated well was expressed as fold increase over the fluorescence in the corresponding vehicle-treated wells.

2.2.6 BODIPY 558/568 C_{12} Assay

Cellular accumulation of BODIPY 558/568 C_{12}, which is a fluorescent lipid analogue, was used as an index of steatosis (Fujimura et al., 2009). Primary cultures of rat hepatocytes were treated with VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, culture medium (vehicle for VPA and (*E*)-2-ene-VPA), acetaminophen, or DMSO (vehicle control for acetaminophen) in the presence of the fluoroprobe BODIPY 558/568 C_{12} (2 µg/ml). At the end of 24 h of drug treatment, the culture medium was aspirated and 1 mL of phosphate-buffered saline was added to wash the cells. After gentle swirling of the plate, the phosphate-buffered saline was aspirated and replaced with 1 mL of fresh phosphate-buffered saline. Fluorescence was measured at a λ_{ex} of 484 nm and λ_{em} of 618 nm in a Biotek Synergy Mx microplate reader (Biotek Instruments, Winooski, VT). The blank wells had culture medium containing BODIPY 558/568 C_{12} and a test compound, but without cells. The fluorescence in each well was expressed as fold increase over the fluorescence values in the corresponding vehicle-treated control wells.

2.2.7 Lactate Dehydrogenase (LDH) Assay

Release of intracellular LDH into the culture supernatant was used as a marker of cellular necrosis (Jauregui et al., 1981), as described previously (Kiang et al., 2010). At the end of the 24 h drug treatment period, the culture supernatant was removed and stored in a refrigerator overnight. Subsequently, 1.25 ml of lysis buffer (containing 20 mM EDTA and 2% Triton X-100 in phosphate-buffered saline, pH 7.4) was added to each well and the plates were subjected

to shaking for 2 h at ambient temperature prior to removing the cell lysate. LDH activity was determined according to the instructions in the Roche LDH Cytotoxicity Kit (Roche Diagnostics). LDH activity in the culture supernatant was expressed as a percentage of the total cellular LDH activity (i.e. sum of the LDH activities in the culture supernatant and cell lysate).

2.2.8 Determination of Cellular Levels of Total GSH

At the end of the 24 h drug treatment period, hepatocytes were washed twice with phosphate-buffered saline (pH 7.4) and stored immediately in a -80^{o}C freezer. Cellular levels of total GSH was determined with the Glutathione Assay Kit (Cayman Chemical Co.), according to the manufacturer's instructions. 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® multiwell plate reader (Thermo Electron Corp., Burlington, ON, Canada). The blank sample consisted of equal volumes / concentrations of phosphate buffered saline (pH 7.4; supplemented with 1 mM EDTA) and metaphosphoric acid but without cell homogenate. A calibration curve was constructed according to the instructions provided in the assay kit (Cayman Chemical Co.).

2.2.9 Benzyloxyresorufin *O*-Dealkylation (BROD) Assay

Benzyloxyresorufin *O*-dealkylation (BROD), which is a marker of cytochrome P450 2B (CYP2B) enzymatic activity (Nerurkar et al., 1993), was determined as detailed previously (Chang et al., 2006). At the end of 24, 48, and 72 h of PB pretreatment, hepatocytes were washed with prewarmed phosphate-buffered saline and the BROD assay was initiated by adding 500 µl of substrate solution containing 15 µM 7-benzyloxy resorufin and 10 µM dicumarol in

supplemented Williams' medium E. The hepatocytes were incubated at 37°C for 60 min. A 75 µl aliquot of the incubation mixture was transferred to a 96-well plate containing 15 Fishman units of β-glucuronidase and 120 Roy units of arylsulfatase. The plate was placed in a shaking incubator at 37°C for 2 h. The enzymatic product was solubilized by adding 200 µl ethanol, the microplate was centrifuged, and fluorescence of the supernatant was read at a λ_{ex} of 530 nm and λ_{em} of 580 nm in a Biotek Synergy Mx microplate reader (Biotek Instruments, Winooski, VT).

2.2.10 Quantification of VPA Metabolites

At the end of 24 h of drug treatment, the culture supernatants were transferred into microfuge tubes and stored at -80°C until analysis. Samples were processed as reported earlier (Kiang et al., 2006), except that the internal standards were: 1) D_6-VPA (m/z 149) for (*E,Z*)-2,3′-diene-VPA, (*E*)-2,4-diene-VPA, (*E,E*)-2,3′-diene-VPA, 4-ene-VPA, 3-ene-VPA, (*E*)-2-ene-VPA, 3-keto-VPA, and 4-keto-VPA; and 2) D_7-5-OH-VPA (m/z 280) for 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA. Levels of VPA metabolites were quantified using a gas chromatography – mass spectrometry assay (Tong et al., 2003). A standard curve was constructed for each metabolite over the concentration range of 1 to 500 ng/ml.

2.2.11 Statistical Analysis

All the statistical analyses were performed using the Sigmaplot for Windows, Version 11.0, Systat Software, Inc. (Chicago, IL). The data on the effects of PB and 1-ABT on the toxicity of VPA and (*E*)-2-ene-VPA were analyzed by two-way analysis of variance and, where appropriate, was followed by the Student-Newman-Keuls multiple comparison test. VPA metabolite levels were analyzed by the independent student's *t*-test. All the other data were

analyzed by one-way analysis of variance and, where appropriate, was followed by the Student-Newman-Keuls multiple comparison test. Statistical significance was set *a priori* at $P < 0.05$. Parametric testing was employed for the statistical analysis of the data. In situations where the requirements for parametric statistical testing (*i.e.*, tests for normality and equal variance) were not met, the data were analyzed by an equivalent non-parametric test.

2.3 Results

2.3.1 Concentration-Response Relationship in the Toxicity of VPA, (*E*)-2-Ene-VPA, and (*E*)-2,4-Diene-VPA in Sandwich-Cultured Rat Hepatocytes

Concentration-response experiments were performed to characterize the effects of VPA, (*E*)-2-ene-VPA, and (*E*)-2,4-diene-VPA on markers of oxidative stress, steatosis, and necrosis in sandwich-cultured rat hepatocytes. VPA, (*E*)-2-ene-VPA, and (*E*)-2,4-diene-VPA increased DCF formation (Fig. 2.2A), BODIPY 558/568 C_{12} accumulation (Fig. 2.2B), and LDH release (Fig. 2.2C) in a concentration-dependent fashion. The concentration-response relationships of VPA and (*E*)-2,4-diene-VPA on DCF and LDH markers were similar to a previous study in sandwich-cultured rat hepatocytes (Kiang et al., 2010), although the duration of hepatocyte culture and the composition of the culture medium were different in that study. Whereas the concentration-response curves for VPA and (*E*)-2-ene-VPA were quite similar in each of the three assays (Fig. 2.2A, 2.2B, and 2.2C), the curve for (*E*)-2,4-diene-VPA was shifted to the left in the DCF (Fig. 2.2A) and LDH (Fig. 2.2C) markers, when compared with those for (*E*)-2-ene-VPA and VPA. Statistically significant increases in DCF formation, BODIPY 558/568 C_{12} accumulation, and LDH release occurred, respectively, at VPA concentrations of $\geq$ 20, 60, and

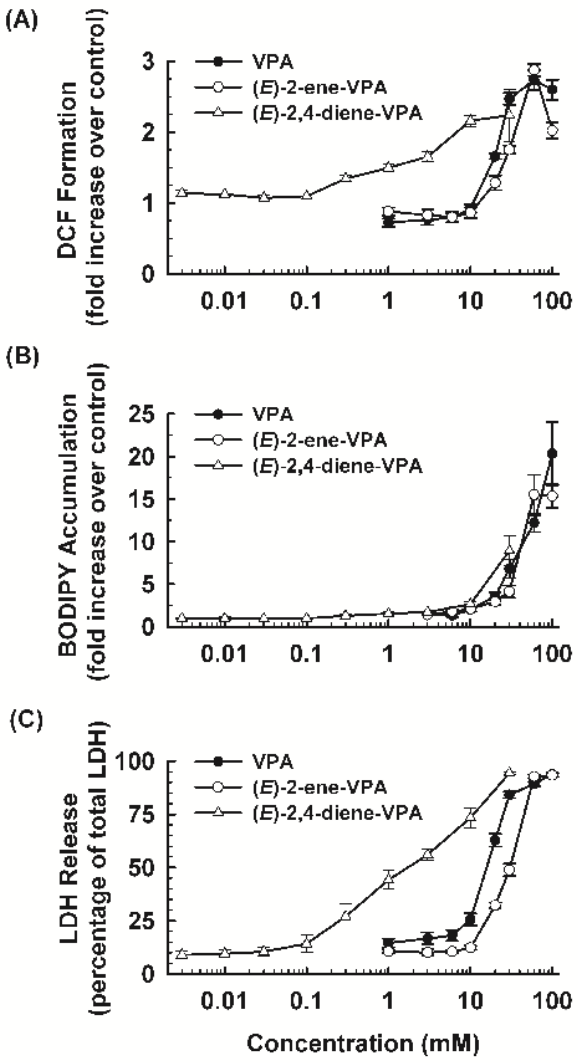

Figure 2.2 Concentration-Response Relationship of VPA, (*E*)-2-Ene-VPA, and (*E*)-2,4-Diene-VPA on (A) DCF Formation, (B) BODIPY 558/568 C_{12} Accumulation, and (C) LDH Release in Sandwich-Cultured Rat Hepatocytes.

At the end of the 120 h culture period, hepatocytes were treated with VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, or culture medium (vehicle) for 24 h. The concentrations of VPA and (*E*)-2-ene-VPA were 1-100 mM for the DCF and LDH assays, and 3-100 mM for the BODIPY assay, whereas those of (*E*)-2,4-diene-VPA were 0.003-30 mM for the DCF, BODIPY, and LDH assays. Data are expressed as mean ± SEM (N = three or four rats). Significant increases ($P < 0.05$) in DCF formation, BODIPY 558/568 C_{12} accumulation, and LDH release occurred at 20, 60, and 10 mM VPA, 20, 60, and 20 mM (*E*)-2-ene-VPA, and 3, 30, and 0.3 mM (*E*)-2,4-diene-VPA, respectively.

10 mM, (*E*)-2-ene-VPA concentrations of ≥ 20, 60, and 20 mM, and (*E*)-2,4-diene-VPA concentrations of ≥ 3, 30, and 0.3 mM. As evident from the minimum toxic concentrations stated above, synthetic (*E*)-2,4-diene-VPA was more potent in its toxicity than VPA and (*E*)-2-ene-VPA. The EC_{50} values were 20.3 ± 1.0 mM (mean ± SEM) and 17.4 ± 0.6 mM in the DCF and LDH assays, respectively, in cultured hepatocytes treated with VPA. By comparison, the EC_{50} values for (*E*)-2-ene-VPA were 33.0 ± 2.5 mM (mean ± SEM), 42.6 ± 5.0 mM, and 30.8 ± 1.1 mM in the DCF, BODIPY, and LDH assays, and that of (*E*)-2,4-diene-VPA were 6.5 ± 4.7 mM (mean ± SEM) and 1.9 ± 0.4 mM, in the DCF and LDH assays, respectively. The EC_{50} values for VPA and (*E*)-2,4-diene-VPA in the BODIPY assay could not be determined as the concentration-response curves did not plateau even at the highest concentration (Fig. 2.2B).

2.3.2 Optimization of Treatment Conditions for PB Induction of VPA-Biotransforming Cytochrome P450 Enzymes

Previously, rat CYP2B enzymes were shown to catalyze the terminal desaturation of VPA to 4-ene-VPA (Rettie et al., 1995), which subsequently undergoes mitochondrial β-oxidation to form (*E*)-2,4-diene-VPA (Fig. 2.1). Therefore, BROD, which is a marker of CYP2B enzyme activity (Nerurkar et al., 1993), was employed to identify the appropriate experimental conditions to induce CYP2B by PB in the sandwich-cultured rat hepatocyte model used in this study. At 48 h after plating, hepatocytes were treated with varying concentrations (10 to 1000 μM) of PB for 24, 48, or 72 h. PB at ≥ 100 μM resulted in maximal induction of BROD activity (Fig. A.1A) and the magnitude of induction was 13-, 14- and 17-fold after 24, 48 and 72 h pretreatment, respectively (Fig. A.1B). Therefore, in subsequent PB experiments, cultured hepatocytes were pretreated with 100 μM PB for 72 h.

2.3.3 Effect of PB Pretreatment on Metabolite Levels in Sandwich-Cultured Rat Hepatocytes Treated with VPA or (*E*)-2-Ene-VPA

In order to determine the effect of PB pretreatment on the *in situ* generated metabolites of VPA and (*E*)-2-ene-VPA, cultured hepatocytes were pretreated with 100 μM PB every 24 h for 72 h and then treated with VPA, (*E*)-2-ene-VPA, or culture medium (vehicle) for the next 24 h. The culture supernatants were then assayed for the oxidative metabolites of VPA or (*E*)-2-ene-VPA. (*E*)-2-ene-VPA, 3-keto-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA were the predominant metabolites after VPA treatment of vehicle-pretreated hepatocytes (Table 2.1). (*E*)-2,4-diene-VPA was not detected in vehicle-pretreated cells, but could be detected at trace levels following PB pretreatment (Table 2.1). PB also increased the levels of 4-ene-VPA, 4-keto-VPA, 4-OH-VPA, and 5-OH-VPA metabolites by 7-, 25-, 8-, and 5-fold, respectively (Table 2.1). PB had little or no effect on the levels of the β-oxidation-mediated metabolites of VPA; namely, (*E*)-2-ene-VPA, (*E,Z*)-2,3′-diene-VPA, (*E,E*)-2,3′-diene-VPA, 3-ene-VPA, 3-keto-VPA, and 3-OH-VPA (Table 2.1).

In (*E*)-2-ene-VPA-treated hepatocytes, (*E*)-2,4-diene-VPA, 3-ene-VPA, and (*E,E*)-2,3′-diene-VPA were the predominant metabolites in the absence of PB pretreatment (Table 2.1). PB pretreatment augmented the levels of (*E*)-2,4-diene-VPA by 16-fold (Table 2.1) and that of (*E,E*)-2,3′-diene-VPA and 3-keto-VPA by 1.3- and 3.5-fold, respectively. In contrast, it did not affect the levels of other oxidative metabolites of (*E*)-2-ene-VPA, such as (*E,Z*)-2,3′-diene-VPA, 3-ene-VPA, 4-keto-VPA, and 3-OH-VPA (Table 2.1).

An additional peak was observed at m/z 271 in the culture supernatants from (*E*)-2-ene-VPA-treated cells, which is believed to be the 4-OH-(*E*)-2-ene-VPA peak previously

Table 2.1 Effect of PB on VPA Metabolite Levels in Sandwich-Cultured Rat Hepatocytes Treated with VPA or (*E*)-2-Ene-VPA

Metabolite	Treatment			
	VPA		(*E*)-2-Ene-VPA	
	Culture Medium	PB	Culture Medium	PB
	Concentration (μM)			
(*E*)-2,4-diene-VPA	None detected	0.01 ± 0.01	3.5 ± 0.59	58 ± 7.5*
(*E,Z*)-2,3′-diene-VPA	0.20 ± 0.01	0.20 ± 0.01	0.33 ± 0.05	0.37 ± 0.08
(*E,E*)-2,3′-diene-VPA	1.2 ± 0.04	1.3 ± 0.03	1.5 ± 0.07	1.9 ± 0.09*
4-ene-VPA	0.21 ± 0.04	1.5 ± 0.26*	None detected	None detected
3-ene-VPA	1.5 ± 0.17	1.7 ± 0.17	4.3 ± 0.20	4.5 ± 0.24
(*E*)-2-ene-VPA	3.6 ± 0.08	3.2 ± 0.03*	Not determined	Not determined
4-keto-VPA	0.17 ± 0.05	4.2 ± 0.92*	0.013 ± 0.010	0.015 ± 0.014
3-keto-VPA	4.6 ± 1.4	3.8 ± 0.88	0.34 ± 0.10	1.2 ± 0.31*
4-OH-VPA	15 ± 3.9	117 ± 21*	None detected	None detected
3-OH-VPA	7.6 ± 1.4	9.2 ± 0.78	0.45 ± 0.15	0.40 ± 0.17
5-OH-VPA	2.4 ± 0.36	12.1 ± 3.2*	None detected	None detected

At the end of the 48 h culture period, hepatocytes were pretreated with 100 μM PB or culture medium (vehicle) every 24 h for 72 h and then treated with 20 mM VPA, 30 mM (*E*)-2-ene-VPA, or culture medium (vehicle) for 24 h. Data are expressed as mean ± SEM (N = five rats). *Significantly different from the corresponding culture medium-pretreated group, $P < 0.05$.

reported by Kassahun and Baillie (1993). While there was no authentic 4-OH-(*E*)-2-ene-VPA standard available for accurate identification and quantification, the chromatographic and mass spectrometric characteristics of the m/z 271 peak were consistent with the reported 4-OH-(*E*)-2-ene-VPA structure (Kassahun and Abbott, 1993). PB pretreatment enhanced the levels of the putative 4-OH-(*E*)-2-ene-VPA peak by several-fold in hepatocytes treated with (*E*)-2-ene-VPA (Table A.1).

2.3.4 Effect of PB on Markers of Oxidative Stress, Steatosis, and Necrosis in Sandwich-Cultured Rat Hepatocytes Treated with VPA or (*E*)-2-Ene-VPA

The next experiment was to determine the effect of increasing the *in situ* levels of (*E*)-2,4-diene-VPA on the toxicity of (*E*)-2-ene-VPA or VPA. Cultured hepatocytes were pretreated with 100 μM PB every 24 h for 72 h and then treated with VPA, (*E*)-2-ene-VPA, or culture medium (vehicle) for the next 24 h. The concentrations were 20, 20, and 15 mM VPA and 30, 30, and 20 mM (*E*)-2-ene-VPA in the DCF, BODIPY, and LDH assays, respectively. These concentrations were chosen from the log-linear portion of the respective concentration-response curves (Fig. 2.2A-C). Treatment with VPA or (*E*)-2-ene-VPA alone increased DCF formation (Fig. 2.3A), BODIPY 558/568 C_{12} accumulation (Fig. 2.3B), and LDH release (Fig. 2.3C) in sandwich-cultured rat hepatocytes. Whereas PB pretreatment did not influence the effects of VPA in DCF (Fig. 2.3A), BODIPY (Fig. 2.3B), or LDH (Fig. 2.3C) assays, it enhanced the effects of (*E*)-2-ene-VPA in all the three assays (Fig. 2.3A, 2.3B, and 2.3C). By comparison, PB enhanced DCF formation (Fig. 2.3A), BODIPY 558/568 C_{12} accumulation (Fig. 2.3B), and LDH release (Fig. 2.3C) in hepatocytes treated with 10 mM acetaminophen, which is a known hepatotoxicant that is subject to cytochrome P450-mediated bioactivation (Jaeschke et al., 2012).

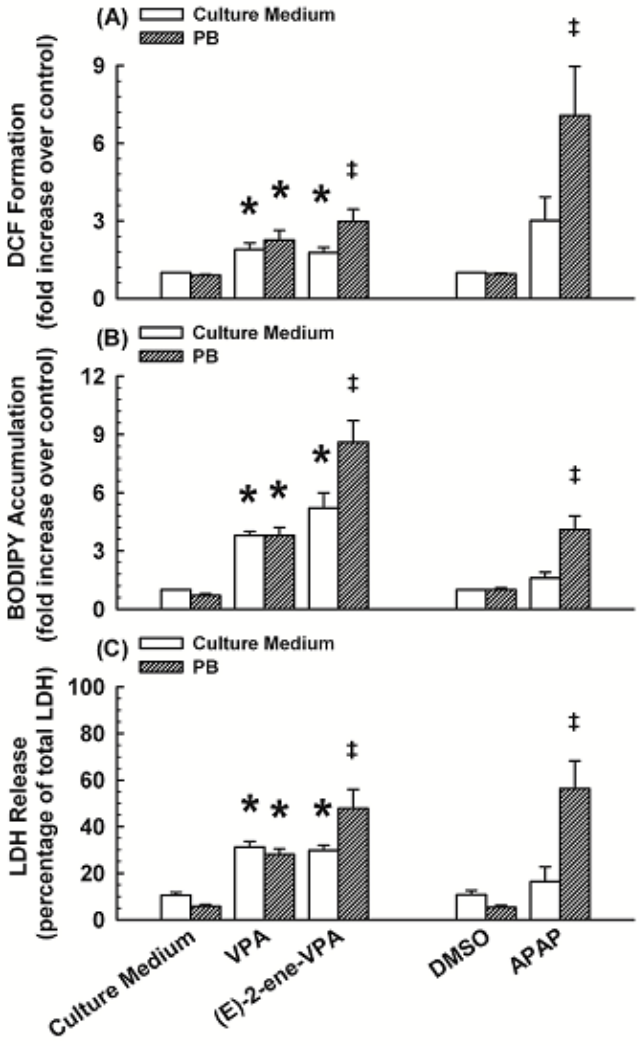

Figure 2.3 Effect of PB on (A) DCF Formation, (B) BODIPY 558/568 C_{12} Accumulation, and (C) LDH Release in Sandwich-Cultured Rat Hepatocytes Treated with VPA or (*E*)-2-Ene-VPA.

At the end of the 48 h culture period, hepatocytes were pretreated with 100 µM PB or culture medium (vehicle control) every 24 h for 72 h. Subsequently, the hepatocytes were treated with VPA, (*E*)-2-ene-VPA, culture medium (vehicle for VPA and (*E*)-2-ene-VPA), acetaminophen (APAP; 10 mM), or DMSO (vehicle for APAP; 0.5% v/v) for 24 h. The concentration of VPA was 20, 20, and 15 mM in the DCF, BODIPY, and LDH assays, respectively, whereas that of (*E*)-2-ene-VPA was 30, 30, and 20 mM, respectively. Data are expressed as mean ± SEM (N = five rats). *Significantly different from the corresponding vehicle-treated control group, $P < 0.05$, ‡significantly different from both the corresponding vehicle-treated control group and the corresponding culture medium-pretreated group, $P < 0.05$.

Acetaminophen toxicity does not typically involve steatosis (Fig. 2.3B), but the purpose of a positive control in these experiments was to verify the effects of pretreatment with PB and/or 1-ABT. As PB pretreatment increased acetaminophen-mediated BODIPY 558/568 C_{12} accumulation in the sandwich-cultured rat hepatocyte model (Fig. 2.3B), acetaminophen was employed as the positive control for the BODIPY assay as well.

2.3.5 Effect of 1-ABT Pretreatment on Metabolite Levels in PB- and Vehicle-Pretreated Sandwich-Cultured Rat Hepatocytes Administered VPA or (*E*)-2-Ene-VPA

To decrease the *in situ* levels of (*E*)-2,4-diene-VPA, a chemical inhibition experiment was performed with 1-ABT, which is a mechanism-based inactivator of cytochrome P450 enzymes (Ortiz de Montellano and Mathews, 1981). Based on the preliminary concentration-response experiments with 1-ABT (Fig. A.2), a concentration of 0.5 mM was chosen because it had a maximal inhibitory effect on BROD activity, and no effect on DCF formation, BODIPY 558/568 C_{12} accumulation, or LDH release. At the end of the 72 h of PB or vehicle pretreatment, cultured hepatocytes were treated with 0.5 mM 1-ABT for 30 min prior to the addition of VPA or (*E*)-2-ene-VPA. 1-ABT substantially inhibited the formation of 4-ene-VPA, 4-keto-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA following VPA treatment of hepatocytes pretreated with PB (Table 2.2) or vehicle (Table 2.3). The levels of other VPA metabolites; namely, (*E,Z*)-2,3′-diene-VPA, (*E,E*)-2,3′-diene-VPA, 3-ene-VPA, (*E*)-2-ene-VPA, and 3-keto-VPA, were not affected by 1-ABT in both PB-pretreated (Table 2.2) and vehicle-pretreated hepatocytes (Table 2.3).

In the case of (*E*)-2-ene-VPA-treated hepatocytes, the *in situ* levels of (*E*)-2,4-diene-VPA were almost completely reduced by 1-ABT in hepatocytes pretreated with PB, in addition to a

Table 2.2 Effect of 1-ABT on VPA Metabolite Levels in Sandwich-Cultured Rat Hepatocytes Treated with PB and VPA or (*E*)-2-Ene-VPA

Metabolite	Treatment			
	VPA		(*E*)-2-Ene-VPA	
	PB	PB + 1-ABT	PB	PB + 1-ABT
	Concentration (μM)			
(*E*)-2,4-diene-VPA	0.01 ± 0.01	None detected	61 ± 5.2	0.15 ± 0.09*
(*E,Z*)-2,3′-diene-VPA	0.17 ± 0.04	0.18 ± 0.04	0.29 ± 0.04	0.21 ± 0.04
(*E,E*)-2,3′-diene-VPA	1.3 ± 0.10	1.2 ± 0.09	1.7 ± 0.05	1.2 ± 0.05*
4-ene-VPA	1.8 ± 0.22	0.03 ± 0.01*	None detected	None detected
3-ene-VPA	1.2 ± 0.12	1.3 ± 0.26	4.6 ± 0.21	5.1 ± 0.31
(*E*)-2-ene-VPA	2.4 ± 0.23	2.8 ± 0.24	Not determined	Not determined
4-keto-VPA	4.2 ± 0.23	0.04 ± 0.01*	None detected	None detected
3-keto-VPA	2.5 ± 0.41	3.0 ± 0.46	1.5 ± 0.51	0.37 ± 0.24
4-OH-VPA	136 ± 8	1.5 ± 0.05*	None detected	None detected
3-OH-VPA	12 ± 1.43	0.76 ± 0.22*	0.21 ± 0.13	0.42 ± 0.18
5-OH-VPA	17 ± 1.7	0.28 ± 0.06*	None detected	None detected

At the end of the 48 h culture period, hepatocytes were pretreated with 100 μM PB every 24 h for 72 h and this was followed by either 0.5mM 1-ABT or culture medium (vehicle) for 30 min. Subsequently, the cells were treated with 20 mM VPA, 30 mM (*E*)-2-ene-VPA, or culture medium (vehicle) and either 0.5 mM 1-ABT or culture medium (vehicle) for 24 h. Data are expressed as mean ± SEM (N = four rats). *Significantly different from the corresponding treatment group without 1-ABT, $P < 0.05$.

Table 2.3 Effect of 1-ABT on VPA Metabolite Levels in Sandwich-Cultured Rat Hepatocytes Treated with Only VPA or (*E*)-2-Ene-VPA (Without PB Pretreatment)

Metabolite	Treatment			
	VPA		(*E*)-2-Ene-VPA	
	Culture medium	Culture medium + 1-ABT	Culture medium	Culture medium + 1-ABT
	Concentration (μM)			
(*E*)-2,4-diene-VPA	None detected	None detected	1.4 ± 0.24	0.06 ± 0.01*
(*E,Z*)-2,3′-diene-VPA	0.18 ± 0.01	0.17 ± 0.01	0.08 ± 0.03	0.07 ± 0.02
(*E,E*)-2,3′-diene-VPA	1.4 ± 0.11	1.4 ± 0.12	0.51 ± 0.11	0.44 ± 0.11
4-ene-VPA	0.16 ± 0.02	0.01 ± 0.00*	Not determined	Not determined
3-ene-VPA	0.56 ± 0.06	0.57 ± 0.05	Not determined	Not determined
(*E*)-2-ene-VPA	1.8 ± 0.05	1.8 ± 0.22	Not determined	Not determined
4-keto-VPA	0.30 ± 0.05	0.06 ± 0.00*	None detected	None detected
3-keto-VPA	1.4 ± 0.80	1.6 ± 0.16	0.11 ± 0.06	0.27 ± 0.19
4-OH-VPA	8.0 ± 1.20	0.25 ± 0.02*	None detected	None detected
3-OH-VPA	1.2 ± 0.55	0.16 ± 0.06	0.05 ± 0.04	0.43 ± 0.25
5-OH-VPA	2.2 ± 0.17	0.09 ± 0.01*	None detected	None detected

At the end of the 48 h culture period, hepatocytes were pretreated with culture medium every 24 h for 72 h and this was followed by either 0.5mM 1-ABT or culture medium (vehicle) for 30 min. Subsequently, the cells were treated with 20 mM VPA, 30 mM (*E*)-2-ene-VPA, or culture medium (vehicle) and either 0.5 mM 1-ABT or culture medium (vehicle) for 24 h. Data are expressed as mean ± SEM (N = three rats). *Significantly different from the corresponding treatment group without 1-ABT, $P < 0.05$.

slight decrease in the levels of (*E,E*)-2,3′-diene-VPA (Table 2.2). As expected, pretreatment with 1-ABT did not affect the levels of β-oxidation-mediated metabolites of (*E*)-2-ene-VPA; namely, (*E,Z*)-2,3′-diene-VPA, 3-ene-VPA, 3-keto-VPA, and 3-OH-VPA (Table 2.2). In vehicle-pretreated hepatocytes, 1-ABT decreased the levels of (*E*)-2,4-diene-VPA following (*E*)-2-ene-VPA treatment (Table 2.3). Furthermore, 1-ABT almost completely reduced the levels of the putative 4-OH-(*E*)-2-ene-VPA peak in both PB- and vehicle-pretreated rat hepatocytes (Table A.2).

2.3.6 Effect of 1-ABT Pretreatment on Markers of Oxidative Stress, Steatosis, and Necrosis in Sandwich-Cultured Rat Hepatocytes Treated with VPA or (*E*)-2-Ene-VPA

Having shown that 1-ABT pretreatment almost eliminates the *in situ* formation of (*E*)-2,4-diene-VPA, the next step was to determine the effect of 1-ABT on the toxicity of VPA and (*E*)-2-ene-VPA in sandwich-cultured rat hepatocytes. 1-ABT did not influence the effects of VPA on DCF formation, BODIPY 558/568 C_{12} accumulation, or LDH release in hepatocytes pretreated with PB (Fig. 2.4A, 2.4B and 2.4C) or with vehicle (Fig. 2.4D, 2.4E, and 2.4F). The lack of effect of 1-ABT on VPA-associated DCF formation and LDH release in vehicle-pretreated hepatocytes is consistent with a previous study from this laboratory that used a slightly different hepatocyte culture model (Kiang et al., 2010).

Contrary to the results observed with VPA, 1-ABT attenuated the increase in (*E*)-2-ene-VPA toxicity in PB-pretreated hepatocytes, and the magnitude of the decrease was 30, 70, and 60% in the DCF (Fig. 2.5A), BODIPY (Fig. 2.5B), and LDH assays (Fig. 2.5C), respectively. However, in contrast to the results obtained with PB pretreatment, 1-ABT did not influence the effects of (*E*)-2-ene-VPA on DCF formation (Fig. 2.5D), BODIPY 558/568 C_{12} accumulation

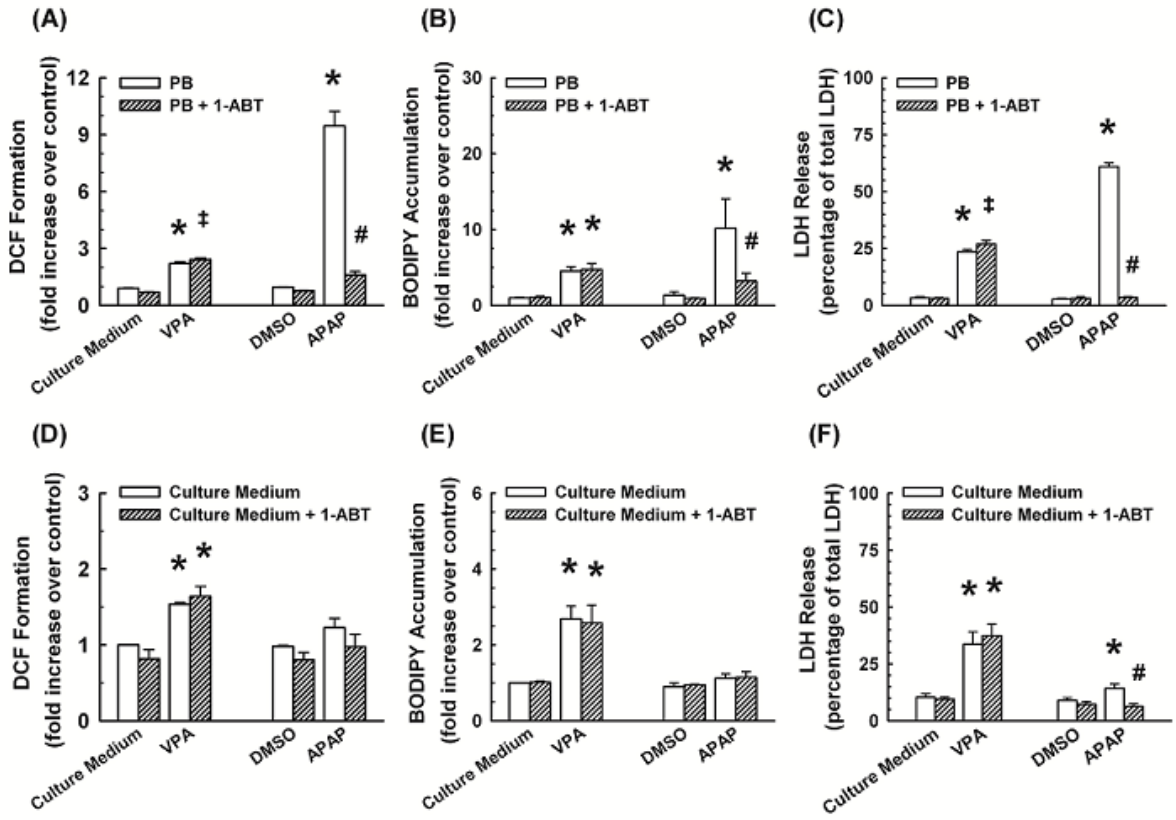

Figure 2.4 Effect of 1-ABT on DCF Formation, BODIPY 558/568 C_{12} Accumulation, and LDH Release in Sandwich-Cultured Rat Hepatocytes Treated with PB and VPA.
At the end of the 48 h culture period, hepatocytes were pretreated with 100 µM PB (Fig. 4A, 5B, and 4C) or culture medium (Fig. 4D, 4E, and 4F) every 24 h for 72 h, and this was followed by either 0.5 mM 1-ABT or culture medium (vehicle) for 30 min. Subsequently, the cells were treated with VPA, culture medium (vehicle for VPA), acetaminophen (APAP; 10 mM), or DMSO (vehicle for APAP; 0.5% v/v) and either 0.5 mM 1-ABT or culture medium (vehicle) for 24 h. The concentration of VPA was 20, 20, and 15 mM in the DCF, BODIPY, and LDH assays, respectively. Data are expressed as mean ± SEM (N = three or four rats). *Significantly different from the corresponding vehicle-treated control group, $P < 0.05$; $^{\#}$significantly different from the corresponding treatment group without 1-ABT, $P < 0.05$; ‡significantly different from both the corresponding vehicle-treated control group and the corresponding treatment group without 1-ABT, $P < 0.05$.

(Fig. 2.5E), or LDH release (Fig. 2.5F) in sandwich-cultured rat hepatocytes not pretreated with PB (i.e., pretreated with the vehicle). Control experiments indicated that 1-ABT greatly attenuated the PB-mediated enhancement in the effects of acetaminophen on DCF (Fig. 2.4A), BODIPY (Fig. 2.4B), and LDH markers (Fig. 2.4C). In the case of vehicle-pretreated hepatocytes, 1-ABT reduced the effects of acetaminophen only in the LDH assay (Fig. 2.4F), but not in the DCF (Fig. 2.4D) or BODIPY (Fig. 2.4E) assays.

2.3.7 Effect of PB and/or 1-ABT Pretreatment on Cellular Levels of Total GSH in Sandwich-Cultured Rat Hepatocytes Treated with VPA or (*E*)-2-Ene-VPA

(*E*)-2,4-diene-VPA is known to conjugate with GSH (Tang et al., 1996) and previous studies have detected GSH conjugates of (*E*)-2,4-diene-VPA in the bile of rats (Kassahun et al., 1991) and NAC conjugates of (*E*)-2,4-diene-VPA in the urine of rats and humans (Gopaul et al., 2000; Kassahun et al., 1991). Consistent with these reports, synthetic (*E*)-2,4-diene-VPA was more potent and more effective than VPA in depleting cellular GSH in sandwich-cultured rat hepatocytes (Kiang et al., 2011). Therefore, the next experiment was to determine the effect of modulating the *in situ* formation of (*E*)-2,4-diene-VPA on cellular levels of total GSH in sandwich-cultured rat hepatocytes. Treatment of rat hepatocytes with VPA or (*E*)-2-ene-VPA resulted in the depletion of total GSH levels by 25% (Fig. 2.6A). Consistent with the results of the DCF, BODIPY, and LDH assays, PB pretreatment did not further influence the GSH depletion in VPA-treated hepatocytes (Fig. 2.6A). Also, 1-ABT did not attenuate the VPA-associated decrease in total GSH levels in both PB- and culture medium-pretreated hepatocytes (Fig. 2.6B and 2.6C). Conversely, PB pretreatment greatly enhanced the decrease in total GSH levels in (*E*)-2-ene-VPA-treated hepatocytes, where the cellular total GSH levels were depleted

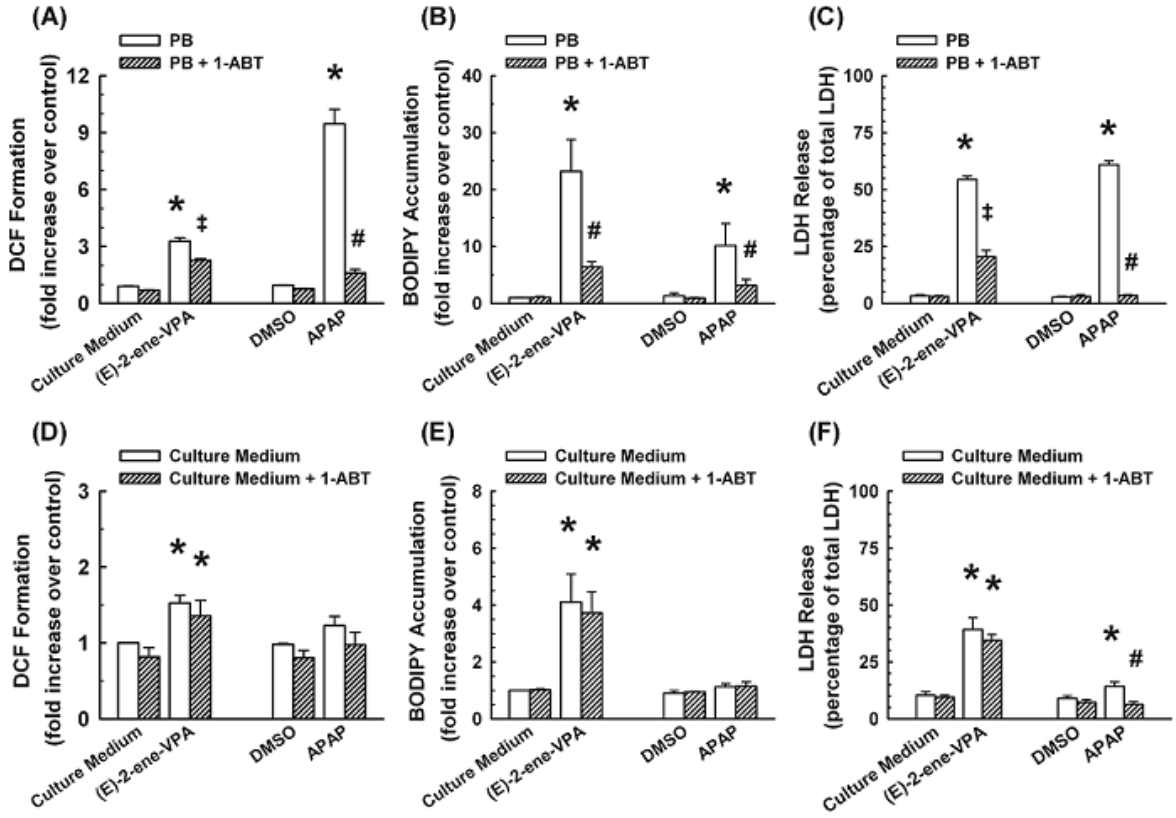

Figure 2.5 Effect of 1-ABT on DCF Formation, BODIPY 558/568 C_{12} Accumulation, and LDH Release in Sandwich-Cultured Rat Hepatocytes Treated with PB and (*E*)-2-Ene-VPA.

At the end of the 48 h culture period, hepatocytes were pretreated with 100 µM PB (Fig. 5A, 5B, and 4C) or culture medium (vehicle; Fig. 5D, 5E, and 5F) every 24 h for 72 h, and this was followed by 0.5 mM 1-ABT or culture medium (vehicle) for 30 min. Subsequently, the cells were treated with (*E*)-2-ene-VPA, culture medium (vehicle for (*E*)-2-ene-VPA), acetaminophen (APAP; 10 mM), or DMSO (vehicle for APAP; 0.5% v/v) and either 0.5 mM 1-ABT or culture medium (vehicle) for the next 24 h. The concentration of (*E*)-2-ene-VPA was 30, 30, and 20 mM in the DCF, BODIPY, and LDH assays, respectively. Data are expressed as mean ± SEM (N = three or four rats). *Significantly different from the corresponding vehicle-treated control group, $P < 0.05$, $^{\#}$significantly different from the corresponding treatment group without 1-ABT, $P < 0.05$, ‡significantly different from both the corresponding vehicle-treated control group and the corresponding treatment group without 1-ABT, $P < 0.05$.

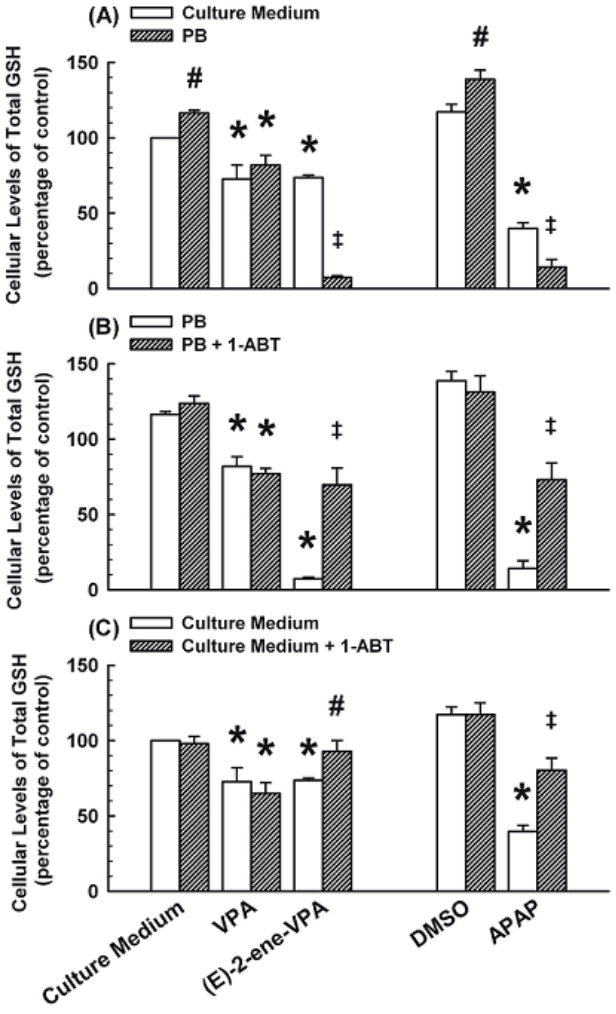

Figure 2.6 Effect of PB and/or 1-ABT on the Levels of Cellular Total GSH in Sandwich-Cultured Rat Hepatocytes Treated with (*E*)-2-Ene-VPA or VPA.

At the end of the 48 h culture period, hepatocytes were pretreated with 100 μM PB or culture medium (indicated in each figure legend), every 24 h for 72 h, and this was followed by either 0.5 mM 1-ABT or culture medium (indicated in each figure legend) for 30 min. Subsequently, the cells were treated with (*E*)-2-ene-VPA (30 mM), VPA (20 mM), culture medium (vehicle for (*E*)-2-ene-VPA and VPA), acetaminophen (APAP; 10 mM), or DMSO (vehicle for APAP; 0.5% v/v) and either 0.5 mM 1-ABT or culture medium (vehicle) for 24 h. Data are expressed as mean ± SEM (N = three rats). *Significantly different from the corresponding vehicle-treated control group, $P < 0.05$; $^{\#}$significantly different from the corresponding treatment group without PB (Fig. 6A) or without 1-ABT (Fig. 6B and 6C), $P < 0.05$; ‡significantly different from the corresponding vehicle-treated control group and the corresponding treatment group without PB (Fig. 6A) or without 1-ABT (Fig. 6B and 6C), $P < 0.05$.

to less than 10% of the control group (Fig. 2.6A). 1-ABT attenuated the depletion of GSH caused by (*E*)-2-ene-VPA in both PB- and culture medium-pretreated hepatocytes with total GSH levels at 70 and 90% of the control group, respectively (Fig. 2.6B and 2.6C). As would be expected, treatment of rat hepatocytes with acetaminophen (positive control) decreased the total GSH levels to 33% of the vehicle control (Fig. 2.6A). Whereas PB enhanced the GSH depletion caused by acetaminophen (Fig. 2.6A), 1-ABT attenuated the decrease in total GSH levels in both PB- and culture medium-pretreated hepatocytes (Fig. 2.6B and 2.6C), although the level did not return to that of vehicle-treated hepatocytes.

2.4 Discussion

(*E*)-2,4-diene-VPA is a reactive compound and products derived from GSH conjugates have been detected in rats (Kassahun et al., 1991) and humans (Gopaul et al., 2000; Kassahun et al., 1991). Synthetic (*E*)-2,4-diene-VPA was found to be more toxic than VPA both *in vivo* in rats (Kesterson et al., 1984) and *in vitro* in cultured rat hepatocytes (Kiang et al., 2010). However, the role of *in situ* generated (*E*)-2,4-diene-VPA in VPA toxicity is still not known. Therefore, the primary goal of the present study was to determine the effects of modulating the levels of *in situ* generated (*E*)-2,4-diene-VPA on the observed toxicity of VPA or (*E*)-2-ene VPA in sandwich-cultured rat hepatocytes.

The results of this study suggest that (*E*)-2,4-diene-VPA was not responsible for the hepatocyte toxicity of VPA either with or without PB pretreatment. This conclusion is based on the following findings: 1) in the absence of PB pretreatment, (*E*)-2,4-diene-VPA was not detected in VPA-treated hepatocytes, 2) PB pretreatment increased the *in situ* formation of (*E*)-

2,4-diene-VPA only to trace levels but had no effect on VPA toxicity, and 3) 1-ABT-mediated attenuation of the trace levels of (*E*)-2,4-diene-VPA did not affect the hepatocyte toxicity of VPA, as determined by markers of oxidative stress, steatosis, necrosis, and by GSH depletion in rat hepatocytes.

Conversely, the experimental findings with (*E*)-2-ene-VPA indicated that *in situ* generated (*E*)-2,4-diene-VPA can add to (*E*)-2-ene-VPA toxicity in PB-pretreated rat hepatocytes. This conclusion is based on evidence from cultured hepatocytes treated with (*E*)-2-ene-VPA that indicated: 1) PB pretreatment substantially increased the levels of *in situ* generated (*E*)-2,4-diene-VPA accompanied by increases in the effects of (*E*)-2-ene-VPA on markers DCF, BODIPY, LDH, and GSH; and 2) a decrease in the *in situ* formation of (*E*)-2,4-diene-VPA by pretreatment with 1-ABT was accompanied by an attenuation in the enhancement of (*E*)-2-ene-VPA toxicity in PB-pretreated hepatocytes. In the absence of PB pretreatment, 1-ABT did not affect the toxicity of (*E*)-2-ene-VPA, although it reduced the *in situ* levels of (*E*)-2,4-diene-VPA and attenuated (*E*)-2-ene-VPA-induced GSH depletion. Thus, the findings with (*E*)-2-ene-VPA confirm the toxic potential of *in situ* generated (*E*)-2,4-diene-VPA, and validate the applicability of the sandwich-cultured hepatocyte model and the markers that were employed here to respond to the *in situ* levels of this reactive metabolite. The results of the present study also illustrate that the levels of the *in situ* formed (*E*)-2,4-diene-VPA were not sufficiently high enough for it to play a role in the hepatocyte toxicity of VPA (with or without PB pretreatment) and (*E*)-2-ene-VPA (without PB pretreatment).

In the present study, (*E*)-2,4-diene-VPA was quantifiable at low micromolar concentrations in (*E*)-2-ene-VPA-treated hepatocytes, whereas it was not detectable in VPA-treated hepatocytes. The observed difference in the extent of *in situ* (*E*)-2,4-diene-VPA

formation from VPA and (*E*)-2-ene-VPA cannot be explained by the modest difference in the concentration of substrates (concentration of (*E*)-2-ene-VPA being 1.5-fold higher than VPA). A benefit of including (*E*)-2-ene-VPA to assess the toxicity of *in situ* generated (*E*)-2,4-diene-VPA is the fact that cytochrome P450-mediated desaturation of (*E*)-2-ene-VPA results in relatively greater amounts of (*E*)-2,4-diene-VPA than that from VPA (Lin et al., 1991). As illustrated in Fig. 2.1, (*E*)-2,4-diene-VPA can be generated from VPA in a two-step reaction that initially involves terminal desaturation to form 4-ene-VPA, which subsequently undergoes β-oxidation to form (*E*)-2,4-diene-VPA. The (*E*)-2,4-diene-VPA metabolite can also be enzymatically formed directly by the terminal desaturation of (*E*)-2-ene-VPA. By comparing the concentrations of the enzymatically-generated (*E*)-2,4-diene-VPA from (*E*)-2-ene-VPA to that of 4-ene-VPA from VPA, it is evident that the terminal desaturation of (*E*)-2-ene-VPA is far more efficient than the terminal desaturation of VPA (Table 1). Therefore, it appears that (*E*)-2-ene-VPA is a better substrate than VPA for the constitutive cytochrome P450 enzymes that catalyze the terminal desaturation of these two chemicals. It has been proposed that the presence of a double bond at C_2 of (*E*)-2-ene-VPA stabilizes the carbon-centered free radical intermediate (Kassahun and Abbott, 1993), which subsequently undergoes either oxygenation to yield 4-OH-(*E*)-2-ene-VPA or dehydrogenation to yield (*E*)-2,4-diene-VPA. If this proposal is correct, differences would also be anticipated in the levels of the 4-OH-(*E*)-2-ene-VPA formed from (*E*)-2-ene-VPA and those of 4-OH-VPA formed from VPA, because these two hydroxy metabolites come from the same carbon-centered free radical intermediate as that of (*E*)-2,4-diene-VPA (Kassahun and Abbott, 1993) and 4-ene-VPA (Rettie et al., 1987). According to preliminary data reported in the present study, the level of 4-OH-(*E*)-2-ene-VPA generated from (*E*)-2-ene-VPA was greater than that of 4-OH-VPA produced from VPA (Table A.3). Furthermore, the stabilization of the

carbon-centered free radical intermediate by the double bond in (*E*)-2-ene-VPA is also shown to result in a much higher partition ratio of (*E*)-2,4-diene-VPA:4-OH-(*E*)-2-ene-VPA (0.45), when compared to the partition ratio of 4-ene-VPA:4-OH-VPA from VPA (0.015) (Kassahun and Baillie, 1993), suggesting that the dehydrogenation of (*E*)-2-ene-VPA is more favored than the dehydrogenation of VPA.

Another novel finding in the present study is that PB increased the levels of (*E*)-2,4-diene-VPA to a substantially greater extent in hepatocytes treated with (*E*)-2-ene-VPA than in those treated with VPA. A possible explanation is that (*E*)-2-ene-VPA is preferred over VPA as a substrate for the PB-inducible cytochrome(s) P450 that catalyzes the terminal desaturation of these two compounds. Another possibility is that the terminal desaturation of (*E*)-2-ene-VPA and that of VPA are catalyzed by distinct PB-inducible cytochrome P450 enzymes in rat hepatocytes. The identity of the specific PB-inducible cytochrome P450 that catalyzes the enzymatic conversion of (*E*)-2-ene-VPA to (*E*)-2,4-diene-VPA is not known. In a previous study, CYP2B1, which is subject to induction by PB (Guengerich et al., 1982), was shown to be a catalyst of the terminal desaturation of VPA to form 4-ene-VPA (Rettie et al., 1995).

The (*E*)-2,4-diene-VPA generated *in situ* from (*E*)-2-ene-VPA appears to be more potent than the exogenously added, synthetic (*E*)-2,4-diene-VPA with respect to observed effects on markers of oxidative stress, steatosis, and necrosis in sandwich-cultured rat hepatocytes. This conclusion is based on the fact that the levels (*i.e.,* 0.06 mM) of this metabolite generated *in situ* in hepatocytes treated with PB and (*E*)-2-ene-VPA were much smaller than the minimum toxic concentrations of synthetic (*E*)-2,4-diene-VPA observed in this study. Although the *in situ* levels of (*E*)-2,4-diene-VPA could be an underestimate owing to the fact that (*E*)-2,4-diene-VPA is known to form thiol-conjugates (Gopaul et al., 2000; Kassahun et al., 1991) and that only free

levels of *in situ* (*E*)-2,4-diene-VPA were quantified in this study, this is unlikely to account for the entire difference between the *in situ* concentration of (*E*)-2,4-diene-VPA and the minimum toxic concentrations of the synthetic (*E*)-2,4-diene-VPA. Hypothetically, the observed differences in toxicity between the *in situ* generated and exogenously added (*E*)-2,4-diene-VPA could be attributed to: 1) the differences in hepatocyte exposure to *in situ* generated (*E*)-2,4-diene-VPA and synthetic (*E*)-2,4-diene-VPA owing to the differences in lipophilicity between the synthetic (*E*)-2,4-diene-VPA and (*E*)-2-ene-VPA which is metabolized to (*E*)-2,4-diene-VPA *in situ*, as observed in studies with enalapril and its metabolite enalaprilat (de Lannoy and Pang, 1986; Pang et al., 1984), and 2) greater proximity of *in situ* generated (*E*)-2,4-diene-VPA to critical cellular organelles than the synthetic (*E*)-2,4-diene-VPA added exogenously to the culture medium. Overall, it is believed that the preformed (synthetic) (*E*)-2,4-diene-VPA might have been less able to permeate hepatocytes to undergo further metabolism and/or to exert its toxicity. Further studies are required to verify this supposition.

Previous studies have assessed the hepatotoxicity potential of synthetic, exogenously administered (*E*)-2-ene-VPA. Administration of (*E*)-2-ene-VPA at a dosage regimen of 100 mg/kg/day ip for five days (Kesterson et al., 1984) or 250 mg/kg ip three times daily for seven days (Loscher et al., 1992) did not yield microvesicular steatosis or increase plasma activity levels of alanine aminotransferase, which is a biochemical marker of hepatotoxicity. In contrast, (*E*)-2-ene-VPA has been shown to be toxic to cultured rat hepatocytes, as assessed by markers of cell viability (Kiang et al., 2010), oxidative stress (Kiang *et al*., 2010), and necrosis (Jurima-Romet *et al*., 1996), and the effects of (*E*)-2-ene-VPA were comparable to those of VPA (Jurima-Romet *et al*., 1996). The reasons for the conflicting results between *in vivo* rat studies and cell culture studies are not known, but they could relate to differences in the concentrations

of (*E*)-2-ene-VPA achieved *in vivo* and those shown to be toxic in cell culture experiments. As an example, the plasma concentration of (*E*)-2-ene-VPA following its ip administration (250 mg/kg) to rats ranges from 0.25 mM (C_{min}; at 2 h) to 3.5 mM (C_{max}; at 0.25 h) (Loscher et al., 1992). By comparison, in cultured rat hepatocytes, 20-100 mM (present study), 30-100 mM (present study), and 10-100 mM (Jurima-Romet *et al*., 1996; Kiang *et al*., 2010; present study) were the concentrations of (*E*)-2-ene-VPA that produced toxicity, as assessed by markers of oxidative stress, steatosis, and necrosis, respectively. For the current study, the concentrations of VPA and (*E*)-2-ene-VPA selected for the modulation experiments were based on the results of the concentration-response experiments (Fig. 2.2). The criteria were such that the chosen concentrations should be equal to or less than the EC_{50} value, and at those concentrations, VPA and (*E*)-2-ene-VPA would have a similar magnitude of effect on DCF, BODIPY, and LDH markers. Even then, the concentrations of VPA employed in this study were much greater than the plasma concentrations of VPA obtained in patients after oral administration, which is reported to range from 0.28 mM to 0.7 or 1.0 mM (Silva et al., 2008; Sztajnkrycer, 2002). However, it is suggested that the *in vitro* evaluation for idiosyncratic hepatotoxicity of orally administered drugs should include concentrations which are 100-fold of the human therapeutic C_{max} values (Xu et al., 2008; Dykens et al., 2008). Therefore, considering the plasma concentrations of VPA in patients (Silva et al., 2008; Sztajnkrycer, 2002) and the C_{max} values of (*E*)-2-ene-VPA in rats (Loscher et al., 1992), the concentrations of VPA and (*E*)-2-ene-VPA employed in the key experiments with PB and 1-ABT (i.e., 15 and 20 mM for VPA; 20 and 30 mM for (*E*)-2-ene-VPA) are well within this threshold. Furthermore, the *in situ* concentrations of (*E*)-2,4-diene-VPA obtained after treatment of hepatocytes with these concentrations of VPA

and (*E*)-2-ene-VPA are very relevant to the reported plasma concentration of this metabolite in patients treated with VPA (Levy et al., 1990).

A potential limitation of this study could be the use of DCF formation as a marker for oxidative stress, owing to the reported disadvantages associated with this fluorescent probe. These include susceptibility to photooxidation, lack of reactive oxygen species specificity, and the requirement of a catalyst to react with H_2O_2 (Murphy et al., 2011; Wardman, 2007). Oxidation of 2′,7′-dichlorofluorescin to 2′,7′-dichlorofluorescein itself is known to generate superoxide and subsequently H_2O_2 (Bonini et al., 2006; Murphy et al., 2011). However, it needs to be emphasized that DCF was used only as a marker of overall oxidative stress in the hepatocytes and not to quantitatively assess the formation of any particular reactive oxygen species. The results from the DCF assay were always interpreted in a relative-manner with the control group and other treatment groups in this study. Also, the observed changes in DCF formation in the experiments with PB and/or 1-ABT were consistent with the changes in cellular levels of total GSH, suggesting that the observed differences in DCF formation were due to cytochrome P450-mediated reactive metabolites. Furthermore, the pattern of change in DCF formation observed in this study was similar to the pattern of change in the other two markers, namely, BODIPY 558/568 C_{12} accumulation and LDH release. Therefore, it was decided that the limitations of the DCF assay would not have significantly affected the interpretation of the data from this study as well as the conclusions drawn thereof.

In summary, using the sandwich-cultured rat hepatocyte model, (*E*)-2,4-diene-VPA does not appear responsible for the hepatocyte toxicity of VPA, as shown in the modulation experiments that used PB and 1-ABT to increase and decrease, respectively, the *in situ* production of (*E*)-2,4-diene-VPA. On the other hand, *in situ* generated (*E*)-2,4-diene-VPA

contributes to the hepatocyte toxicity of (*E*)-2-ene-VPA, but this occurs only under conditions (e.g. PB treatment) in which relatively high *in situ* levels of this reactive metabolite occur. Based on these results it would appear unlikely that (*E*)-2,4-diene-VPA will contribute significantly to the clinical hepatotoxicity of VPA. Nevertheless, the results from the experiments with PB and (*E*)-2-ene-VPA indicate the toxic potential of (*E*)-2,4-diene-VPA if generated *in situ* at very high levels, and hence, it remains to be seen if this metabolite would contribute to VPA hepatotoxicity in patients with metabolic idiosyncrasies. Based on the markers monitored in this study, both VPA and (*E*)-2-ene-VPA exhibit hepatocyte toxicity that is unrelated to P450-mediated metabolism to (*E*)-2,4-diene-VPA. However, biotransformation appears to play a role in the hepatotoxicity of VPA, as suggested by studies with alpha-fluoro-VPA, a fluorinated analogue of VPA that has a limited ability to undergo biotransformation via glucuronidation (Tang and Abbott, 1997) and mitochondrial β-oxidation (Grillo et al., 2001). Alpha-fluoro-VPA did not produce microvesicular steatosis in rats (Tang et al., 1995) and was not toxic to cultured rat hepatocytes, as assessed by markers of oxidative stress and necrosis (Kiang et al., 2010). Whether the toxicity of VPA and (*E*)-2-ene-VPA involves other pathways of biotransformation such as glucuronidation and mitochondrial β-oxidation is currently under investigation.

Chapter 3: A Rapid and Sensitive Assay to Quantify Valproyl 1-*O*-Acyl Glucuronide in Supernatants of Sandwich-Cultured Rat Hepatocytes Using Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

3.1 Introduction

Clinical use of valproic acid (VPA), which is a commonly used anticonvulsant drug, is associated with a rare, but fatal, idiosyncratic hepatotoxicity (Gerstner et al., 2008). Among the different pathways of VPA biotransformation, glucuronidation is a major pathway in humans, accounting for 30 to 50% of the administered dose (Silva et al., 2008). VPA is biotransformed to valproyl-1-*O*-acyl glucuronide (VPA-G) in a reaction catalyzed by specific uridine diphospho-glucuronosyltransferases (UGTs), such as the human UGT1A3, UGT1A6, UGT1A8, UGT1A9, UGT1A10, and UGT2B7 (Argikar and Remmel, 2009; Ethell et al., 2003; Green et al., 1998; Sakaguchi et al., 2004). In VPA-treated rats, the formation of VPA-G is associated with an elevation of plasma and hepatic levels of 15-F_{2t}-isoprostanes (Tong et al., 2005c), which is an *in vivo* marker of lipid peroxidation (Halliwell and Whiteman, 2004). Therefore, VPA-G is a metabolite of potential toxicological significance.

Studies are currently underway to investigate the role of VPA-G in the toxicity of VPA using a sandwich-cultured rat hepatocyte model. The experimental approach involves evaluating markers of hepatocyte toxicity in relation to VPA-G levels in the cell culture supernatants. There is currently no bioanalytical method in the literature for the direct quantification of VPA-G in

cell culture supernatants. Many of the reported *in vitro* and *in vivo* studies that have quantified VPA-G used an indirect method of determining the difference in the levels of unconjugated VPA (aglycone) before and after alkaline hydrolysis of the sample (Lee et al., 2009; Wong et al., 2001; Wright and Dickinson, 2004; Yu and Shen, 2002). Only a few studies have reported a direct quantification of VPA-G in matrices, such as sheep liver microsomes (Wong et al., 2007) and rat liver homogenate (Tong et al., 2005c), with lower limits of quantification at approximately 0.125 and 0.5 µg/mL, respectively. Furthermore, these assays require larger sample volumes (0.2 and 0.5 mL). However, in cell culture studies, metabolite levels tend to be less and they have to be quantified accurately in relatively small sample volumes, necessitating a sensitive bioanalytical method for the quantification of VPA-G in cell culture samples.

In the present study, a rapid and sensitive ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) method was developed and validated for the quantification of VPA-G in hepatocyte culture medium. The advantages of this method over the reported methods (Tong et al., 2005c; Wong et al., 2007) in the literature include a shorter run time and a 250- to 1000-fold greater sensitivity that is beneficial when assaying small volumes of sample. This method was applied successfully to the quantification of VPA-G levels in culture supernatants of sandwich-cultured rat hepatocytes.

3.2 Materials and Methods

3.2.1 Chemicals, Reagents, and Solvents

Sodium VPA, Williams' medium E, insulin, dexamethasone, collagenase (type IV), trypsin inhibitor (type II-S), dimethyl sulfoxide, and trypan blue, were obtained from Sigma-Aldrich (St. Louis, MO). 2-(Propyl-3,3,3-d3) pentanoic-5,5,5-d3 acid ([$^2H_6$]-VPA) was obtained

from CDN Isotopes (Pointe-Claire, QC, Canada). Liver perfusion medium, hepatocyte wash medium, heat-inactivated fetal bovine serum, 10× Hank's balanced salt solution, 10× Dulbecco's phosphate buffered saline, penicillin-streptomycin, and L-glutamine 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 (HPLC grade), acetonitrile (HPLC grade), and diethyl ether were obtained from Fisher Scientific (Ottawa, ON, Canada). Ultrapure water (0.22 μm filtered deionized water, with a resistivity of 18.2 MΩ.cm at 25°C and total organic carbon content of < 5 ppb) was obtained *via* a Milli-Q Synthesis system (Millipore, Billerica, MA).

3.2.2 Synthesis of VPA-G and [$^2H_6$]-VPA-G

VPA-G and [$^2H_6$]-VPA-G were prepared as described previously (Tong et al., 2005c). Briefly, rats were dosed with either VPA or [$^2H_6$]-VPA at a dose of 100 mg/kg i.p. once every 2 h up to a maximum of four doses, and bile was collected on ice up to 8 h (*i.e.*, 2 h after last dose). Bile samples were acidified with 5 M phosphoric acid to pH 2 and centrifuged at 3000 × *g* for 10 min at room temperature. The supernatants were extracted thrice using 1-chlorobutane to remove the free acid (VPA or [$^2H_6$]-VPA) and the aqueous phase was further extracted with diethylether. The diethylether extracts were dried in vacuum to a dark green residue, which was then reconstituted in mobile phase (15% v/v of 10 mM ammonium acetate in acetonitrile and 85% v/v of 10 mM ammonium acetate in water), and further purified by high performance liquid chromatography. Chromatographic separation was achieved using a Zorbax 300SB-C8 semipreparative column (9.4 mm i.d. × 250 mm), a flow rate of 3 mL/min, and a linear gradient

of 15% to 33% v/v of acetonitrile in water containing 10 mM ammonium acetate The total run time was 12 min. The fractions obtained after HPLC purification were collected, pooled, lyophilized, and stored at -80^{o}C.

3.2.3 Instrumentation and Experimental Conditions

The UHPLC-MS/MS system used for this study consisted of an Agilent 1290 Infinity Binary Pump, a 1290 Infinity Autosampler, and a 1290 Infinity Thermostatted Column Compartment (Agilent, 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. The Analyst® v. 1.5.2 software program (AB Sciex, Concord, Ontario, Canada) was used to operate the UHPLC-MS/MS system and for data acquisition and processing. Chromatographic separation was achieved using a Waters Acquity UPLC® BEH C_{18} column (1.7 µm, 2.1 × 50 mm), which was maintained at 30^{o}C. The autosampler tray was at ambient temperature. The mobile phase was composed of solvent A (water containing 2 mM ammonium acetate) and solvent B (a mixture of acetonitrile/water; 90/10 v/v, containing 2 mM ammonium acetate). The flow rate was 0.4 mL/min and the gradient elution was as follows: 95% solvent A and 5% solvent B from 0 min to 0.8 min, followed by a linear change to 5% solvent A and 95% solvent B from 0.8 to 1.5 min, and this was maintained until 2 min. At 2.1 min, the mobile phase composition was returned to 95% solvent A and 5% solvent B, and the column was re-equilibrated with this mobile phase composition. The total run time was 4 min.

The QTRAP® was operated in multiple reaction monitoring (MRM) mode, using the following instrument settings: curtain gas, 30 units; ion source gas 1, 40 units; ion source gas 2, 60 units; collision-activated dissociation gas level, medium; ion source temperature, 500°C; ion spray voltage, -4500 V; collision cell exit potential, -12 V; entrance potential, -10 V, dwell time, 200 msec. VPA-G was quantified by adding the total ion currents (TIC) of the two MRM transitions m/z 319.1→142.7 and m/z 319.1→175.2, whereas the quantity of internal standard (IS), [$^2H_6$]-VPA-G, was determined using the sum of the TIC of the two MRM transitions of m/z 325.1→149.3 and m/z 325.1→174.9. The declustering potential and collision energy settings were -65 V and -24 V for VPA-G, and -40 V and -20 V for [$^2H_6$]-VPA-G, respectively.

3.2.4 Preparation of Calibration Standard and Internal Standard Solutions

A primary stock solution of VPA-G was prepared at a concentration of 1 mg/mL in ultrapure water and stored in an ultra-low temperature freezer set at -80°C. A series of working stock solutions were prepared by diluting the primary stock solution of VPA-G in Williams' medium E supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1 µM insulin, and 100 nM dexamethasone (Surendradoss et al., 2012), henceforth referred to as hepatocyte culture medium. They were aliquoted and stored at -80°C. Each aliquot was thawed and used only once. The calibration standards were prepared fresh by adding 10 µL of the appropriate working stock solution into 990 µL ultrapure water. The concentrations of the VPA-G calibration standards were 0.5, 1, 5, 10, 50, 100, and 500 ng/mL. The working stock solution of the IS was prepared at a concentration of 50 µg/mL in ultrapure water. Ten µL of the working stock solution was spiked into calibration standards, quality control (QC)

samples, and hepatocyte culture supernatant samples during sample preparation. The final concentration of the IS was 495 ng/mL in each of the samples.

3.2.5 Preparation of QC Samples

A stock solution of VPA-G (1 mg/mL) was diluted in hepatocyte culture medium to prepare the working stock solutions for the low QC (LQC; 1.5 ng/mL), middle QC (MQC; 75 ng/mL), and high QC (HQC; 400 ng/mL) samples. To prepare the QC samples used for determining accuracy, 10 µL of the appropriate working stock solution was spiked into 990 µL ultrapure water. The QC samples were freshly prepared for each batch of analysis. To prepare the QC samples used for determining precision, 0.3 mL of the appropriate working stock solution was spiked into 29.7 mL ultrapure water. The LQC, MQC, and HQC samples were aliquoted into 8 mL volumes and stored at -80^{o}C until use. At the time of analysis, the samples were thawed, further aliquoted in 1 mL volume (six replicates), and processed as described in the next section.

3.2.6 Sample Preparation

Each sample contained 990 µL of ultrapure water and 10 µL of culture medium (blank samples), culture medium containing a known concentration of VPA-G (calibration and validation samples), or culture supernatants (test samples) from sandwich-cultured rat hepatocytes (further described in Section 2.7). To each tube, 10 µL of 50 µg/mL solution of the IS and 20 µL of *ortho*-phosphoric acid were added. After mixing the tubes briefly, 4 mL of ethyl acetate and 4 mL of diethyl ether were added. Each tube was subjected to shaking for 5 min and centrifugation at 2060 × *g* for 10 min at ambient temperature. The organic layer was transferred

to another tube and evaporated to dryness under nitrogen stream in a sample evaporator maintained at 30°C. The dried residue was reconstituted in 0.5 mL of assay diluent (15% solvent A and 85% solvent B), vortex-mixed for 15 seconds, and centrifuged at 2060 × *g* for 10 min at ambient temperature. A 250 µL volume of the sample was transferred to a 96-well polypropylene plate. Fifteen (15) µL of the sample was injected onto the UHPLC-MS/MS system.

3.2.7 Method Validation

The VPA-G assay was validated according to the U.S. Food and Drug Administration guidance on bioanalytical method validation (U.S.Department of Health and Human Services et al., 2001) for the following parameters: calibration curves, lower limit of quantification (LLOQ), selectivity, accuracy, precision, matrix effects, extraction recovery, dilution integrity, carry over, and analyte stability. Hepatocyte culture medium was used as the blank matrix for method validation.

3.2.7.1 Selectivity

Aliquots of hepatocyte culture medium from six different lots were processed as blank samples without adding VPA-G or the IS. The peak area at the retention time associated with VPA-G was compared with that of culture medium samples spiked with VPA-G at the level of LLOQ. The acceptance criterion was that the mean peak area response of blank samples at the retention time of VPA-G should be ≤ 20% of the mean VPA-G peak area response at the LLOQ (U.S.Department of Health and Human Services et al., 2001).

3.2.7.2 Calibration Curves

A blank sample (hepatocyte culture medium without IS), zero sample (hepatocyte culture medium with IS), and seven concentrations of VPA-G ranging from 0.5 to 500 ng/mL were prepared for each batch of analysis. Calibration curves were constructed by plotting the concentrations of VPA-G on the x-axis against the peak area ratio of VPA-G to the IS on the y-axis. Polynomial first order equation for a straight line ($Y = mX + C$) with $1/X^2$ weighting (GraphPad Prism® 5 program, version 5.01, GraphPad Software, Inc., La Jolla, CA) was used to generate the calibration curves. The acceptance criteria for the calibration curves were: i) the accuracy (% deviation) of the back-calculated concentrations of VPA-G for each of the calibration curve standards should be within ± 15% of the nominal concentration, except at the LLOQ where it may be within ± 20% of the nominal concentration (U.S.Department of Health and Human Services et al., 2001), and ii) at least 4 out of 6 calibration standards, including the LLOQ and the highest concentration of the calibration standard, should meet the accuracy criteria described above (U.S.Department of Health and Human Services et al., 2001).

3.2.7.3 LLOQ

The lowest standard in the calibration curve that met the following criteria was deemed to be the LLOQ: i) the VPA-G response (peak area) should be at least ≥ 5 times the response in the blank sample, and ii) the VPA-G peak should be identifiable, discrete, and reproducible with an accuracy (% deviation) of ± 20% and a precision (% RSD) of ≤ 20% (U.S.Department of Health and Human Services et al., 2001).

3.2.7.4 Accuracy

Accuracy was expressed as % deviation of the measured concentration of VPA-G from the nominal concentration, and was calculated as follows: % deviation = [(measured concentration / nominal concentration) x 100] minus 100. The acceptance criterion for accuracy was that the % deviation values should be within ± 15% (U.S.Department of Health and Human Services et al., 2001). Intra-day accuracy was determined by replicate analysis (six each) of freshly prepared LQC, MQC, and HQC samples of VPA-G in hepatocyte culture medium. Inter-day accuracy was determined by preparing and analyzing six replicates of LQC, MQC, and HQC samples of VPA-G on three consecutive days.

3.2.7.5 Precision

Precision of the assay was expressed as the relative standard deviation (% RSD) of the measured concentration of VPA-G for LQC, MQC, and HQC samples, and the % RSD value should be ≤ 15% (U.S.Department of Health and Human Services et al., 2001). Single bulk-spiked samples (30 mL) were prepared for LQC, MQC, and HQC and stored at -80^{o}C as single-use eight mL aliquots. On the day of analysis, an eight mL aliquot from each QC level was thawed, further aliquoted into 1 mL volumes, and processed for analysis to estimate the intra-day precision of the assay for each of the three QC levels. Inter-day precision was determined by preparing and analyzing six replicates of LQC, MQC, and HQC samples of VPA-G on three consecutive days.

3.2.7.6 Matrix Effects and Extraction Recovery

LQC, MQC, and HQC samples of VPA-G were used to assess matrix effects and extraction recovery. Matrix effects were calculated as follows: matrix effect (%) = (peak area of analyte added post-extraction to processed and reconstituted blank culture supernatant sample / peak area of analyte in unextracted neat standard at corresponding concentration) × 100. Neat standards were prepared in diluent by adding working stock solutions of VPA-G and IS that were prepared in water. The % RSD of matrix effects at each concentration should be ≤ 15% (Zhang and Fan, 2012). Recovery was calculated as: recovery (%) = (peak area of analyte in spiked hepatocyte culture medium sample subsquently extracted and analyzed / peak area of analyte in unextracted neat standard at corresponding concentration) × 100. Matrix effects and extraction recovery of the IS were determined by the procedures described above for VPA-G.

3.2.7.7 Dilution Integrity and Carry-Over

The purpose of the dilution integrity test was to validate the dilution step that may be required for some of the actual culture supernatant samples with VPA-G levels greater than ULOQ. Twelve aliquots of HQC (400 ng/mL) were diluted with hepatocyte culture medium by 5- and 10-fold to 80 and 40 ng/mL concentrations (6 aliquots each), respectively, and were processed and analyzed as described in Section 2.5. The accuracy (% deviation) and precision (% RSD) of these diluted samples should be within ± 15% (Zhang and Fan, 2012). Assessment of carry-over was performed by analyzing three injections of ULOQ (500 ng/mL) sample followed by three injections of a processed blank sample. The carry-over in the blank sample should not be greater than 20% of VPA-G peak area response at the LLOQ and 5% of the IS peak area response (Zhang and Fan, 2012).

3.2.7.8 Stability of VPA-G

The stability of VPA-G in hepatocyte culture medium was determined with LQC and HQC samples (five replicates for each sample) under various conditions: cell culture conditions, freeze-thaw, bench top, short term, and post-preparative storage conditions. To evaluate the stability of VPA-G under cell culture conditions, hepatocyte culture medium spiked with VPA-G was incubated in 12-well plates (without cells) at 37^{o}C for 24 h in a CO_2 incubator with 5% CO_2 and 95% air. To investigate freeze-thaw stability, the samples were subjected to three freeze-thaw cycles. In each cycle, the spiked samples were stored at -80^{o}C for >24 h and then thawed unassisted at room temperature for 30 min. To determine bench top stability, the samples were removed from the -80^{o}C freezer, thawed unassisted at room temperature, and stored at this temperature for 6 h. The short term stability of VPA-G in hepatocyte culture medium was assessed after storage of LQC and HQC samples at -80^{o}C for 14 consecutive days. The samples from freeze-thaw, bench top, and short term stability studies were then processed and analyzed. For the post-preparative stability evaluation, the processed and reconstituted LQC and HQC samples were analyzed immediately and again after 12 h of storage at room temperature in the autosampler. To assess re-injection reproducibility, the calibration standards along with LQC and HQC samples were re-analyzed at 24 h after storage at room temperature in the autosampler. In each of these samples, the measured concentration of VPA-G was compared to the nominal concentration of VPA-G, and expressed as % deviation from the nominal value. The criterion to assess the stability of VPA-G was that the % deviation of measured concentration of VPA-G should be $\leq \pm 15\%$ of the nominal value (Zhang and Fan, 2012).

3.2.7.9 Modified Chromatographic Separation to Resolve the Positional Isomers of VPA-G

VPA-G has been shown to undergo pH- and temperature-dependent acyl migration resulting in the formation of positional isomers of VPA-G (Dickinson et al., 1984), henceforth referred to as VPA-G *iso*-glucuronides (Dickinson, 2011). Given the lack of reference standards for VPA-G *iso*-glucuronides, VPA-G was incubated at a concentration of 400 ng/mL (HQC) in hepatocyte culture medium at pH 9.2 (using 10 M sodium hydroxide) for 24 h in a 37^{o}C water bath to generate VPA-G *iso*-glucuronides (Dickinson et al., 1984). These samples were subsequently processed for analysis as described for VPA-G in Section 2.5. The UHPLC-MS/MS method for quantifying VPA-G was modified to resolve VPA-G *iso*-glucuronides from 1-*O*-β-VPA-G using a Waters Acquity® UPLC BEH C_{18} (1.7 µm, 2.1 x 150 mm) column at a flow rate of 0.2 mL/min. The gradient elution was as follows: 95% solvent A and 5% solvent B from 0 min to 0.8 min, followed by a linear change to 5% solvent A and 95% solvent B from 0.8 to 8 min, and this was maintained until 8.5 min. At 8.6 min, the mobile phase composition was returned to 95% solvent A and 5% solvent B, and the column was re-equilibrated with this mobile phase composition. The total run time was 11 min. The eluate was monitored for VPA-G and VPA-G *iso*-glucuronides in both selected ion monitoring (SIM; m/z 319.2) and MRM (transitions m/z 319.1→142.7 and m/z 319.1→175.2) modes. To investigate whether VPA-G *iso*-glucuronides were formed under the cell culture conditions used, VPA-G samples were incubated at 37^{o}C for 24 h in a CO_2 incubator (5% CO_2 / 95% air) and were re-analyzed using this modified method.

3.2.8 Isolation, Culturing, and Treatment of Rat Hepatocytes

Adult male Sprague-Dawley rats (200-250 g) were obtained from Charles River laboratories, Inc. (Senneville, Quebec, Canada), and were housed in the University of British Columbia Animal Resource Unit facility at 22^{o}C in 12 h dark-light cycles. The animals were provided *ad libitum* with food (Labdiet 5001 rodent diet, PMI Feeds Inc., Richmond, IN, USA) and water. 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. Rat hepatocytes were isolated by a two-step collagenase perfusion method (Seglen, 1993), as detailed previously (Tong et al., 2005b). Cells were plated in Matrigel-coated 12-well plates at a density of 0.7×10^6 cells/well in 0.8 mL volume. Coating of Matrigel and medium supplementation were as outlined earlier (Surendradoss et al., 2012). At 4 h after plating, the medium was removed and replaced with fresh supplemented William's medium E (0.8 mL) containing Matrigel (2.5% v/v). Thereafter, the culture medium was replaced every 24 h with 1 mL of serum-free supplemented William's medium E. At 4, 24, 48, 72, 96, or 120 h after plating, hepatocytes were treated with VPA (1 mM) or culture medium (vehicle for VPA) for 24 h. At the end of the 24 h treatment period, culture supernatants were collected and stored at nominal -80^{o}C until analysis.

3.2.9 Statistical Analysis

Data on the effect of culture age on VPA-G levels were analyzed statistically by parametric one-way analysis of variance and, where appropriate, was followed by the Student-Newman-Keuls multiple comparison test (Sigmaplot for Windows, Version 11.0, Systat Software, Inc., Chicago, IL, USA). Statistical significance was set *a priori* at $P < 0.05$.

3.3 Results and Discussion

3.3.1 Method Optimization

The mass spectrometry parameters, such as source temperature, ion spray voltage, focusing potential, declustering potential, entrance potential, collision energy, and collision cell exit potential were optimized by direct infusion of 0.1 µg/mL solutions of VPA-G and the IS into the source of the mass spectrometer. As shown by the product ion spectra, the product ions of VPA-G were m/z 113.1, 142.7, and 175.2 (Fig. 3.1A) and those of IS were m/z 112.9, 149.3, and 174.9 (Fig. 3.1B). The MRM transition m/z 319.1→113.1 did not provide sufficiently abundant signal, particularly at the lower concentrations of VPA-G. Therefore, this MRM transition was not used for quantification. Similarly, the corresponding pair m/z 325.1→112.9 for the IS was also not used. Thus, VPA-G was analyzed using the total of the TIC of the two MRM transitions of m/z 319.1→142.7 and m/z 319.1→175.2, with the IS detected using the MRM transition pairs m/z 325.1→149.3 and 325.1→174.9, which are similar to a previous study (Tong et al., 2005c). In an experiment conducted under SIM mode, the in-source fragmentation of VPA-G to VPA was approximately 1%, as determined by comparison of the peak area of VPA-G with that of VPA occurring at the retention time associated with VPA-G. The UHPLC gradient conditions were optimized to the values stated in Section 3.2.2 to achieve a shorter run time. VPA-G and IS eluted at 1.79 and 1.78 min, respectively. The total run time was 4 min.

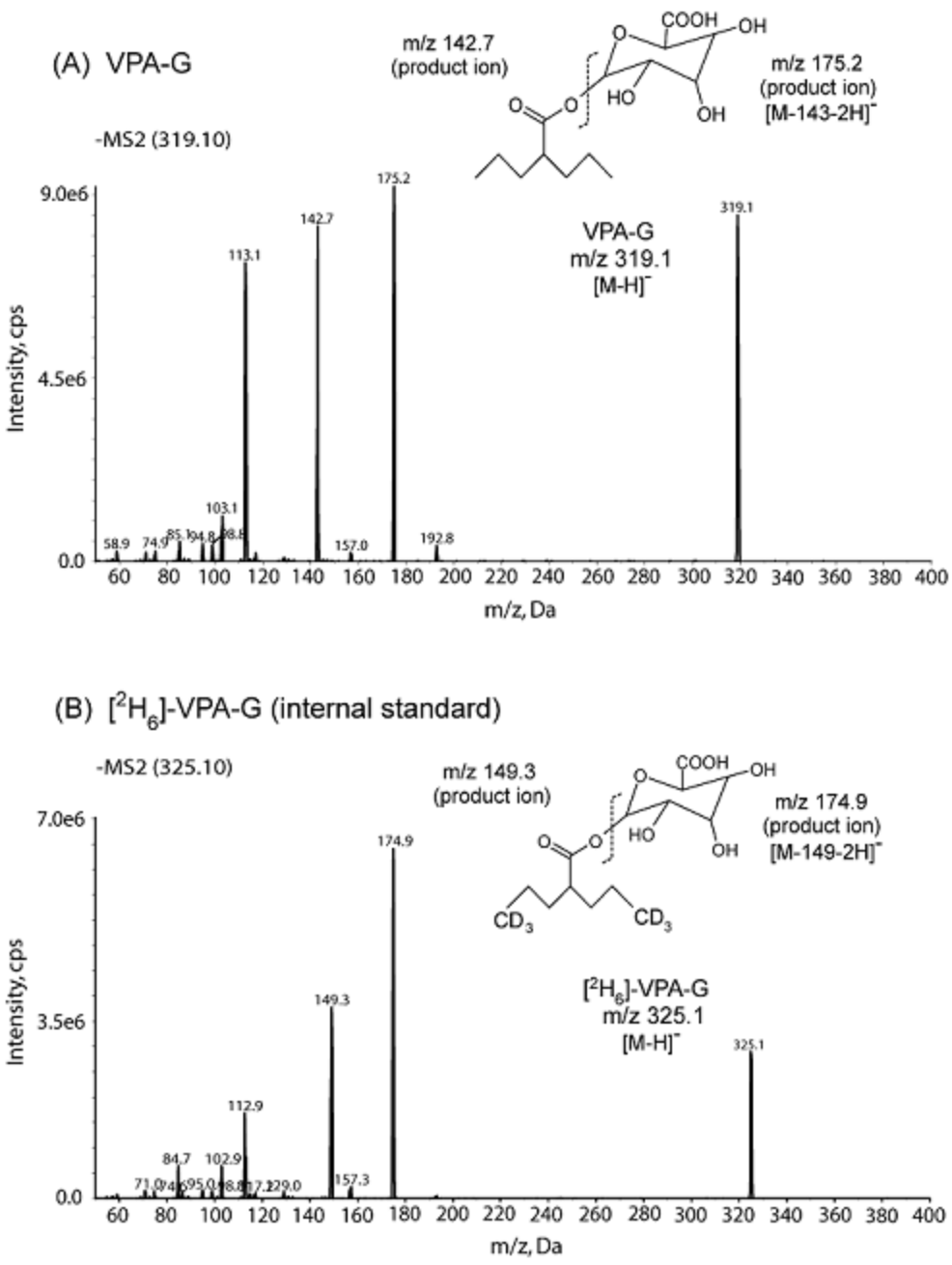

Figure 3.1 Full Scan Product Ion Mass Spectra and Chemical Structures of VPA-G (m/z 319.1) (A) and [$^2H_6$]-VPA-G (Internal Standard; m/z 325.1) (B).

3.3.2 Method Validation

3.3.2.1 Selectivity

Hepatocyte culture media from six different lots were processed as described under Section 3.2.5 and analyzed to determine the selectivity of the method for the quantification of VPA-G. Typical chromatograms of a blank sample (*i.e.*, hepatocyte culture medium without VPA-G or the IS) are shown in Fig. 3.2A (VPA-G) and Fig. 3.2B (IS), and those of LLOQ (*i.e.*, culture medium containing 0.5 ng/mL VPA-G) are shown in Fig. 3.2C (VPA-G) and Fig. 3.2D (IS). The response for the VPA-G peak at the level of LLOQ (Fig. 3.2C) was much greater when compared to the response in the blank sample (Fig. 3.2A). However, the IS contained a small amount of non-deuterated VPA-G (~ 0.2 % as determined by peak area counts). This interfering peak corresponding to VPA-G was observed only in the zero sample (blank containing IS), but not in the blank sample processed without IS. This could most likely be due to the presence of non-labeled VPA in the [$^2H_6$]-VPA sample that was used to generate [$^2H_6$]-VPA-G. Therefore, to avoid any interference in the quantification of VPA-G, the peak area ratio of the zero sample in each batch was subtracted from the peak area ratios of all the samples (except the blank sample) in the batch. In order to further evaluate the selectivity of the assay, blank culture supernatants (*i.e.*, from sandwich-cultured rat hepatocytes treated with hepatocyte culture medium alone) from three independent experiments with 6 replicates for each experiment were processed with IS and analyzed. After subtracting the VPA-G peak area ratio in the zero sample from the peak area ratios of blank culture supernatant samples, the response at the retention time of VPA-G (response ratio of 0.00049 ± 0.00013 (mean ± SD)) was at least 8-fold less than that of the VPA-G peak response at the level of LLOQ (response ratio of 0.0038 ± 0.00012 (mean ± SD)).

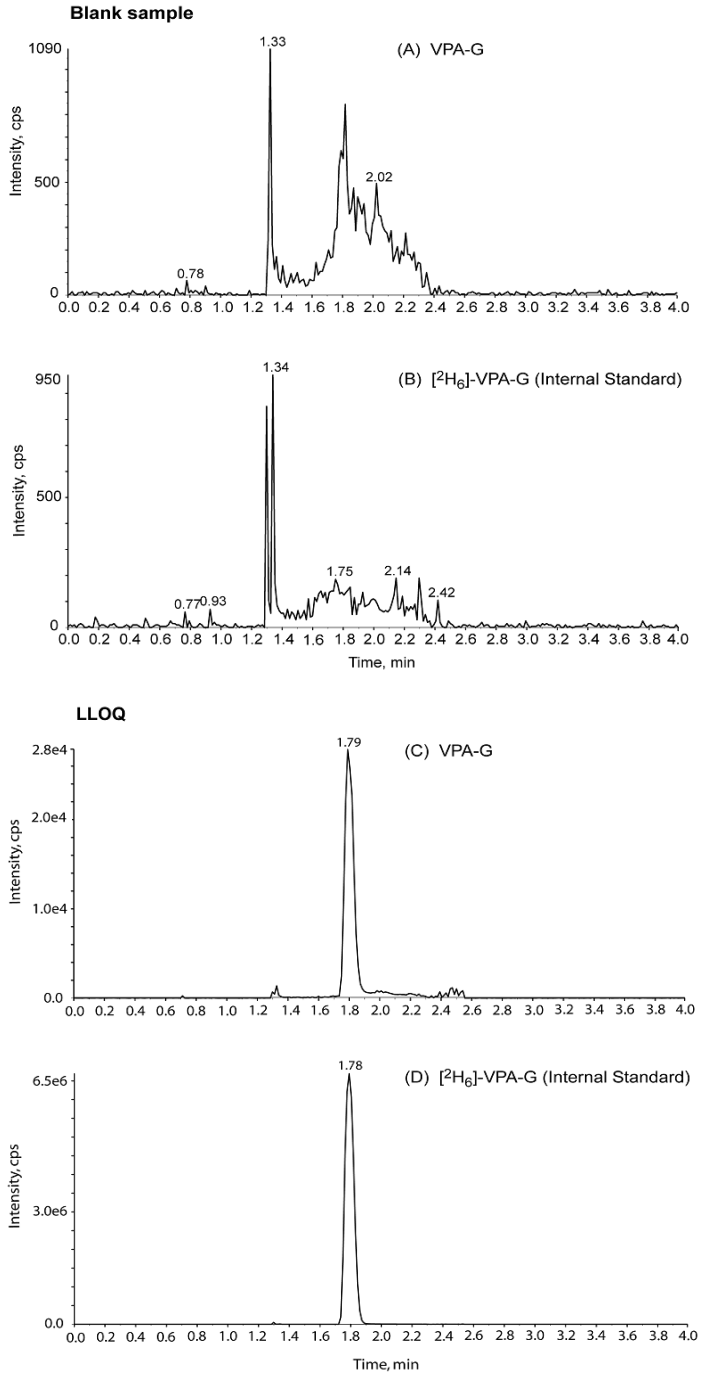

Figure 3.2 Representative Total Ion Current (TIC) Chromatograms of 2 Multiple Reaction Monitoring (MRM) Pairs for VPA-G (A and C) and [$^2H_6$]-VPA-G (Internal Standard; B and D) in Blank Hepatocyte Culture Medium Without VPA-G or IS and in the LLOQ Sample Containing 0.5 ng/mL VPA-G.

The MRM transitions for VPA-G were m/z 319.1→142.7 and m/z 319.1→175.2, and those for [$^2H_6$]-VPA-G were m/z 325.1→149.3 and 325.1→174.9.

3.3.2.2 Calibration Curve

The calibration curve of VPA-G was linear over the concentration range examined (0.5 to 500 ng/mL) and the coefficient of determination (r^2) was 0.995 ± 0.002 (mean ± SD; n = 4). As shown in Table 3.1, the accuracy (% deviation) of the calibration curve standards ranged from -6.8 to + 8.2%, whereas the precision (% RSD) ranged from 2.1 to 6.5%. These data indicate that the calibration curve is linear, accurate, and precise over the VPA-G concentration range of 0.5 to 500 ng/mL.

3.3.2.3 LLOQ

The peak area ratio of VPA-G at the LLOQ (0.5 ng/mL) was several-fold greater than the peak area ratio in the blank hepatocyte culture medium samples that were processed without IS. As stated earlier, the approach of subtracting the peak area ratio in the zero sample resulted in a VPA-G peak area ratio at LLOQ that was 8-fold greater than the peak area ratio of vehicle-treated culture supernatant samples (incurred blank samples) processed with IS. Thus, the method had good selectivity at the level of LLOQ. As shown in Table 3.2, the intra-day accuracy (% deviation) and precision (% RSD) of the assay at the level of LLOQ were 8.9% and 7.4%, whereas the inter-day accuracy and precision were -10 and 5, respectively. This method offers 250- and 1000-fold greater sensitivity than previous methods reported in the literature (Tong et al., 2005c; Wong et al., 2007). Furthermore, the fact that this method requires only 10 µL of sample makes it well suited for application to microplate-based cell culture studies where the total culture supernatant volume will be ≤ 100 µL. These results indicate that the method is selective, accurate, and precise at the LLOQ (0.5 ng/ml).

Table 3.1 Linear Range and Inter-Day Accuracy and Precision of VPA-G Calibration Standards

Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy (% deviation)	Precision (% RSD)
0.5	0.50 ± 0.02	-0.43	3.4
1.0	1.0 ± 0.04	2.4	4.0
5.0	5.0 ± 0.33	0.79	6.5
10	11 ± 0.58	8.2	5.3
50	50 ± 2.2	-0.67	4.4
100	99 ± 5.1	-1.5	5.2
500	466 ± 9.8	-6.8	2.1

Measured concentration of VPA-G is expressed as mean ± SD (n = 4). The slopes of the calibration curves were 0.003 ± 0.0002, whereas, the Y-intercept ranged from -0.0006 to 0.0002. Linearity of VPA-G calibration curves was evaluated by regression analysis. The coefficient of determination (r^2) was 0.995 ± 0.002.

Table 3.2 Intra-Day and Inter-Day Accuracy and Precision for the Quantification of LLOQ and QC Samples of VPA-G

Nominal Concentration of VPA-G (ng/mL)	Intra-Day			Inter-Day		
	Measured Concentration of VPA-G (ng/mL)	Accuracy (% deviation)	Precision (% RSD)	Measured Concentration of VPA-G (ng/mL)	Accuracy (% deviation)	Precision (% RSD)
0.5 (LLOQ)	0.54 ± 0.01	8.9	7.4	0.45 ± 0.02	-10	5.0
1.5 (LQC)	1.6 ± 0.05	9.8	2.7	1.7 ± 0.06	11	2.5
75 (MQC)	75 ± 1.7	0.34	1.2	75 ± 1.7	0.26	3.3
400 (HQC)	386 ± 6.0	-3.5	0.90	390 ± 9.5	-2.4	3.1

Measured concentration of VPA-G is expressed as mean ± SD. The number of replicates for intra-day experiments was 6, whereas that for inter-day experiments was 5 or 6 per day for 3 days.

3.3.2.4 Accuracy and Precision

The intra-day and inter-day accuracy and precision were determined by quantifying VPA-G concentrations in LQC (1.5 ng/mL), MQC (75 ng/mL), and HQC (400 ng/mL) samples (six determinations per concentration in a day on three consecutive days). The concentration of VPA-G was calculated from the corresponding calibration curve. The accuracy and precision data are shown in Table 3.2. The intra-day accuracy (% deviation) of LQC, MQC, and HQC was 9.8%, 0.34%, and -3.5%, respectively. By comparison, the inter-day accuracy was 11%, 0.26%, and -2.4%, respectively. The precision (% RSD) of the method was ≤ 2.7% (intra-day) and 3.3% (inter-day). These data demonstrate that the method can accurately and reproducibly quantify VPA-G levels in hepatocyte culture medium.

3.3.2.5 Matrix Effects and Extraction Recovery

Matrix effects and extraction recovery of VPA-G and the IS are shown in Table 3.3. The % RSD of matrix effects for VPA-G and the IS was ≤ 2.8% and 3.3%, respectively, for LQC, MQC, and HQC samples. Therefore, the hepatocyte culture medium, as a matrix, did not have any substantive effect on the ionization of VPA-G and the IS. The mean % recovery of VPA-G ranged from 91% to 100% for the LQC, MQC, and HQC concentrations and that of IS ranged from 89 to 100%, indicating that the liquid-liquid extraction procedure was efficient.

3.3.2.6 Dilution Integrity and Evaluation of Carry-Over

To test the dilution integrity, multiple aliquots of 400 ng/mL of VPA-G (HQC) at six replicates of each were diluted 5- and 10-fold in hepatocyte culture medium, processed, and analyzed. As shown in Table 3.4, the accuracy (% deviation) after 10- and 5-fold dilution was

Table 3.3 Determination of Matrix Effects and Extraction Recovery of VPA-G

QC Sample	Matrix Effect (%)		Extraction Recovery (%)	
	VPA-G	$[^2H_6]$-VPA-G (internal standard)	VPA-G	$[^2H_6]$-VPA-G (internal standard)
LQC (1.5 ng/mL)	96 ± 1.8	95 ± 1.8	100 ± 2.6	100 ± 1.7
MQC (75 ng/mL)	96 ± 1.7	95 ± 1.2	95 ± 2.1	95 ± 2.2
HQC (400 ng/mL)	90 ± 2.6	90 ± 3.0	91 ± 4.8	89 ± 4.5

Matrix effects and extraction recovery were calculated as described in Section 2.6.6. Data are expressed as mean ± SD for 5 or 6 replicates per QC sample.

-3.8% and -6.4%, respectively. The precision (% RSD) was 2.1% and 4.5% in the HQC sample diluted by 10- and 5-fold, respectively (Table 3.4). Analysis of three injections of blank sample immediately after three injections of ULOQ sample (500 ng/mL of VPA-G) revealed a carry-over that was equivalent to 12% of the peak area response of VPA-G at the LLOQ and 0.02% of the peak area response of the IS.

3.3.2.7 Stability of VPA-G

The data on the stability of VPA-G under various experimental / storage conditions are summarized in Table 3.5. After incubation in hepatocyte culture medium at 37^{o}C for 24 h in a 5% CO_2 incubator, VPA-G levels decreased slightly in the HQC samples (400 ng/mL) with a % deviation of -19% (the criterion is ± 15% from the nominal concentration), whereas the % deviation of VPA-G levels in the LQC samples (1.5 ng/mL) was -6.4% (Table 3.5). VPA-G was stable after three freeze-thaw cycles, storage at room temperature for 6 h, and storage at -80^{o}C for 14 days (Table 3.5). Similarly, after extraction and reconstitution, VPA-G was stable for up to 12 h at room temperature in the autosampler (Table 3.5). The re-injection reproducibility results indicated that the samples may be re-analyzed for at least 24 h after preparation (Table 3.5).

3.3.2.7.1 Positional Isomers of VPA-G

As shown in a MRM chromatogram (Fig. 3.3A), the UHPLC-MS/MS modified method as described in Section 3.2.6.9 was capable of resolving the putative VPA-G *iso*-glucuronide peaks (retention times of 5.56, 5.94, and 6.30 min) from the 1-*O*-β VPA-G peak eluting at 6.12 min. Although authentic standards were not available to verify the identity of these peaks, their

Table 3.4 Dilution Integrity of the VPA-G Assay

Diluted HQC Sample of VPA-G (ng/mL)	Measured Concentration (ng/mL)	Accuracy (% deviation)	Precision (% RSD)
40	38 ± 0.82	-3.8	2.1
80	75 ± 3.3	-6.4	4.5

The HQC sample (400 ng/mL) was diluted 5- and 10-fold to yield 80 and 40 ng/mL, respectively. Measured concentration of VPA-G is expressed as mean ± SD for six replicates.

Table 3.5 Stability of LQC and HQC Samples of VPA-G Under Various Conditions

Stability Test Condition	LQC (1.5 ng/mL)			HQC (400 ng/mL)		
	Measured Concentration of VPA-G (ng/mL)	Accuracy (% deviation)	Precision (% RSD)	Measured Concentration of VPA-G (ng/mL)	Accuracy (% deviation)	Precision (% RSD)
VPA-G in hepatocyte culture medium analyzed immediately after spiking	1.4 ± 0.04	-6.4	2.5	350 ± 3.7	-12	1.1
Cell culture conditions	1.3 ± 0.16	-11	12	324 ± 14	-19	4.5
Freeze-thaw cycles	1.4 ± 0.04	-6.0	2.5	357 ± 3.4	-11	0.9
Bench top storage	1.3 ± 0.04	-12	3.3	342 ± 11	-15	3.2
Short term storage	1.5 ± 0.08	-0.19	5.5	366 ± 4.5	-8.5	1.2
Post-preparative storage	1.4 ± 0.05	-3.9	3.5	358 ± 2.3	-10	0.6
Re-injection reproducibility	1.4 ± 0.04	-6.1	2.7	358 ± 2.6	-10	0.7

The stability of the LQC and HQC samples of VPA-G was assessed under various stability test conditions as described in Section 2.6.8. Measured concentration of VPA-G is expressed as mean ± SD for five or six replicates per QC sample.

detection in both SIM and MRM modes and their appearance at the expense of 1-*O*-β VPA-G peak suggest that these are likely to be VPA-G *iso*-glucuronides. However, except for a small peak at 5.94 min, the putative VPA-G *iso*-glucuronide peaks were not detected following a 24 h incubation of VPA-G in hepatocyte culture medium (without cells) under the cell culture conditions *i.e.*, at 37^{o}C in a CO_2 incubator with 5% CO_2 and 95% air (Fig. 3.3B). Similarly, there were no additional peaks other than the *in situ* generated 1-*O*-β VPA-G peak (Fig. 3.3C) in the culture supernatant samples of sandwich-cultured rat hepatocytes treated with VPA for 24 h, indicating that the intra-molecular acyl migration of VPA-G was not evident under the cell culture conditions employed in the present study.

3.3.3 Effect of Age of Hepatocyte Culture on Glucuronidation of VPA in Sandwich-Cultured Rat Hepatocytes

The validated method was applied to the quantification of VPA-G levels in the supernatants of sandwich-cultured rat hepatocytes treated with VPA in an experiment that investigated the effect of age of hepatocyte culture on VPA glucuronidation. The aim of this experiment was to examine whether there was any decrease over time in the capacity of sandwich-cultured rat hepatocytes to glucuronidate VPA. A representative chromatogram of the culture supernatant sample from hepatocytes treated with 1 mM VPA is shown in Fig. 3.4. The levels of VPA-G in these samples were higher than the linear range of the method and were analyzed after a 5-fold dilution in hepatocyte culture medium. As evident in Fig. 3.5, there was no significant difference in the VPA-G levels over the 6 day culture period in hepatocytes treated with 1 mM VPA for 24 h.

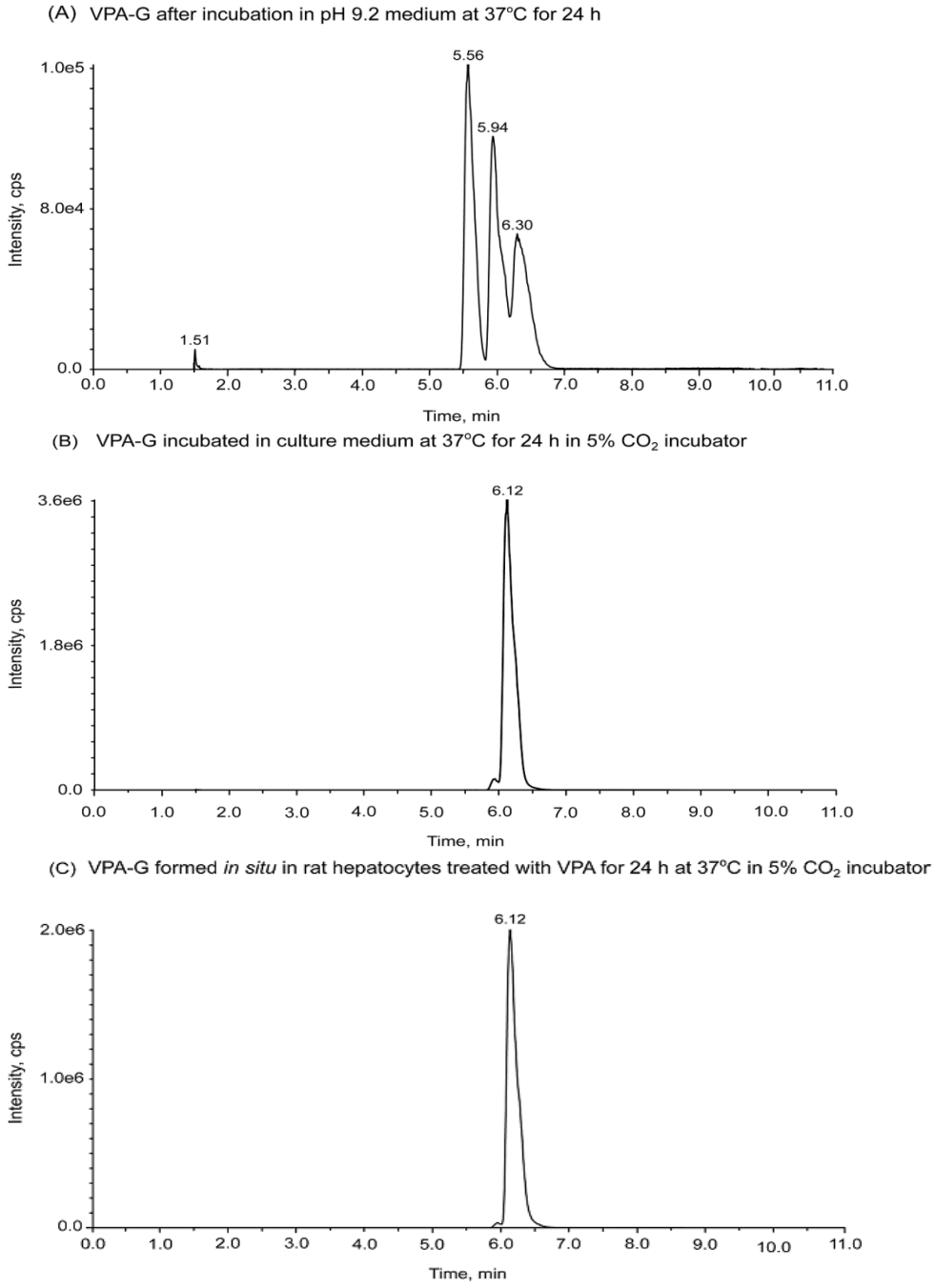

Figure 3.3 Evaluation of VPA-G *Iso*-Glucuronide Formation in Various Conditions.
Shown are the representative total ion current (TIC) chromatograms of VPA-G in hepatocyte culture medium (adjusted to pH 9.2) incubated at 37°C for 24 h (A), VPA-G in hepatocyte culture medium incubated under cell culture conditions, i.e., at 37°C for 24 h in a 5% CO_2 incubator (B), and *in situ* generated VPA-G in the supernatants of sandwich-cultured rat hepatocytes after 24 h treatment with 1 mM VPA at 37°C in a 5% CO_2 incubator (C). The MRM transitions for VPA-G were m/z 319.1→142.7 and m/z 319.1→175.2. These samples were analyzed using a modified UHPLC-MS/MS method described in Section 2.6.9.

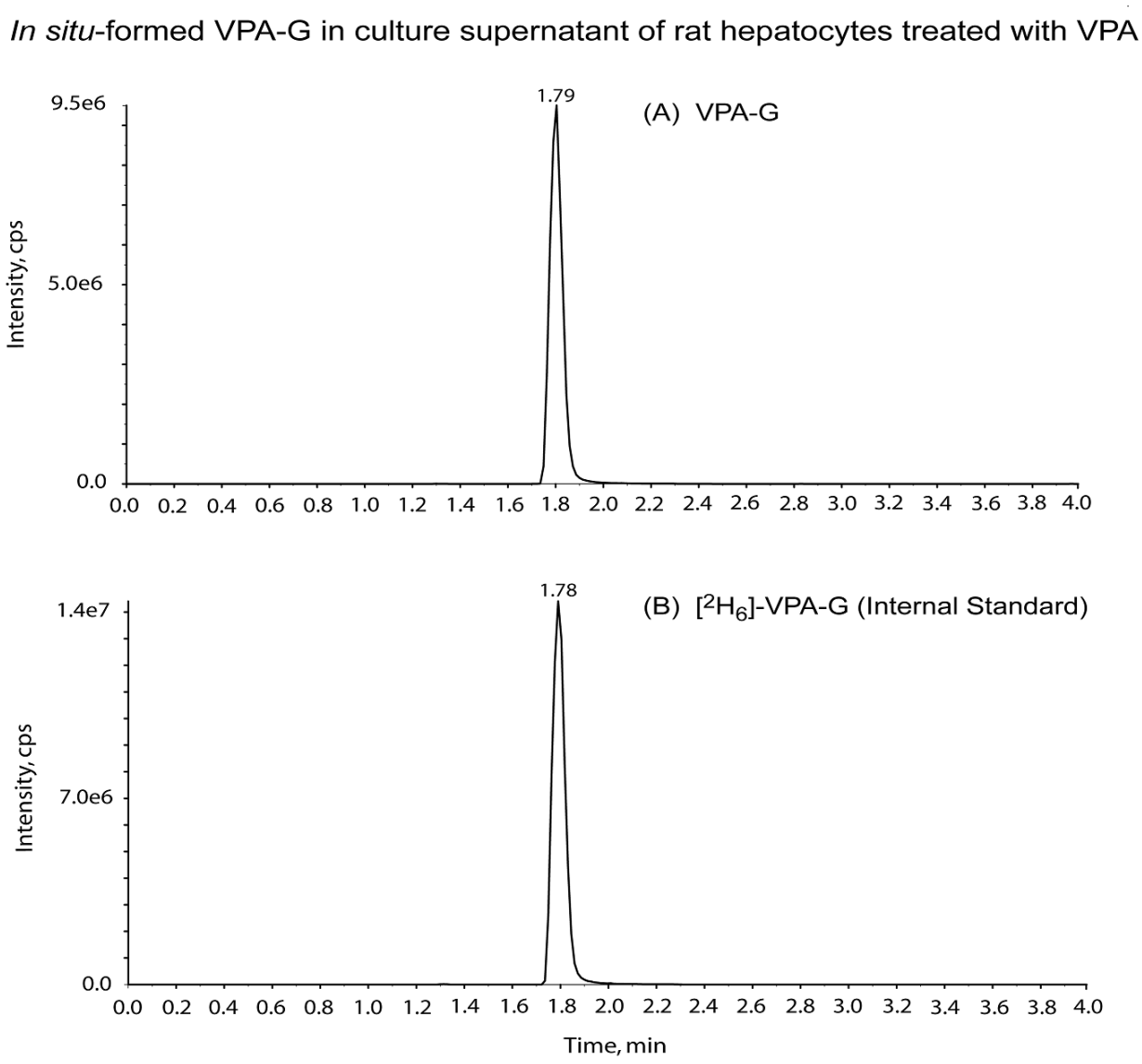

Figure 3.4 *In Situ* Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with 1 mM VPA for 24 h.

Shown are representative total ion current (TIC) chromatograms of VPA-G (A) and [$^2H_6$]-VPA-G (internal standard; B). The MRM transitions for VPA-G were m/z 319.1→142.7 and m/z 319.1→175.2, and those of [$^2H_6$]-VPA-G were m/z 325.1→149.3 and 325.1→174.9.

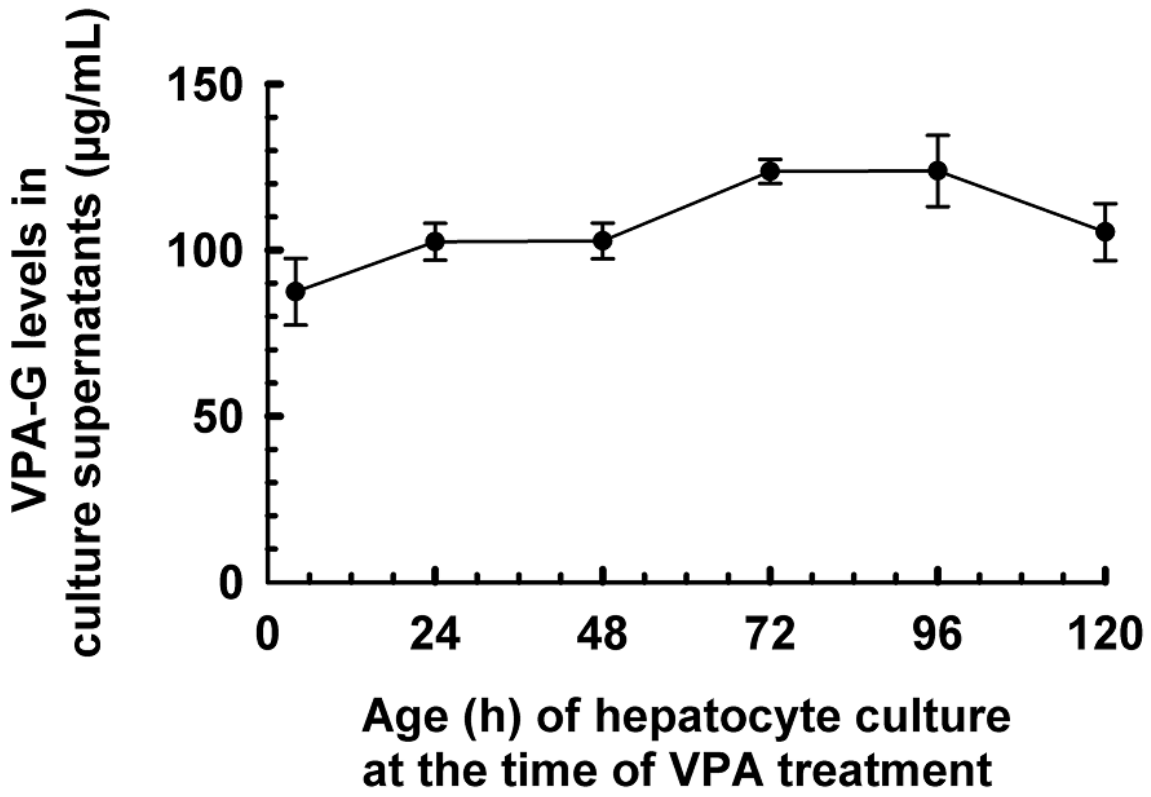

Figure 3.5 Effect of Age of Hepatocyte Culture on VPA-G Levels in Culture Supernatants of Sandwich-Cultured Rat Hepatocytes Treated with VPA.
At 4, 24, 48, 72, 96, or 120 h of culture, hepatocytes were treated with 1 mM VPA or culture medium (vehicle for VPA) for 24 h. At the end of the drug treatment period, culture supernatants were collected and stored at -80^{o}C until UHPLC-MS/MS analysis for VPA-G levels. Data are expressed as mean ± SEM (N = three rats). VPA-G levels were not significantly different at 4, 24, 48, 72, 96, and 120 h of hepatocyte culture.

3.4 Conclusions

A sensitive UHPLC-MS/MS method was developed and validated for the quantification of VPA-G in hepatocyte culture medium. This method builds upon existing methods (Tong et al., 2005c; Wong et al., 2007) for assaying VPA-G levels while offering much greater sensitivity and a shorter run time than previous methods (Tong et al., 2005c; Wong et al., 2007). The increased level of sensitivity with the current method enables the use of smaller sample volumes, which makes the method suitable for microplate-based cell culture studies. The accuracy and precision of this method were within allowed limits. The sample preparation method was efficient in recovering VPA-G and IS, with no matrix effects on their ionization. VPA-G was stable at various storage conditions. The method was applied successfully to the quantification of VPA-G levels in supernatants of sandwich-cultured rat hepatocytes in an experiment that investigated the effect of the age of the culture on VPA-G levels in hepatocytes treated with VPA. This method should be adaptable for the quantification of VPA-G in other matrices, such as plasma, urine, and bile.

Chapter 4: Evaluation of the Toxicity of *In Situ* Generated Valproyl 1-*O*-β-Acyl Glucuronide in Sandwich-Cultured Rat Hepatocytes Treated with Valproic Acid

4.1 Introduction

Acyl glucuronides, in general, are reactive electrophilic metabolites (Boelsterli and Ramirez-Alcantara, 2011), which are capable of undergoing: 1) hydrolysis to parent aglycone mediated by β-glucuronidases, non-specific esterases, hydroxide ion, or serum albumin; 2) intra-molecular 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). Even though acyl glucuronides are hypothesized to be involved in the toxicities associated with carboxylic acid-containing drugs, a causal role for these reactive species is yet to be established (Boelsterli, 2011). Postulated mechanisms for the toxicity of acyl glucuronides include: i) 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), and the consequent GSH depletion and possibly covalent binding to proteins (Shipkova et al., 2003; Skonberg et al., 2008).

Valproic acid (VPA) is a commonly used antiepileptic drug, which is effective against various types of seizures and epileptic syndromes (Aldenkamp et al., 2006). Clinical use of VPA is associated with the occurrence of a rare, but potentially fatal, idiosyncratic hepatotoxicity (Nanau and Neuman, 2013). The exact mechanism of VPA hepatotoxicity is not fully understood; however, the biotransformation of VPA and the subsequent formation of reactive metabolites are implicated in VPA-associated liver injury (Kiang et al., 2010). VPA biotransformation includes both phase-I (mitochondrial β-oxidation and cytochrome P450 (CYP)-mediated oxidation) and phase-II (glucuronidation) pathways (Abbott and Anari, 1999). Glucuronidation is a major metabolic pathway for VPA, and contributes to the metabolism of about 30 to 50% of the administered dose of VPA in humans (Silva et al., 2008). Uridine 5'-diphospho-glucuronosyltransferases (UGTs) catalyze the conjugation of VPA with glucuronic acid, yielding valproyl 1-*O*-β-acyl glucuronide (VPA-G) (Ethell et al., 2003). VPA-G is one of the least reactive acyl glucuronides investigated so far (Stachulski et al., 2006). Yet, it undergoes intra-molecular 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). An *in vivo* study in rats implicated VPA-G in VPA-associated elevation of plasma and hepatic levels of 15-F_{2t}-isoprostane (Tong et al., 2005c), which is an *in vivo* marker of lipid peroxidation (Halliwell and Whiteman, 2004). Furthermore, the findings from the recent studies that investigated the role of VPA metabolites, 4-ene-VPA and (*E*)-2,4-diene-VPA in the hepatocyte toxicity of VPA appear to postulate a possible role for VPA-G and/or VPA-CoA in VPA toxicity (Kiang et al., 2010; Surendradoss et al., 2012). However, the toxicity of VPA-G and its role in VPA hepatotoxicity still remain to be established.

The present study was performed to investigate the toxicity of *in situ* generated VPA-G in sandwich-cultured rat hepatocytes treated with VPA. Hepatocytes cultured in sandwich-configuration are valuable models to obtain mechanistic insights on hepatotoxicity (Soldatow et al., 2013). The objectives of this study were: 1) to evaluate the effects of various known inducers of UGT enzymes on increasing the *in situ* formation of VPA-G in sandwich-cultured rat hepatocytes treated with VPA; and 2) to determine the effects of modulating *in situ* formation of VPA-G on toxicity in VPA-treated sandwich-cultured rat hepatocytes. The results are discussed in the context of whether *in situ* generated VPA-G is toxic to sandwich-cultured rat hepatocytes. Diclofenac, which is a well-known carboxylic acid drug that forms an acyl glucuronide and is associated with idiosyncratic hepatotoxicity (Tang, 2003), was also included in the key experiments as a means to compare and contrast the results obtained with VPA.

4.2 Materials and Methods

4.2.1 Chemicals, Reagents, and Solvents

Sodium VPA, sodium phenobarbital, sodium diclofenac, β-naphthoflavone, 3-methylcholanthrene, L-sulforaphane, quercetin, dexamethasone, clofibrate, pregnenolone 16α-carbonitrile (PCN), *trans*-stilbene oxide, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Ritonavir, diclofenac acyl-β-D-glucuronide (DFN-G), 4'-hydroxy-diclofenac (4'-OH-DFN), and 5-hydroxy diclofenac (5-OH-DFN) were purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Culture media, chemicals, and reagents for hepatocyte isolation and culture, chemicals and solvents for metabolite

quantification, and various cytotoxicity assay kits were purchased from suppliers indicated previously (Surendradoss et al., 2012; Surendradoss et al., 2013).

4.2.2 Animals

Adult male Sprague-Dawley rats (175-200 g) were obtained from Charles River Laboratories, Inc. (Senneville, QC, Canada), and were housed and cared for in the University of British Columbia Animal Resource Unit 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.

4.2.3 Isolation, Culture, and Treatment of Rat Hepatocytes

Rat hepatocytes were isolated by a two-step collagenase perfusion method (Seglen, 1993), as described previously (Tong et al., 2005c). Plating of hepatocytes (0.7×10^6 cells per well), preparing the sandwich-culture configuration, and culturing of hepatocytes were performed as reported earlier (Surendradoss et al., 2012). At 120 h after plating, sandwich-cultured rat hepatocytes were treated with VPA, culture medium (vehicle for VPA), diclofenac, or DMSO (vehicle for diclofenac) for the next 24 h at the concentrations indicated in each figure legend. In other experiments, at 48 h after plating, cultured hepatocytes were pretreated with an inducer of UGT or an inhibitor of glucuronidation (i.e. borneol) and then treated with VPA, diclofenac, or vehicle as described in each figure legend.

4.2.4 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 phosphate-buffered saline (PBS; pH 7.4) containing 20 mM EDTA. Each sample was transferred into a microfuge tube and stored at -80^{o}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 [$^{2}H_{6}$]-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 Thermostatted 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.

4.2.5 Quantification of Diclofenac Acyl-β-D-Glucuronide (DFN-G) Concentration

To quantify DFN-G concentrations in culture supernatant and cell lysate from diclofenac-treated hepatocytes, 10 µl of the sample and 10 µl of a 50 µg/ml solution of [$^{2}H_{6}$]-VPA-G (internal standard) were added to 480 µl of assay diluent (85%: 2 mM ammonium acetate in water and 15% 2 mM ammonium acetate in 9:1 mixture of acetonitrile and water), vortex-mixed for 10 s, and centrifuged at 10600 × *g* for 5 min at 4^{o}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 as described previously for VPA-G assay (Surendradoss et al., 2013). 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 [$^2H_6$]-VPA-G transition pairs being m/z 325.1 → 149.3 and 325.1 → 174.9.

4.2.6 Lactate Dehydrogenase (LDH) Assay

LDH activity was determined using the Cytotoxicity Detection Kit (Roche Diagnostics, Indianapolis, IN) (Surendradoss et al., 2012). LDH released in to culture supernatant was expressed as a percentage of the total cellular LDH activity (i.e. sum of the LDH activity in the culture supernatant and cell lysate).

4.2.7 BODIPY 558/568 C_{12} Assay

Cellular accumulation of BODIPY 558/568 C_{12} was determined as reported previously (Surendradoss et al., 2012). Fluorescence was measured at a λ_{ex} of 484 nm and λ_{em} of 618 nm in a Biotek Synergy Mx microplate reader (Biotek Instruments, Winooski, VT). Each blank well had culture medium containing BODIPY 558/568 C_{12} and a test compound, but without cells. BODIPY 558/568 C_{12} accumulation was expressed as fold increase in fluorescence in drug-treated wells over that in vehicle-treated control wells.

4.2.8 Glutathione (GSH) Assay

Cellular content of total GSH was quantified using the Glutathione Assay Kit (Cayman Chemical Co.) 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® multiwell plate reader

(Thermo Electron Corp., Burlington, ON, Canada). The blank sample consisted of equal volumes of phosphate buffered saline (pH 7.4; supplemented with 1 mM EDTA) and metaphosphoric acid, but without cell homogenate.

4.2.9 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 (GC-MS) assay (Surendradoss et al., 2012).

4.2.10 Quantification of 4'-OH-DFN and 5-OH-DFN.

Concentrations of 4'-OH-DFN and 5-OH-DFN in the culture supernatants of diclofenac-treated hepatocytes were determined using a LC-MS/MS assay adapted from Sparidans 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 s, and centrifuged at 10600 × *g* for 5 min at 4^{o}C. A 5 µl volume of the supernatant was injected onto 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 as described previously (Sparidans et al., 2008). Analysis was done 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 units; ion source gas 1, 60 units; ion source gas 2, 40 units; 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. 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 with no internal standard. 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.

4.2.11 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, Inc., Chicago, IL). The level of statistical significance was set *a priori* at $P < 0.05$. Parametric testing was employed for the statistical analysis of the data. In situations where the requirements for parametric statistical testing (*i.e.*, tests for normality and equal variance) were not met, the data were analyzed by an equivalent non-parametric test.

4.3 Results

4.3.1 Concentration of *In Situ* Generated VPA-G in Culture Supernatant and Cell Lysate of Sandwich-Cultured Rat Hepatocytes Treated with VPA

The concentration of VPA-G (mean ± SEM; n = 4 independent experiments) was 251 ± 12 and 7 ± 2 µM in the culture supernatant and cell lysate, respectively. As more than 97% of the *in situ* generated VPA-G is localized in the culture supernatant, VPA-G concentration was quantified in culture supernatant in all the subsequent experiments, unless indicated otherwise.

4.3.2 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. 4.1A, the *in situ* concentration of VPA-G continued to increase in the culture supernatants in a linear fashion over the 1 to 24 h period. Concentration-response experiments indicated that the *in situ* formation of VPA-G increased from 19.7 to 348 μM in response to increases in VPA concentration from 0.03 mM to 3 mM (Fig. 4.1B), with plateauing of VPA-G concentrations occurring over 3 to 10 mM VPA. *In situ* concentrations of VPA-G began to decrease with VPA concentrations ≥ 20 mM (Fig. 4.1B), which could be due to the toxicity of VPA in sandwich-cultured rat hepatocytes (Surendradoss et al., 2012). 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 would result in near maximal formation of VPA-G (Fig. 4.1B) and also elicit a measurable response in the toxicity markers (Surendradoss et al., 2012).

4.3.3 Effect of Various Known UGT Inducers on *In Situ* Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA

β-Naphthoflavone (Viollon-Abadie et al., 2000), 3-methylcholanthrene (Jemnitz et al., 2000), L-sulforaphane (Kohle and Bock, 2006), quercetin (Soars et al., 2004), clofibrate (Jemnitz et al., 2000), dexamethasone (Jemnitz et al., 2000), PCN (Shelby and Klaassen, 2006), phenobarbital (Soars et al., 2004), *trans*-stilbene oxide (Shelby and Klaassen, 2006), and ritonavir (Foisy et al., 2008) have been shown to induce specific UGT enzymes in mice, rats, or humans. Therefore, the present study determined whether any of these chemicals at

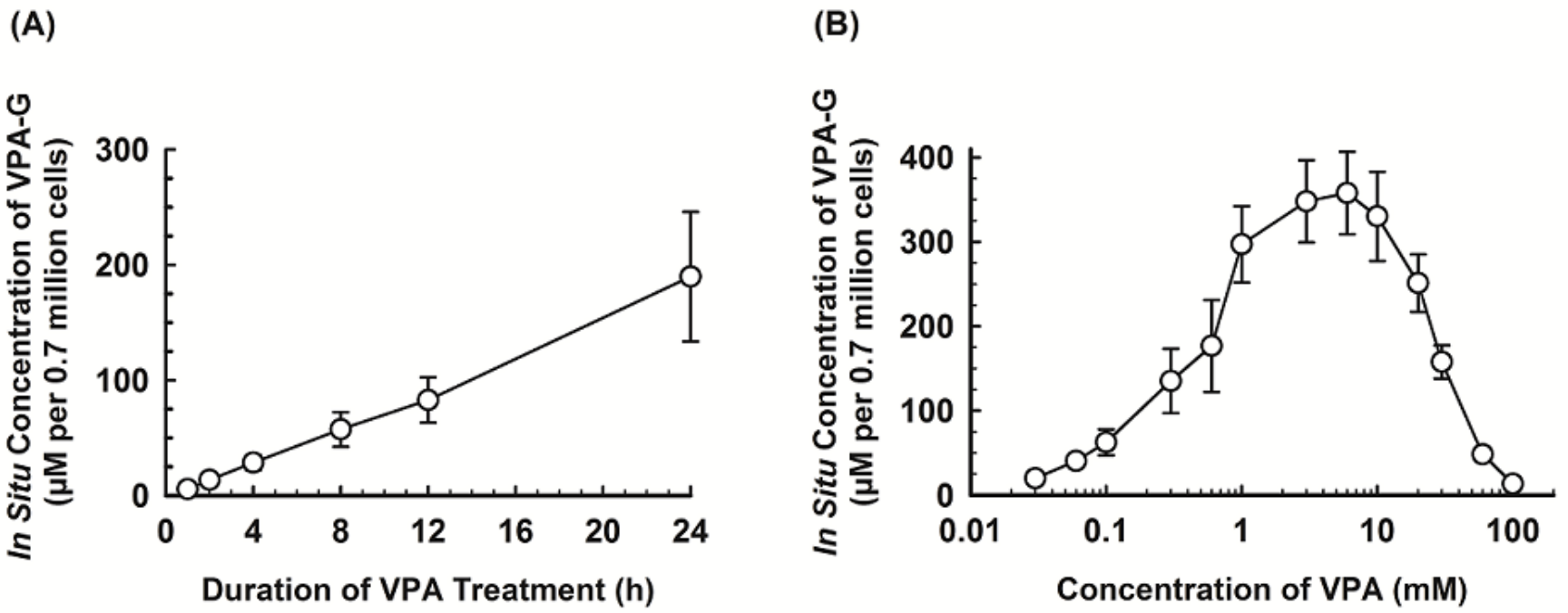

Figure 4.1 Time Course and Concentration-Response Relationship in the *In Situ* Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

(A) Hepatocytes were cultured for 120 h and then treated with VPA (1 mM) or culture medium (vehicle). At 1, 2, 4, 8, 12, and 24 h after drug treatment, an aliquot of culture supernatant was collected and VPA-G concentrations were quantified by UHPLC-MS/MS. Data are expressed as mean ± SEM (N = three rats). (B) Hepatocytes were cultured for 120 h and then treated with VPA (0.03-100 mM) or culture medium (vehicle) for 24 h. Data are expressed as mean ± SEM (N = three or four rats).

concentrations reported in the literature increases the *in situ* formation of VPA-G in sandwich-cultured rat hepatocytes treated with VPA. β-Naphthoflavone, L-sulforaphane, and phenobarbital were effective in increasing the *in situ* formation of VPA-G (Fig. 4.2A), whereas none of the other chemicals had an effect (Fig. A.3). For comparison, β-naphthoflavone, L-sulforaphane, and phenobarbital were also capable of increasing the glucuronidation of diclofenac (Fig. 4.2A), which is known to form an acyl glucuronide (Tang, 2003). Among the three chemical modulators, β-naphthoflavone, which produced the maximal increase in *in situ* VPA-G concentration, was chosen to investigate the effects of increased *in situ* formation of VPA-G on toxicity markers in sandwich-cultured rat hepatocytes.

4.3.4 Effect of β-Naphthoflavone Pretreatment on Markers of Toxicity in Sandwich-Cultured Rat Hepatocytes Treated with VPA

To investigate the toxicity associated with β-naphthoflavone-mediated increase in the *in situ* concentrations of VPA-G, sandwich-cultured rat hepatocytes were pretreated with 20 μM β-naphthoflavone or 0.1% DMSO (vehicle) once every 24 h for 72 h, followed by 24 h treatment with VPA (10 or 15 mM) or culture medium (vehicle). At the end of the treatment period, LDH release, which is a marker of cell necrosis (Jauregui et al., 1981), BODIPY 558/568 C_{12} accumulation, which is an index of steatosis (Fujimura et al., 2009), and cellular concentration of total GSH were measured. As shown in Fig. 4.2B, β-naphthoflavone alone did not affect LDH release, whereas 10 and 15 mM VPA increased it by 1.3-fold and 1.5-fold, respectively. β-naphthoflavone pretreatment did not further increase the LDH release by VPA (Fig. 4.2B). In the same samples, β-naphthoflavone pretreatment increased the extracellular and intracellular

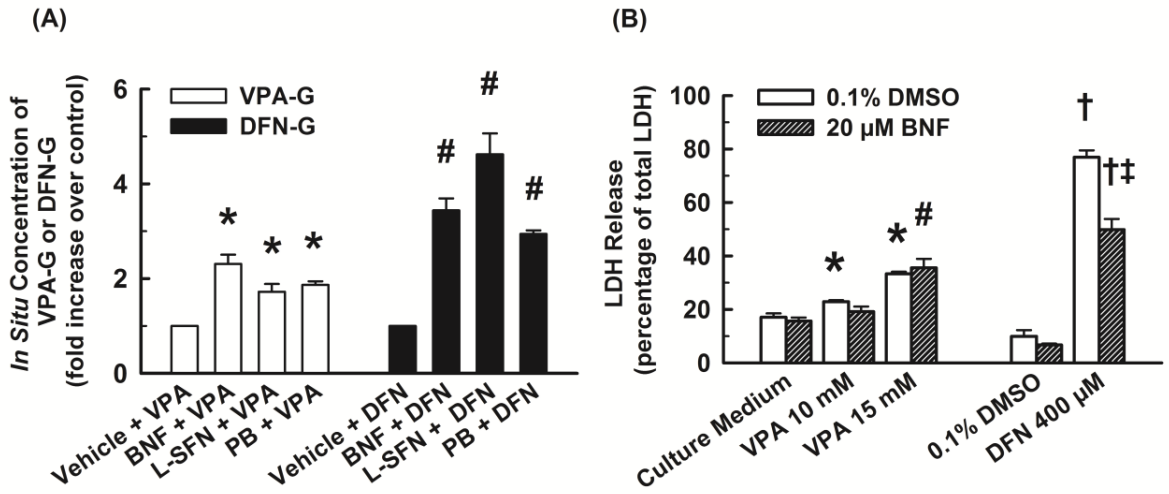

Figure 4.2 Effect of UGT Enzyme Inducers on *In Situ* Formation of VPA-G and LDH Release in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

(A) Hepatocytes were cultured for 48 h and then pretreated with β-naphthoflavone (BNF; 20 μM), L-sulforaphane (L-SFN; 5 μM), sodium phenobarbital (PB; 2 mM), or vehicle (0.1% DMSO for BNF and L-SFN; culture medium for PB) once every 24 h for 72 h. 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 h. Data are expressed as mean ± SEM (N = three rats). *Significantly different from the vehicle + VPA control group, $P < 0.05$; #significantly different from the vehicle + DFN control group, $P < 0.05$. The *in situ* concentrations (mean ± SEM) of VPA-G were 256 ± 45 and 213 ± 35 μM per 0.7 million cells in the culture medium- and 0.1% DMSO-pretreated groups, respectively. The *in situ* concentrations (mean ± SEM) of DFN-G were 23 ± 2 and 20 ± 2 μM per 0.7 million cells in the culture medium- and 0.1% DMSO-pretreated groups, respectively. (B) Hepatocytes were cultured for 48 h and then pretreated with BNF (20 μM) or DMSO (0.1% v/v; vehicle) once every 24 h for 72 h. Subsequently, the hepatocytes were treated with VPA (10 or 15 mM), culture medium (vehicle for VPA), DFN (400 μM), or 0.1% DMSO (vehicle for DFN) for the next 24 h. LDH content in culture supernatant and cell lysate were quantified. Data are expressed as mean ± SEM (N = three rats). *Significantly different from the culture medium-treated group that received 0.1% DMSO pretreatment, $P < 0.05$; #significantly different from the culture medium-treated group that received 20 μM BNF pretreatment, $P < 0.05$; †Significantly different from the 0.1% DMSO-treated group that received 0.1% DMSO pretreatment, $P < 0.05$; ‡significantly different from DFN 400 μM-treated group that had 0.1% DMSO pretreatment, $P < 0.05$.

concentrations of VPA-G in sandwich-cultured rat hepatocytes treated with 10 or 15 mM VPA (Table 4.1). In a comparative experiment, β-naphthoflavone increased DFN-G concentrations by 3.7-fold (Fig. 4.2A) and this was accompanied by an attenuation of LDH release in diclofenac-treated hepatocytes (Fig. 4.2B). In the BODIPY assay, pretreatment with β-naphthoflavone did not affect BODIPY 558/568 C_{12} accumulation in rat hepatocytes, whereas 10 and 15 mM VPA increased BODIPY 558/568 C_{12} accumulation (Fig. 4.3A). This increase in BODIPY 558/568 C_{12} accumulation by VPA was attenuated by β-naphthoflavone pretreatment (Fig. 4.3A). In contrast to the increases observed for the LDH and BODIPY markers, VPA treatment (10 and 15 mM) had no effect on total GSH content in sandwich-cultured rat hepatocytes (Fig. 4.3B). As shown in Fig. 4.3B, β-naphthoflavone pretreatment *per se* led to an increase in the concentration of total GSH in rat hepatocytes. However, treatment with 15 mM VPA, but not 10 mM VPA, reduced the concentration of total GSH (by ~ 25%) in the β-naphthoflavone-pretreated rat hepatocytes.

4.3.5 Effect of Borneol on *In Situ* Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA

Another approach to investigate the effect of *in situ* formed VPA-G is by the use of borneol, which is a known inhibitor of glucuronidation (Watkins and Klaassen, 1983). Borneol, over the concentration range of 0.25 to 1 mM, was found to attenuate both extracellular and intracellular concentrations of VPA-G in VPA-treated hepatocytes (Fig. A.4). As shown in Fig. 4.4A, borneol (1 mM) decreased the *in situ* formation of VPA-G in VPA-treated hepatocytes by 35% in comparison to vehicle control. In a comparative experiment, however, borneol did not affect the *in situ* formation of DFN-G in hepatocytes treated with diclofenac (Fig. 4.4A).

Table 4.1 Effect of β-Naphthoflavone and Borneol on *In Situ* Formation of VPA-G in the Culture Supernatants and Cell Lysates of Sandwich-Cultured Rat Hepatocytes Treated with VPA

Experiment 1. At 48 h after plating, hepatocytes were pretreated with 20 μM BNF or 0.1% DMSO (vehicle) every 24 h for 72 h and then treated with VPA (10 or 15 mM) or culture medium (vehicle) for the next 24 h. *Experiment 2*. At 120 h after plating, hepatocytes were pretreated with 1 mM BRN or 0.1% DMSO (vehicle) for 0.5 h. Subsequently, hepatocytes were treated with VPA (10 or 15 mM) or culture medium (vehicle) in the presence of 1 mM BRN or 0.1% DMSO for 24 h. In both experiments, culture supernatants and cell lysates were collected at the end of the 24 h treatment period and subjected to UHPLC-MS/MS assay for VPA-G concentrations. Data are expressed as mean ± SEM (N = three or four rats).

Pretreatment	*In situ* Concentration of VPA-G (μM / 0.7×10^6 cells)			
	Culture Supernatant		Cell Lysate	
	VPA 10 mM	VPA 15 mM	VPA 10 mM	VPA 15 mM
Experiment 1				
DMSO	276 ± 63	252 ± 57	3.5 ± 0.2	3.3 ± 0.3
BNF	617 ± 99*	516 ± 82*	8.4 ± 0.5*	7.2 ± 0.5*
Experiment 2				
DMSO	313 ± 8	279 ± 14	1.4 ± 0.3	1.5 ± 0.2
BRN	233 ± 10*	207 ± 11*	1.2 ± 0.1	1.2 ± 0.1

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

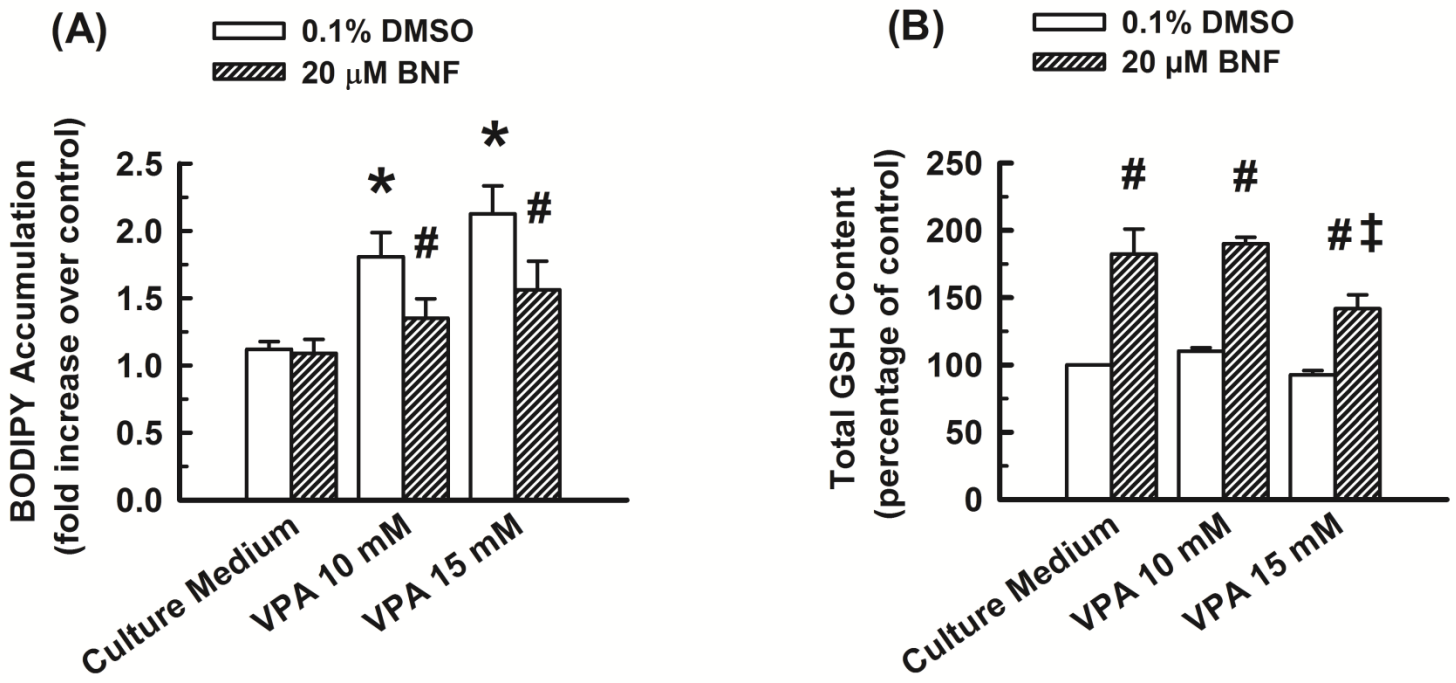

Figure 4.3 Effect of β-Naphthoflavone on (A) BODIPY 558/568 C_{12} Accumulation and (B) Total GSH Content in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

Hepatocytes were cultured for 48 h and then pretreated with β-naphthoflavone (BNF; 20 μM) or DMSO (0.1% v/v; vehicle) once every 24 h for 72 h. Subsequently, the hepatocytes were treated with VPA (10 or 15 mM) or culture medium (vehicle) for the next 24 h. Data are expressed as mean ± SEM (N = three to five rats). *Significantly different from the culture medium-treated group that received 0.1% DMSO pretreatment, $P < 0.05$; #significantly different from the 0.1% DMSO pretreatment group that received the same treatment; ‡Significantly different from the culture medium-treated group that received BNF 20 μM pretreatment, $P < 0.05$. The total GSH content (mean ± SEM) was 7.4 ± 1.6 μM per 0.7 million cells in the culture medium-treated group that received 0.1% DMSO pretreatment.

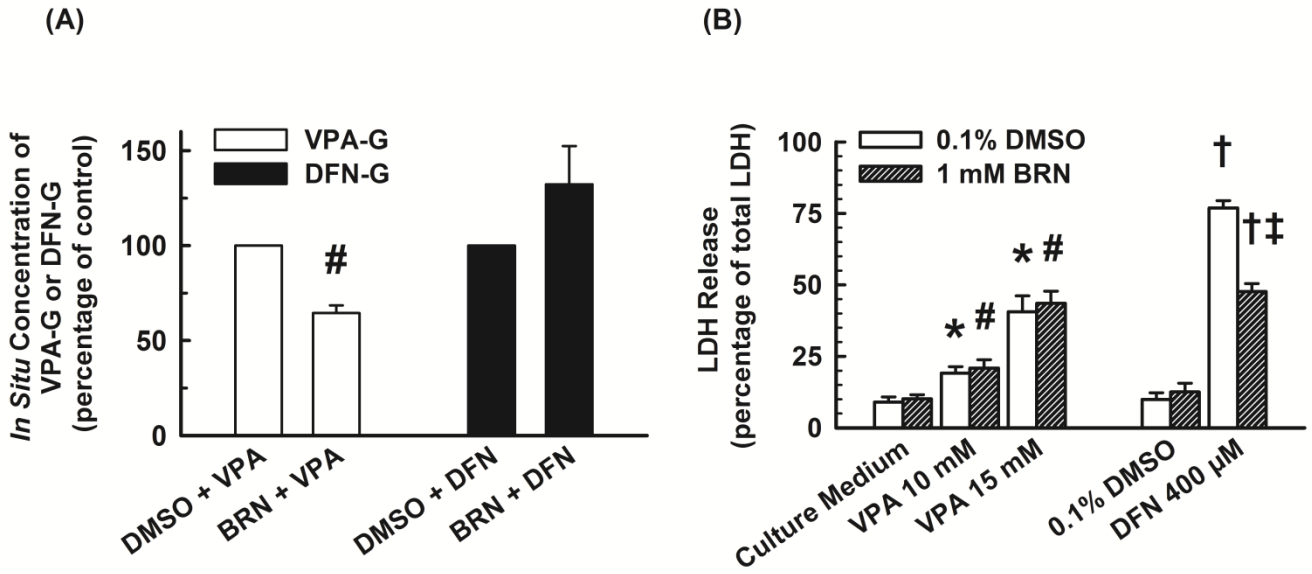

Figure 4.4 Effect of Borneol on *In Situ* Formation of VPA-G and LDH Release in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

(A) Hepatocytes were cultured for 120 h and then pretreated with borneol (BRN; 1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 h. 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 h in the presence of BRN (1 mM) or DMSO (0.1% v/v). Data are expressed as mean ± SEM (N = three rats). #Significantly different from the DMSO + VPA treatment group, $P < 0.05$. The *in situ* concentration (mean ± SEM) of VPA-G in the DMSO co-treatment group was 283 ± 41 µM per 0.7 million cells, whereas that of DFN-G was 20 ± 2 µM per 0.7 million cells. (B) Hepatocytes were cultured for 120 h and then pretreated with borneol (BRN; 1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 h. Subsequently, the hepatocytes were treated with VPA (10 or 15 mM), culture medium (vehicle for VPA), DFN (400 µM), or DMSO (0.1% v/v; vehicle for DFN) for the next 24 h in the presence of borneol (1 mM) or DMSO (0.1% v/v). Data are expressed as mean ± SEM (N = three rats). *Significantly different from the culture medium-treated control group that had 0.1% DMSO co-treatment, $P < 0.05$; #Significantly different from the culture medium-treated control group that had BRN 1 mM co-treatment, $P < 0.05$; †significantly different from the 0.1% DMSO-treated control group that had 0.1% DMSO co-treatment, $P < 0.05$; ‡significantly different from the DFN 400 µM-treated group that had 0.1% DMSO co-treatment, $P < 0.05$.

4.3.6 Effect of Borneol on Markers of Toxicity in Sandwich-Cultured Rat Hepatocytes Treated with VPA

Having shown that borneol is able to attenuate VPA-G formation, the next experiment was to investigate the effects of borneol on toxicity markers in sandwich-cultured rat hepatocytes treated with VPA. Hepatocytes were pretreated with borneol (1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 h, followed by a 24 h treatment with VPA (10 or 15 mM) or culture medium (vehicle) in the presence of borneol or DMSO. As shown in Fig. 4.4B, borneol alone did not affect LDH release in rat hepatocytes, whereas 10 and 15 mM VPA treatment increased LDH release by 2- and 4.5-fold, respectively. Co-treatment with borneol (1 mM) had no effect on VPA-associated LDH release in sandwich-cultured rat hepatocytes (Fig. 4.4B). In the same experiment, borneol (1 mM) was able to attenuate the *in situ* concentration of VPA-G in the culture supernatants, but not cell lysates, from hepatocytes treated with 10 or 15 mM VPA (Table 4.1). By comparison, borneol (1 mM) was capable of attenuating LDH release in hepatocytes treated with 400 μM diclofenac (Fig. 4.4B), although there was no effect on the *in situ* formation of DFN-G. In Fig 4.5A, treatment with 10 and 15 mM VPA increased BODIPY 558/568 C_{12} accumulation by 1.9- and 2.5-fold, respectively, in rat hepatocytes, whereas the presence of borneol had no effect on BODIPY 558/568 C_{12} accumulation caused by VPA. Treatment with 15 mM VPA, but not 10 mM VPA, reduced the total GSH content in sandwich-cultured rat hepatocytes by 35% (Fig. 4.5B). Whereas borneol did not affect the total GSH content in hepatocytes treated with 10 mM VPA, it further enhanced the GSH depletion caused by 15 mM VPA (Fig. 4.5B).

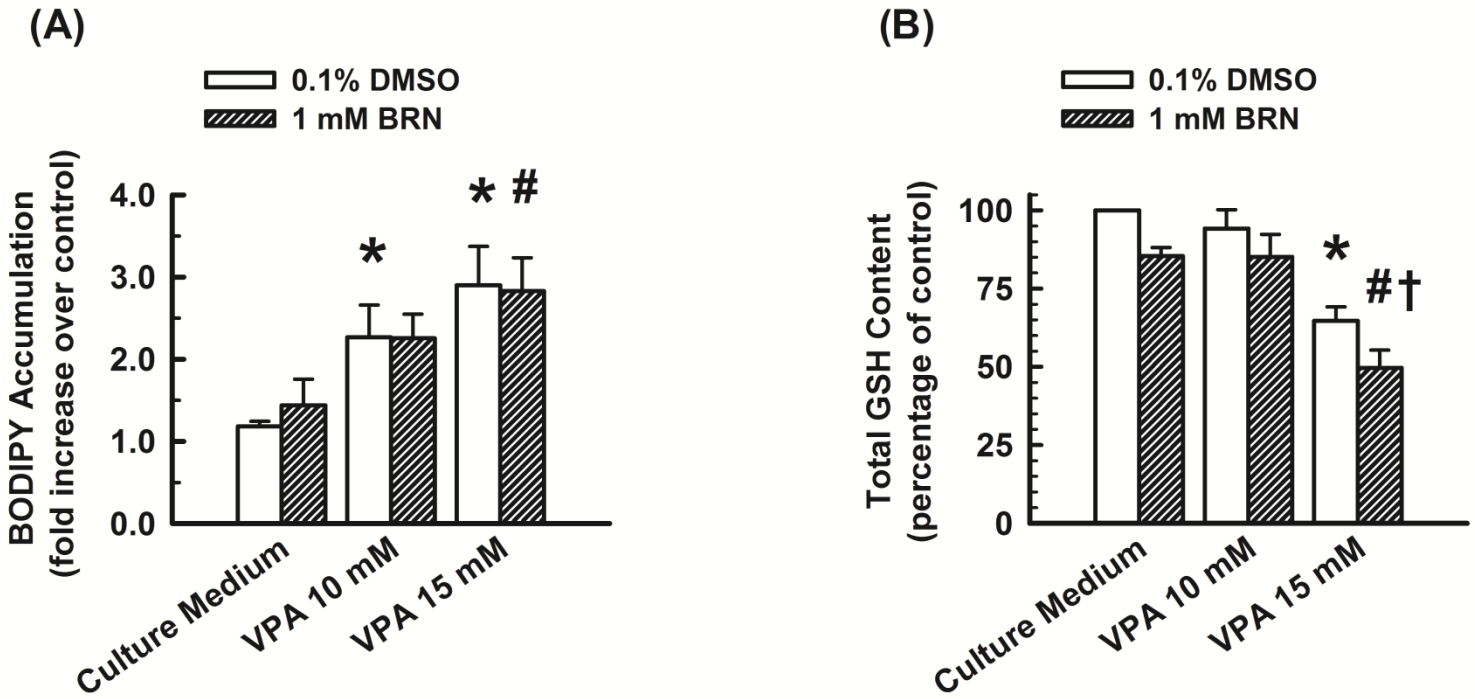

Figure 4.5 Effect of Borneol on (A) BODIPY 558/568 C_{12} Accumulation and (B) Total GSH Content in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

Hepatocytes were cultured for 120 h and then pretreated with borneol (BRN; 1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 h. Subsequently, the hepatocytes were treated with VPA (10 or 15 mM) or culture medium (vehicle) for 24 h in the presence of borneol (1 mM) or DMSO (0.1% v/v). Data are expressed as mean ± SEM (N = three to four rats). *Significantly different from the culture medium-treated control group that received 0.1% DMSO co-treatment, $P < 0.05$; #Significantly different from the culture medium-treated control group that received BRN 1 mM co-treatment, $P < 0.05$; †significantly different from the VPA 15 mM-treated group that had 0.1% DMSO co-treatment. The total GSH content (mean ± SEM) was 13.1 ± 1.1 µM per 0.7 million cells in the group co-treated with 0.1% DMSO and culture medium.

4.3.7 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. Whereas VPA treatment increased VPA-G concentrations in the culture supernatants over a 2 to 24 h period (Fig. 4.6A), an increase in LDH release was seen only at 24 h (Fig. 4.6B). Borneol co-treatment attenuated *in situ* formation of VPA-G by approximately 69, 66, 59, and 40% at 2, 4, 8, and 24 h, respectively, in cultured hepatocytes treated with VPA–(Fig. 4.6A). Yet again, an increase in VPA-associated LDH release was observed only at the 24 h time point (Fig. 4.6B). In a comparative experiment, diclofenac treatment resulted in an increase in extracellular DFN-G concentrations over the 2 to 24 h time period (Fig. 4.6C), with an increase in LDH release occurring after 8 h of treatment (Fig. 4.6D). Borneol decreased the *in situ* formation of DFN-G by 65, 49, and 29% at 2, 4, and 8 h, respectively, but no difference was observed at 24 h (Fig. 4.6C). The effect of borneol on LDH release in rat hepatocytes treated with diclofenac was not seen until 8 h, and the LDH release at 8 and 24 h was significantly less than the group treated with diclofenac alone (Fig. 4.6D).

4.3.8 Effect of β-Naphthoflavone and Borneol on *In Situ* Generated Oxidative Metabolites of VPA in Sandwich-Cultured Rat Hepatocytes Treated with VPA

To ascertain whether the effects of β-naphthoflavone and borneol on VPA metabolic pathways other than glucuronidation could have influenced the effects on toxicity, a GC-MS assay was performed to quantify the concentrations of oxidative metabolites of VPA in culture supernatants obtained from the experiments concerning β-naphthoflavone and borneol. As

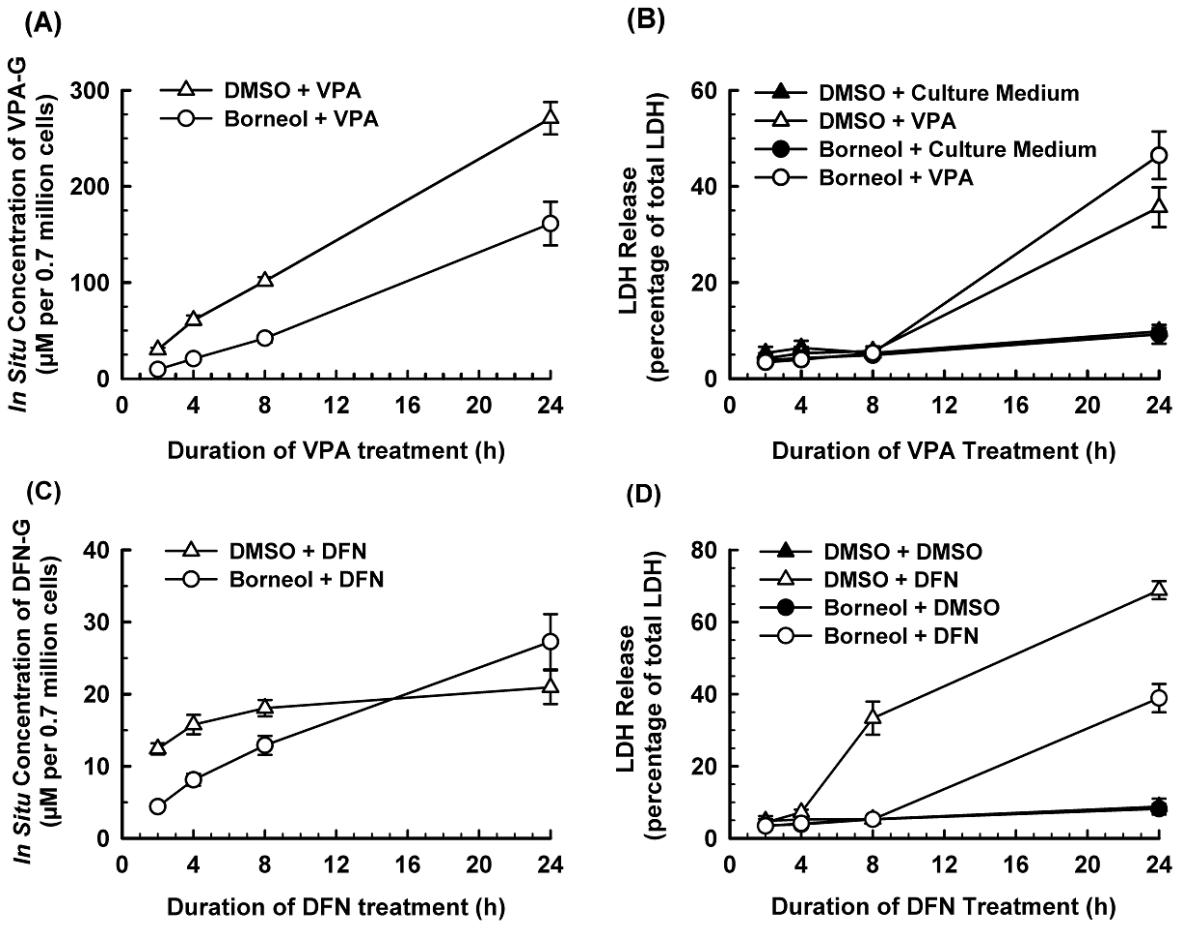

Figure 4.6 Time Course on the Effect of Borneol on (A) VPA-G Concentration, (B) LDH Release After VPA Treatment, (C) DFN-G Concentration, and (D) LDH Release Following Diclofenac Treatment in Sandwich-Cultured Rat Hepatocytes.
Hepatocytes were cultured for 120 h and then pretreated with borneol (BRN; 1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 h. Subsequently, the hepatocytes were treated with diclofenac (DFN; 400 μM), DMSO (0.1% v/v; vehicle for DFN), VPA (15 mM), or culture medium (vehicle for VPA) for the next 2, 4, 8, and 24 h in the presence of BRN (1 mM) or DMSO (0.1% v/v). Data are expressed as mean ± SEM (N = four rats).

shown in Table 4.2, β-naphthoflavone increased the concentrations of the metabolites 4-keto-VPA, 4-OH-VPA, and 3-OH-VPA by 2- to 3-fold, whereas it did not affect the concentrations of other oxidative metabolites of VPA in VPA-treated hepatocytes. In the case of borneol, there was a decrease in the concentrations of the metabolites 4-OH-VPA and 5-OH-VPA by 60- and 40%, respectively (Table 4.3), in hepatocytes treated with 10 mM VPA. However, borneol did not affect the concentrations of other oxidative metabolites of VPA in sandwich-cultured rat hepatocytes (Table 4.3).

4.3.9 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.

UHPLC-MS/MS assays were done to quantify the concentrations of 4'-OH-DFN and 5-OH-DFN in culture supernatants obtained from the experiments concerning β-naphthoflavone and borneol in order to determine the effects of these modulators on the oxidative metabolism of diclofenac. Interestingly, β-naphthoflavone pretreatment was found to have a differential effect on the two hydroxy metabolites of diclofenac, where it increased the formation of 4'-OH-DFN by over 2-fold but attenuated the concentration of 5-OH-DFN by 30% (Fig. A.5). Borneol, on the other hand, did not affect the concentration of 4'-OH-DFN, but attenuated the formation of 5-OH-DFN by 5-fold (Fig. A.5).

4.4 Discussion

To my knowledge, the present study is the first attempt to investigate the toxicity of *in situ* generated VPA-G in an experimental model. Toxicity was assessed using markers of necrosis

Table 4.2 Effect of β-Naphthoflavone on *In Situ* Formation of Oxidative Metabolites of VPA in the Culture Supernatants of Sandwich-Cultured Rat Hepatocytes Treated with VPA

At 48 h after plating, hepatocytes were pretreated with 20 μM BNF or 0.1% DMSO (vehicle) once every 24 h for 72 h and then treated with VPA (10 or 15 mM) or culture medium (vehicle) for the next 24 h. Culture supernatants were collected at the end of the 24 h treatment period and subjected to GC-MS assay for the quantification of the concentrations of oxidative metabolites of VPA. Data are expressed as mean ± SEM (N = three rats).

Metabolite	*In situ* Concentration of VPA Metabolites (μM / 0.7 × 10^6 cells)			
	10 mM VPA		15 mM VPA	
	0.1% DMSO	20 μM BNF	0.1% DMSO	20 μM BNF
(*E*)-2,4-diene-VPA	None detected	None detected	None detected	None detected
(*E,Z*)-2,3′-diene-VPA	0.16 ± 0.01	0.19 ± 0.01	0.15 ± 0.01	0.19 ± 0.02
(*E,E*)-2,3′-diene-VPA	1.7 ± 0.27	1.7 ± 0.21	1.7 ± 0.34	1.8 ± 0.34
4-ene-VPA	0.06 ± 0.01	0.09 ± 0.01	0.05 ± 0.01	0.08 ± 0.01
3-ene-VPA	1.2 ± 0.20	1.3 ± 0.14	1.0 ± 0.15	1.1 ± 0.13
(*E*)-2-ene-VPA	1.7 ± 0.13	1.5 ± 0.06	1.8 ± 0.21	1.8 ± 0.20
4-keto-VPA	0.15 ± 0.01	0.50 ± 0.06*	0.22 ± 0.02	0.58 ± 0.08*
3-keto-VPA	2.0 ± 0.63	1.7 ± 0.32	2.1 ± 0.99	2.2 ± 0.79
4-OH-VPA	2.5 ± 0.30	4.9 ± 0.54*	2.1 ± 0.25	5.0 ± 0. 46*
3-OH-VPA	5.6 ± 1.2	11 ± 4.19	5.5 ± 1.5	18.2 ± 5.0*
5-OH-VPA	1.4 ± 0.27	1.2 ± 0.17	1.3 ± 0.25	1.2 ± 0.24

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

Table 4.3 Effect of Borneol on *In Situ* Formation of Oxidative Metabolites of VPA in the Culture Supernatants of Sandwich-Cultured Rat Hepatocytes Treated with VPA

At 120 h after plating, hepatocytes were pretreated with 1 mM borneol (BRN) or 0.1% DMSO (vehicle) for 0.5 h. Subsequently, hepatocytes were treated with VPA (10 or 15 mM) or culture medium (vehicle) in the presence of 1 mM BRN or 0.1% DMSO for 24 h. . Culture supernatants were collected at the end of the 24 h treatment period and subjected to GC-MS assay for the quantification of the concentrations of oxidative metabolites of VPA. Data are expressed as mean ± SEM (N = four rats).

Metabolite	*In situ* Concentration of VPA Metabolites (μM / 0.7 × 10^6 cells)			
	10 mM VPA		15 mM VPA	
	0.1% DMSO	1 mM BRN	0.1% DMSO	1 mM BRN
(*E*)-2,4-diene-VPA	None detected	None detected	None detected	None detected
(*E,Z*)-2,3′-diene-VPA	0.19 ± 0.02	0.19 ± 0.02	0.18 ± 0.01	0.20 ± 0.01
(*E,E*)-2,3′-diene-VPA	1.5 ± 0.09	1.3 ± 0.20	1.6 ± 0.22	1.5 ± 0.14
4-ene-VPA	0.08 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
3-ene-VPA	1. 3 ± 0.13	1.2 ± 0.16	1.1 ± 0.13	1.0 ± 0.10
(*E*)-2-ene-VPA	1.8 ± 0.16	1.9 ± 0.20	2.2 ± 0.29	2.2 ± 0.23
4-keto-VPA	0.09 ± 0.01	0.09 ± 0.02	0.13 ± 0.01	0.16 ± 0.02
3-keto-VPA	1.4 ± 0.33	1.1 ± 0.35	2.8 ± 0.75	2.1 ± 0.97
4-OH-VPA	3.4 ± 0.56	1.4 ± 0.45*	2.2 ± 0.22	1.9 ± 0. 21
3-OH-VPA	7.1 ± 0.36	6.2 ± 1.5	11 ± 3.1	7.2 ± 1.10
5-OH-VPA	1.6 ± 0.10	0.94 ± 0.05*	1.7 ± 0.23	1.3 ± 0.19

*Significantly different from the DMSO-co-treated vehicle control group, $P < 0.05$.

(LDH release) and steatosis (BODIPY 558/568 C_{12} accumulation), and cellular total glutathione content, all of which are relevant to VPA hepatotoxicity (Kiang et al., 2011; Silva et al., 2008). A major finding of this study 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 evidences: 1) an over two-fold increase in the *in situ* formation of VPA-G by β-naphthoflavone was not accompanied by an increase in toxicity in VPA-treated hepatocytes, 2) the borneol-mediated decrease in the *in situ* concentration of VPA-G did not result in an attenuation of VPA toxicity as determined by LDH, BODIPY, and GSH markers. Previous studies have identified VPA-G as one of the least reactive and the most stable of acyl glucuronides, based on its long degradation half-life (in the range of 60 – 79 h) in pH 7.4 buffer at 37^{o}C (Bailey and Dickinson, 1996; Stachulski et al., 2006). Even though VPA-G has been reported to undergo pH-dependent intra-molecular acyl migration to form β-glucuronidase-resistant positional isomers of VPA-G (Dickinson et al., 1984), acyl migration of VPA-G was not significant under the cell culture conditions employed in this study (Surendradoss et al., 2013). Although the *in situ* concentrations of some of the cytochrome P450-mediated oxidative metabolites were affected by β-naphthoflavone and borneol in this study, these metabolites have previously been shown to have no influence on the toxicity of VPA in sandwich-cultured rat hepatocytes (Kiang et al., 2010; Surendradoss et al., 2012). Furthermore, in the present study, a β-naphthoflavone-mediated increase in VPA-G formation was accompanied by an attenuation of BODIPY 558/568 C_{12} accumulation, which is a marker of steatosis, in VPA-treated hepatocytes. Overall, based on the results of the current study, VPA glucuronidation appears to be a typical detoxification pathway, and not that of bioactivation, in sandwich-cultured rat hepatocytes.

The aforementioned finding is in contrast to a previous study (Tong et al., 2005c), which showed that borneol was able to effectively attenuate VPA-associated elevation of plasma and hepatic levels of 15-F_{2t}-isoprostane (marker of oxidative stress) in rats and thereby, implicated VPA-G in the oxidative stress caused by VPA. Whereas borneol was able to attenuate metabolic formation of VPA-G by 95% in that study (Tong et al., 2005c), the *in situ* concentration of VPA-G was decreased only modestly (25–40%) by borneol in the present study. The reasons for the observed differences in the effectiveness of borneol and the divergent conclusions of the two studies are not known, but could possibly be attributed to enterohepatic recirculation and thereby, a higher hepatic exposure to VPA-G in the *in vivo* study (Tong et al., 2005c). Furthermore, the attenuation of *in situ* formation of VPA-G by borneol appeared to be time-dependent, with the effect decreasing over time as indicated by the percentage decrease of *in situ* VPA-G concentration such as, 69, 66, 59, and 40% at 2, 4, 8, and 24 h of VPA treatment, respectively (Fig. 4.6A). The reasons for the observed time-dependence in the effects of borneol are not known, but could possibly be due to the ability of sandwich-cultured rat hepatocytes to overcome the effects of borneol by an increased synthesis of the cofactor uridine 5'-diphospho-glucuronic acid (UDP-GA). Although this proposition needs to be verified experimentally, other studies that have used borneol to inhibit *in vitro* glucuronidation of drugs other than VPA in cultured rat, mouse, or human hepatocytes have assessed the effects only after shorter durations of treatment, i.e., 2, 6, or 18 h (Koga et al., 2011; Kretz-Rommel and Boelsterli, 1993; Ghaoui et al., 2003). However, in the present study, toxicity was not observed at the 2, 4, or 8 h time points. Therefore, it was not possible to assess the toxicological consequence of the observed attenuation of *in situ* VPA-G formation by borneol (Fig. 4.6B). A relevant observation from the current study is that VPA-G formed *in situ* within hepatocytes is effectively eliminated into

culture medium, with less than 3% of the total VPA-G remaining inside the hepatocytes. Multidrug-resistance-associated protein 2 (Mrp2), which is one of the hepatic efflux transporters (Kock and Brouwer, 2012), has been shown to mediate the canalicular efflux of VPA-G in rats (Wright and Dickinson, 2004). It would be worthwhile for future studies to evaluate the toxicity of VPA-G in experimental models with compromised hepatobiliary transport, such as Mrp2-knockout rats (Zamek-Gliszczynski et al., 2013).

Another novel finding of the present study came from evaluating the effects of various known inducers of rat and human UGTs on increasing *in situ* formation of VPA-G in sandwich-cultured rat hepatocytes treated with VPA. The compounds screened in this study were comprised of agonists / activators of the aryl hydrocarbon receptor, the nuclear factor erythroid 2-related factor 2 (Nrf2), the constitutive androstane receptor, the pregnane X receptor, and the peroxisome proliferator-activated receptor α, all transcriptional pathways shown to be involved in the induction of UGTs in rats (Shelby and Klaassen, 2006). Among the various inducers screened in this study, only β-naphthoflavone, L-sulforaphane, and phenobarbital were effective in increasing the *in situ* formation of VPA-G in sandwich-cultured rat hepatocytes. Whereas phenobarbital and clofibrate increased the urinary levels of VPA-G by 4.3- and 2.2-fold, respectively, in one study (Heinemeyer et al., 1985), another study showed that phenobarbital increased the hepatic concentration of VPA-G by only 1.4-fold in VPA-treated rats (Tong et al., 2005c). In the present study, β-naphthoflavone produced the maximal increase of *in situ* VPA-G formation, but the magnitude of the effect was relatively modest (just over 2-fold). This finding is consistent with the general understanding that the UGTs are less responsive to induction than cytochrome P450 enzymes (Soars et al., 2004) and the increases in glucuronidation in response

to prototypical UGT inducers are usually less than 2-fold (Lin and Wong, 2002; Soars et al., 2004).

As a means to corroborate whether the sandwich-cultured rat hepatocyte model was responding appropriately, this study investigated the effects of various modulators on *in situ* formation of DFN-G and toxicity in diclofenac-treated hepatocytes. Diclofenac toxicity in rat hepatocytes *in vitro* has been attributed to cytochrome P450 2C11-mediated formation of oxidative metabolites of diclofenac, and not the glucuronide, DFN-G (Kretz-Rommel and Boelsterli, 1993). Consistent with that hypothesis, the observed β-naphthoflavone-mediated increase in the *in situ* formation of DFN-G was not accompanied by an increase in toxicity, but rather, an attenuation of diclofenac toxicity. Although β-naphthoflavone attenuated the formation of 5-OH-DFN, it also increased the concentration of 4'-OH-DFN by 2.3-fold (Fig. A.5). Rat CYP2B, CYP2C and CYP3A enzymes have been shown to mediate the bioactivation of diclofenac *via* hydroxylation and subsequent oxidation resulting in the formation of highly electrophilic benzoquinone imine intermediates (Tang et al., 1999). Previous studies have shown that these reactive metabolites are detoxified by conjugation with GSH (Tang et al., 1999) and subsequently, eliminated as mercapturic acid derivatives in rats and humans (Poon et al., 2001). Interestingly, β-naphthoflavone pretreatment *per se* was found to increase the cellular content of GSH by almost 2-fold in the present study (Fig. 4.3B). It is therefore possible that the β-naphthoflavone-mediated increase in GSH concentration could have contributed to the observed attenuation of diclofenac toxicity. Borneol had no significant effect on the *in situ* concentration of DFN-G in the present study, but was shown previously to attenuate DFN-G formation by >70% in diclofenac-treated rat hepatocytes (Kretz-Rommel and Boelsterli, 1993). The lack of effect of borneol in inhibiting DFN-G formation observed here appears to relate to the

differences in the duration of treatment i.e., 2 h in the previous study (Kretz-Rommel and Boelsterli, 1993) vs. 24 h in the present study. Accordingly, in the time course experiments shown in Fig. 4.6C, borneol was able to decrease DFN-G formation by 65% after 2 h of diclofenac treatment. However, after 24 h of diclofenac treatment, the *in situ* DFN-G concentration in borneol-treated group was in fact slightly greater than the DMSO-treated group (Fig. 4.6C). The reason for this observation is not known, but this appears to suggest a possible rebound increase in UDP-GA concentration in borneol-treated hepatocytes in the 8 to 24 h treatment period. Similarly, the observed attenuation of diclofenac toxicity by borneol is in disagreement with the previous report (Kretz-Rommel and Boelsterli, 1993), whereby borneol was found to enhance diclofenac toxicity. Again, the reasons for this difference are not known, but it is possible that the observed decrease in toxicity could be due to inhibition by borneol of the formation of cytochrome P450-mediated oxidative metabolites of diclofenac. Consistent with this hypothesis, borneol attenuated the formation of 5-OH-DFN by more than 80% in the present study (Fig. A.5). Borneol has previously been shown to inhibit human cytochrome P450 2B6 (Kim et al., 2008), and also was found to attenuate the formation of the cytochrome P450-mediated metabolites 4'-OH-VPA and 5-OH-VPA in the present study (Table 4.3).

In conclusion, the present study investigated the toxicity of *in situ* generated VPA-G in sandwich-cultured rat hepatocytes treated with VPA. As evident from the experiments with β-naphthoflavone and borneol, *in situ* generated VPA-G did not appear to be toxic to sandwich-cultured rat hepatocytes. The findings of this study, especially when considered in the light of the available information regarding the chemical nature of VPA-G, suggest that *in situ* generated VPA-G is less likely to contribute to the hepatocyte toxicity of VPA. Recent studies have indicated that the potentially reactive phase I metabolites 4-ene-VPA and (*E*)-2,4-diene-VPA, are

also unlikely to play a role in the hepatocyte toxicity of VPA (Kiang et al., 2010; Surendradoss et al., 2012). Future studies should investigate the role of VPA-CoA and other downstream β-oxidation metabolites of VPA in VPA hepatotoxicity.

Chapter 5: Concluding Remarks

5.1 Summary of the Major Findings

1. According to the results from the experiments involving PB and/or 1-ABT (Chapter 2), *in situ* generated (*E*)-2,4-diene-VPA did not appear to contribute to VPA toxicity in sandwich-cultured rat hepatocytes. The use of (*E*)-2-ene-VPA as a substrate enabled this study to demonstrate, for the first time, the toxic potential of *in situ* generated (*E*)-2,4-diene-VPA, when produced at high concentrations such as those obtained in PB-pretreated hepatocytes. Based on the findings of this study, the chances of (*E*)-2,4-diene-VPA playing a role in VPA hepatotoxicity in patients appear remote. However, further studies are required to see whether *in situ* formed (*E*)-2,4-diene-VPA would become a contributing factor to VPA hepatotoxicity in patients with metabolic idiosyncrasies.
2. A sensitive UHPLC-MS/MS method was developed and validated for the quantification of VPA-G in hepatocyte culture medium (Chapter 3). In comparison to the existing methods for the quantification of VPA-G (Tong et al., 2005c; Wong et al., 2007), the advantages of this method include a shorter run time, a 250- to 1000-fold improvement in sensitivity and thereby, requiring only a smaller volume of the sample for analysis. This method was applied successfully to quantify the concentrations of *in situ* generated VPA-G in a study that investigated the effect of age of hepatocyte culture on the extent of glucuronidation of VPA in sandwich-cultured rat hepatocytes. Another significant finding was the lack of formation of VPA-G *iso*-glucuronides from both preformed and *in situ* generated VPA-G under the cell culture conditions employed in the study.

3. Lastly, the toxicity of *in situ* generated VPA-G in sandwich-cultured rat hepatocytes was investigated (Chapter 4). A significant outcome of this study is the comprehensive screening of various known inducers of UGT enzymes, including activators of nuclear receptors / transcription factors such as, aryl hydrocarbon receptor, nuclear factor erythroid 2-related factor 2, peroxisome proliferator-activated receptor α, constitutive androstane receptor, pregnane X receptor, and glucocorticoid receptor for their effects in increasing *in situ* formation of VPA-G. Among the modulators evaluated in this study, β-naphthoflavone, L-sulforaphane, and phenobarbital were alone effective, although modestly, in increasing the *in situ* concentrations of VPA-G. Borneol was used to attenuate the *in situ* formation of VPA-G from VPA. The findings of the experiments with β-naphthoflavone and borneol appear to suggest that the *in situ* generated VPA-G was not toxic to sandwich-cultured rat hepatocytes, and hence, was unlikely to contribute to the hepatocyte toxicity of VPA. Given the available knowledge on VPA-G, which is one of the most stable and least reactive acyl glucuronide known so far (Stachulski et al., 2006), glucuronidation of VPA appears to be a typical detoxification pathway and not bioactivation.

5.2 Strengths and Limitations of the Present Work

5.2.1 Strengths

1. Using sandwich-cultured rat hepatocyte model, the present study investigated the toxicity of *in situ* generated (*E*)-2,4-diene-VPA and VPA-G, instead of using the preformed (synthetic) metabolites. It is now well recognized that the preformed and

metabolically-generated metabolites could differ in their kinetic and toxicological behavior (Prueksaritanont et al., 2006). To the best of my knowledge, this study is the first to investigate the overall toxicity of *in situ* generated (*E*)-2,4-diene-VPA and VPA-G in any experimental model.

2. This study employed a diverse set of toxicity markers, such as DCF formation, BODIPY 558/568 C_{12} accumulation, LDH release, and cellular content of total GSH, as markers of oxidative stress (Bartosz, 2006), steatosis (Fujimura et al., 2009), cellular necrosis (Jauregui et al., 1981), and cellular antioxidant status (Han et al., 2006), respectively. VPA hepatotoxicity in humans is reported to be accompanied by hepatic microvesicular steatosis, hepatocellular necrosis, and oxidative stress (Dreifuss et al., 1987; Michoulas et al., 2006; Pippenger et al., 1991; Zimmerman and Ishak, 1982). With the choice of the aforementioned markers, all major manifestations of VPA hepatotoxicity in humans have been included in the assessment of hepatocyte toxicity of VPA in the present study.
3. In addition, the evaluation of the toxicological consequences of both the increased and decreased formation of the *in situ* generated metabolites enabled a better understanding of the toxicological potential of those metabolites e.g., the importance of the results from the study involving PB / 1-ABT and (*E*)-2-ene-VPA on the toxicological significance of (*E*)-2,4-diene-VPA as a reactive metabolite stemming from VPA.
4. The possible roles of two pathways of VPA biotransformation (i.e., cytochrome P450-mediated oxidation and glucuronidation) in the metabolic bioactivation and hepatocyte toxicity of VPA were evaluated in the same experimental system, i.e., sandwich-cultured rat hepatocytes (Chapter 2 and Chapter 4). Besides understanding the relative

contribution of these two metabolic pathways, the use of the same experimental model was helpful in the interpretation of the toxicological significance of the effects of chemical modulators on multiple biotransformation pathways of VPA, e.g., β-naphthoflavone-mediated increase in cytochrome P450-mediated oxidative metabolites of VPA in Chapter 4 (Table 4.2) is not likely to be toxicologically significant given the previous findings with PB in Chapter 2 (Table 2.1 and Figure 2.3).

5.2.2 Limitations

1. Although VPA is known to cause dose-dependent hepatotoxicity in patients, VPA-associated idiosyncratic hepatotoxicity, which occurs rarely only in very few patients and is not dose-dependent, is a more severe and clinically important adverse effect of VPA. Various studies have suggested that (*E*)-2,4-diene-VPA and VPA-G could play a role in VPA-associated idiosyncratic hepatotoxicity (Baillie, 1988; Gopaul et al., 2003; Kassahun et al., 1991; Tang et al., 1995; Tang and Abbott, 1996; Tang et al., 1996; Tong et al., 2003) However, there is no experimental model available to investigate VPA-associated idiosyncratic hepatotoxicity. The objective of this study was to investigate the overall toxicity of *in situ* generated (*E*)-2,4-diene-VPA and VPA-G in sandwich-cultured rat hepatocytes and to evaluate if these metabolites could be responsible for the concentration-dependent toxicity of VPA observed in this experimental model. The findings are not directly pertaining to idiosyncratic toxicity; however, they could possibly become relevant in idiosyncratic hepatotoxicity in the presence of a predisposing factor; for example, *in situ* generated (*E*)-2,4-diene-VPA did not appear to be responsible for the hepatocyte toxicity of VPA (Chapter 2); however,

the experiments with PB and (*E*)-2-ene-VPA indicate the toxic potential of this metabolite, when generated *in situ* at very high concentrations. There is a possibility that (*E*)-2,4-diene-VPA could attain levels high enough to be a factor in the idiosyncratic hepatotoxicity of VPA in the event of a metabolic idiosyncrasy resulting in either overproduction of this metabolite or poor antioxidant defense.

2. The similarities / differences in the biotransformation and/or toxicity of VPA in rat and human hepatocytes, and their abilities to respond to various modulators employed in the present work have not yet been investigated. Available evidence suggests several key differences in the metabolite profile of VPA in sandwich-cultured rat hepatocytes from the metabolite profile reported in patients undergoing VPA therapy. For example, whereas the reactive metabolite (*E*)-2,4-diene-VPA was quantifiable in the plasma (Levy et al., 1990) and urine samples (Anderson et al., 1992; Levy et al., 1990) of patients undergoing VPA monotherapy, it was detected only in trace levels in sandwich-cultured rat hepatocytes even after phenobarbital pretreatment (Chapter 2). Therefore, the findings of the present study need to be verified in human hepatocytes, in order to determine the influence of species-specific factors in the observed results and the relevance of using rat hepatocytes as a surrogate for human hepatocytes in future studies. A subsequent step would be to verify the *in vivo* relevance of the findings obtained in sandwich-cultured human hepatocytes.

3. In the experiments that investigated the toxicity of *in situ* generated VPA-G (Chapter 4), the chemical modulators employed, such as β-naphthoflavone and borneol, had only a modest effect on *in situ* VPA-G concentrations (i.e., an increase by over two-fold and a decrease by 25 to 35%, respectively). It is well recognized that the induction of UGT

enzymes, unlike cytochrome P450 enzymes, is quite modest with the usual range being only ~ 2-fold (Lin and Wong, 2002; Soars et al., 2004). Whether the conclusions would have been different if there had been a greater modulation of the *in situ* concentrations of VPA-G is not known.

4. The concentrations of VPA that were used in the key experiments (i.e., 10 to 20 mM VPA) are much higher than the plasma concentrations of VPA observed in humans, which range from 0.28 mM to 0.7 or 1.0 mM (Silva et al., 2008; Sztajnkrycer, 2002). However, concentrations up to 100-fold of the therapeutic C_{max} values are justified while investigating *in vitro* the idiosyncratic hepatotoxicity of orally-administered drugs (Xu et al., 2008; Dykens et al., 2008), and the concentrations of VPA employed in this study were well within the 100-fold limit.
5. Considering the reactive nature of (*E*)-2,4-diene-VPA and that it has been shown to form GSH conjugates in rats (Kassahun et al., 1991) and humans (Gopaul et al., 2000; Kassahun et al., 1991), it would have been more informative to determine the effects of GSH depletion on the toxicity of *in situ* generated (*E*)-2,4-diene-VPA in hepatocytes treated with VPA or (*E*)-2-ene-VPA. However, due to the toxicity of chemical modulators used to deplete GSH, such as BSO, it was not feasible to achieve GSH depletion in sandwich-cultured rat hepatocytes, without an accompanying increase in toxicity, as shown in a preliminary experiment.
6. The chemical modulators used in the experiments described in Chapter 2 and Chapter 4 are not specific to the metabolic pathway that they were intended to modulate. While an effort was made to characterize the effects of selected chemical modulators on other biotransformation pathways of VPA and to account for their influence in the observed

results (Tables 2.1, 2.2, 2.3, 4.2, and 4.3), it is difficult to rule out the role of any non-specific effects of these chemical modulators on cellular functions and/or toxicity markers used in the present study. For example, the effects of β-naphthoflavone *per se* on cellular content of total GSH (Fig. 4.3B). Enzyme-specific chemical modulators could not be used due to the lack of availability of rat cytochrome P450 / UGT isoform-specific chemical modulators and the lack of information on the specific rat cytochrome P450 and UGT enzymes that mediate the formation of (*E*)-2,4-diene-VPA and VPA-G, respectively.

7. Despite the advantages associated with the sandwich-cultured hepatocyte model for screening and mechanistic studies, an *in vitro* model such as this lacks the complex and dynamic interplay of various factors that determine the disposition and toxicity of a drug or metabolite *in vivo* e.g., the effect of enterohepatic recirculation on the hepatic exposure to a drug and/or its metabolites.

5.3 Future Studies

1. Using cryopreserved human hepatocytes, future studies could compare the biotransformation and toxicity of VPA in sandwich-cultured rat and human hepatocytes. Such studies could help verify the usefulness of sandwich-cultured rat hepatocytes as a surrogate experimental model to investigate the biotransformation and hepatotoxicity of VPA in human hepatocytes. The results from human hepatocyte studies will still need to be verified for *in vivo* relevance in humans by analyzing blood and tissue samples obtained from future cases of idiosyncratic hepatotoxicity in patients undergoing VPA therapy.

2. Considering the efficient efflux of VPA-G (see Chapter 4) and a previous report on the involvement of Mrp2 transporters in the biliary excretion of VPA-G (Wright and Dickinson, 2004), it would be worthwhile to investigate the toxicity of *in situ* generated VPA-G in hepatocytes obtained from Mrp2-knockout rats. In the present study an attempt was made to investigate the toxicological consequence of the inhibition of VPA-G efflux and the consequent increase in intracellular concentrations of VPA-G, by using the approach of chemical inhibition of ATP-binding cassette (ABC) efflux transporters. Benzbromarone, prazosin, MK571, and bromosulfalein, which are known chemical inhibitors of the ABC efflux transporters P-glycoprotein, MRP2, and/or breast cancer resistance protein (BCRP) (Matsson et al., 2009), were evaluated for their effects on the efflux of VPA-G in sandwich-cultured rat hepatocytes. Non-cytotoxic concentrations of benzbromarone (1, 5, and 10 μM), prazosin (5 and 10 μM), MK571 (25, 50, and 75 μM), or bromosulfalein (10 and 25 μM) (Fig. A.6) were evaluated for their effects on VPA-G efflux in sandwich-cultured rat hepatocytes treated with 10 mM VPA. As shown in Fig. A.7, the *in situ* concentration of VPA-G in culture supernatants (extracellular concentrations) was attenuated by these chemical inhibitors, with benzbromarone (10 μM) and MK571 (75 μM) producing near-complete attenuation of extracellular VPA-G concentrations. However, the decrease in the extracellular concentrations of VPA-G was not accompanied by an increase in the intracellular concentrations of VPA-G (Fig. A.7). The reasons for this lack of increase in intracellular VPA-G concentrations are not known, but could possibly be due to the inhibition of VPA (parent) uptake into hepatocytes and/or inhibition of VPA glucuronidation. Therefore, to avoid the promiscuous effects associated with the

currently available chemical inhibitors of ABC efflux transporters, it would be better to evaluate the toxicological consequence of high intracellular concentrations of VPA-G in sandwich-cultured rat hepatocytes obtained from Mrp2 knockout rats. As Mrp2 transporters have been reported to mediate the efflux of VPA-G in rats, high intracellular concentrations of VPA-G could be expected in hepatocytes isolated from Mrp2 knockout rats due to reduced efflux of VPA-G. In addition to investigating the toxicity associated with high intracellular concentrations of *in situ* generated VPA-G, this study would address the effect of at least one possible metabolic idiosyncrasy in VPA hepatotoxicity. Mrp2 knockout rats have been reported to have compensatory increase in the expression of MRP3 transporters (Zamek-Gliszczynski et al., 2013) and high intracellular concentrations of GSH (Silva et al., 2005). Whether these changes would influence the toxicological outcome of an attenuated efflux of VPA-G is not known.

3. When the identity of the rat UGT enzymes that mediate VPA glucuronidation becomes known, a future study could determine the effects of more selective and complete attenuation of the *in situ* formation of VPA-G on VPA toxicity in sandwich-cultured rat hepatocytes with the use of RNA interference technology.
4. The findings reported in Chapter 2 and Chapter 4 appear to suggest that *in situ* generated (*E*)-2,4-diene-VPA and VPA-G play little or no role in the toxicity of VPA in sandwich-cultured rat hepatocytes. Therefore, a logical next step would be to investigate the β-oxidative pathway of VPA metabolism and the role of *in situ* generated VPA-CoA thioester in the hepatocyte toxicity of VPA. Acyl-CoA thioesters are known to be highly electrophilic and even more reactive than acyl glucuronides (Skonberg et

al., 2008). Accordingly, formation of VPA-CoA thioester was implicated in the covalent binding of VPA observed *in vitro* in rat hepatocytes (Porubek et al., 1989). A potential study could determine the effect of modulators known to inhibit acyl-CoA formation such as α-lipoic acid (Phua et al., 2008) or lauric acid (Grillo et al., 2012) on VPA toxicity in sandwich-cultured rat hepatocytes.

5. Peroxisome proliferator-activated receptor α (PPARα) is known to be involved in the basal and inducible expression of several enzymes involved in mitochondrial β-oxidation of fatty acids (Aoyama et al., 1998). Thus, it would be interesting to use PPARα agonists such as, clofibrate, ciprofibrate, fenofibrate, or Wy-14643 and/or PPARα antagonist such as GW1069 to modulate the *in situ* formation of VPA-CoA thioester and determine the effects of increasing or decreasing the *in situ* concentrations of VPA-CoA thioester on the toxicity of VPA in sandwich-cultured rat hepatocytes. Furthermore, mitochondria-specific markers could be used in these experiments to understand the role of mitochondrial dysfunction in the observed toxicity of VPA in sandwich-cultured rat hepatocyte model.
6. Short-term primary cultures of hepatocytes are reported to derive most of their energy requirements from mitochondrial oxidative phosphorylation, whereas immortalized cell lines switch to glycolysis to meet their energy demand (Marroquin et al., 2007), which makes primary cultures of hepatocytes a better model to study mitochondrial toxicity. However, in the sandwich-cultured rat hepatocyte model used in this study, it is not known whether rat hepatocytes still retain their metabolic poise after six days of culture. A future experiment could compare the hepatocyte toxicity of VPA and other known mitochondrial-toxicant drugs in this sandwich-cultured rat hepatocyte model in the

presence of glucose- and galactose-containing medium. A lack of difference in toxicity between the two media would indicate that the metabolic poise is preserved until six days of culture.

7. Recently, mutations in mitochondrial DNA polymerase γ (*POLG*) have been reported to predispose individuals to VPA-associated hepatotoxicity and testing for *POLG* mutations has been proposed prior to the commencement of VPA therapy especially in pediatric patients (Saneto et al., 2010; Stewart et al., 2010). If possible, a future study could investigate the role of VPA biotransformation and the reactive metabolites of VPA in VPA toxicity using sandwich-cultures of human hepatocytes isolated from liver samples of individuals known to have *POLG* mutations and control liver samples. It will be very valuable to know if *POLG* mutations render hepatocytes more susceptible to the toxicity of reactive metabolites than the hepatocytes obtained from control liver samples.

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Appendix

Table A.1 Effect of PB on *In Situ* Formation of Putative 4-OH-(*E*)-2-ene-VPA in Sandwich-Cultured Rat Hepatocytes Treated with (*E*)-2-Ene-VPA

Metabolite	Pretreatment	
	Culture Medium	PB
	Peak Area Response Ratio	
4-OH-(*E*)-2-ene-VPA	3.2 ± 0.50	16 ± 2.7*

At the end of the 48 h culture period, hepatocytes were pretreated with 100 µM PB or culture medium (vehicle) every 24 h for 72 h and then treated with 30 mM (*E*)-2-ene-VPA, or culture medium (vehicle) for 24 h. Data are expressed as mean ± SEM (N = five rats). *Significantly different from the corresponding culture medium-pretreated group, $P < 0.05$.

$$\text{Peak area response ratio} = \frac{\text{Peak area of putative 4-OH-(}E\text{)-2-ene-VPA}}{\text{Peak area of [}^{2}H_{7}\text{]-5-OH-VPA (IS)}}$$

Table A.2 Effect of 1-ABT on *In Situ* Formation of Putative 4-OH-(*E*)-2-ene-VPA in Culture Medium- or PB-Pretreated Sandwich-Cultured Rat Hepatocytes Treated with (*E*)-2-Ene-VPA

Metabolite	Pretreatment			
	Culture Medium	Culture Medium + 1-ABT	PB	PB + 1-ABT
	Peak Area Response Ratio			
4-OH-(*E*)-2-ene-VPA	1.5 ± 0.31	0.02 ± 0.01*	16 ± 0.88	0.14 ± 0.01*

At the end of the 48 h culture period, hepatocytes were pretreated with 100 µM PB or culture medium (vehicle) once every 24 h for 72 h and then with 0.5 mM 1-ABT or culture medium for 0.5 h. Subsequently, the cells were treated with 30 mM (*E*)-2-ene-VPA, or culture medium (vehicle) in the presence of either 1-ABT or culture medium for the next 24 h. Data are expressed as mean ± SEM (N = three or four rats). *Significantly different from the corresponding treatment group without 1-ABT, $P < 0.05$.

$$\text{Peak area response ratio} = \frac{\text{Peak area of putative 4-OH-(}E\text{)-2-ene-VPA}}{\text{Peak area of [}^2H_7\text{]-5-OH-VPA (IS)}}$$

Table A.3 Comparison of *In Situ* Formation of 4-OH-VPA from VPA and Putative 4-OH-(*E*)-2-ene-VPA from (*E*)-2-Ene-VPA in Culture Medium- or PB-Pretreated Sandwich-Cultured Rat Hepatocytes

Pretreatment / Treatment	4-OH-VPA		4-OH-(*E*)-2-ene-VPA	
	Culture Medium + VPA	PB + VPA	Culture Medium + (*E*)-2-Ene-VPA	PB + (*E*)-2-Ene-VPA
	Peak Area Response Ratio			
VPA / (*E*)-2-ene-VPA	0.68 ± 0.19	5.4 ± 1.0	3.2 ± 0.50*	16 ± 2.7#

At the end of the 48 h culture period, hepatocytes were pretreated with 100 µM PB or culture medium (vehicle) once every 24 h for 72 h. Subsequently, the cells were treated with 20 mM VPA, 30 mM (*E*)-2-ene-VPA, or culture medium (vehicle) for the next 24 h. Data are expressed as mean ± SEM (N = five rats). *Significantly different from the Culture Medium + VPA treatment group, $P < 0.05$. #Significantly different from the PB + VPA treatment group, $P < 0.05$.

$$\text{Peak area response ratio of 4-OH-VPA} = \frac{\text{Peak area of 4-OH-VPA}}{\text{Peak area of } [^2H_7]\text{-5-OH-VPA}}$$

$$\text{Peak area response ratio of 4-OH-(}E\text{)-2-ene-VPA} = \frac{\text{Peak area of putative 4-OH-(}E\text{)-2-ene-VPA}}{\text{Peak area of } [^2H_7]\text{-5-OH-VPA}}$$

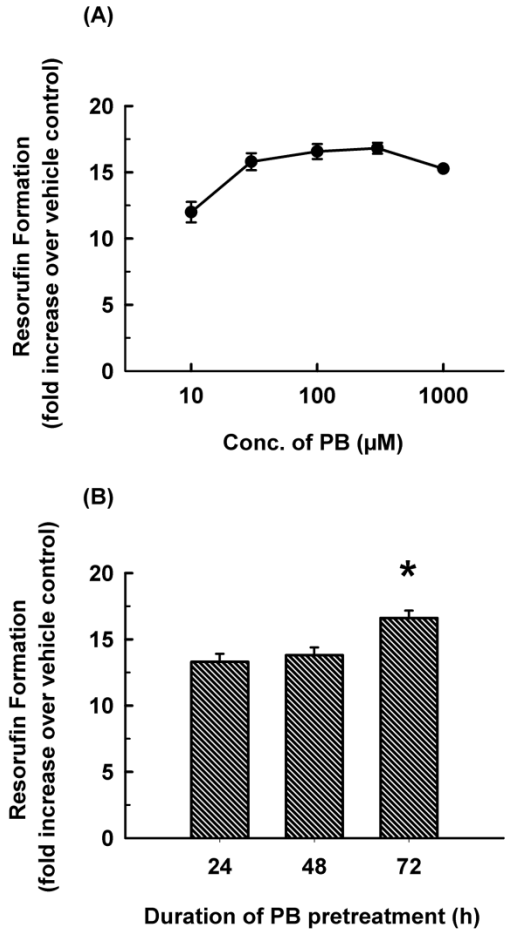

Figure A.1. Effect of (A) Concentration and (B) Duration of Phenobarbital Pretreatment on Resorufin Formation in Sandwich-Cultured Rat Hepatocytes.
(A) At 48 h after plating, hepatocytes were pretreated with PB (10, 30, 100, 300, or 1000 μM), or culture medium (vehicle) once every 24 h for up to 72 h. At the end of the 72 h pretreatment period, hepatocytes were subjected to BROD assay. (B) At 48 h after plating, hepatocytes were pretreated with 100 μM PB or culture medium (vehicle) for 24, 48, or 72 h. At the end of the pretreatment period, hepatocytes were subjected to BROD assay. Data are expressed as mean ± SEM (N = three rats). *Significantly different from the groups pretreated with PB for 24 and 48 h, $P < 0.05$.
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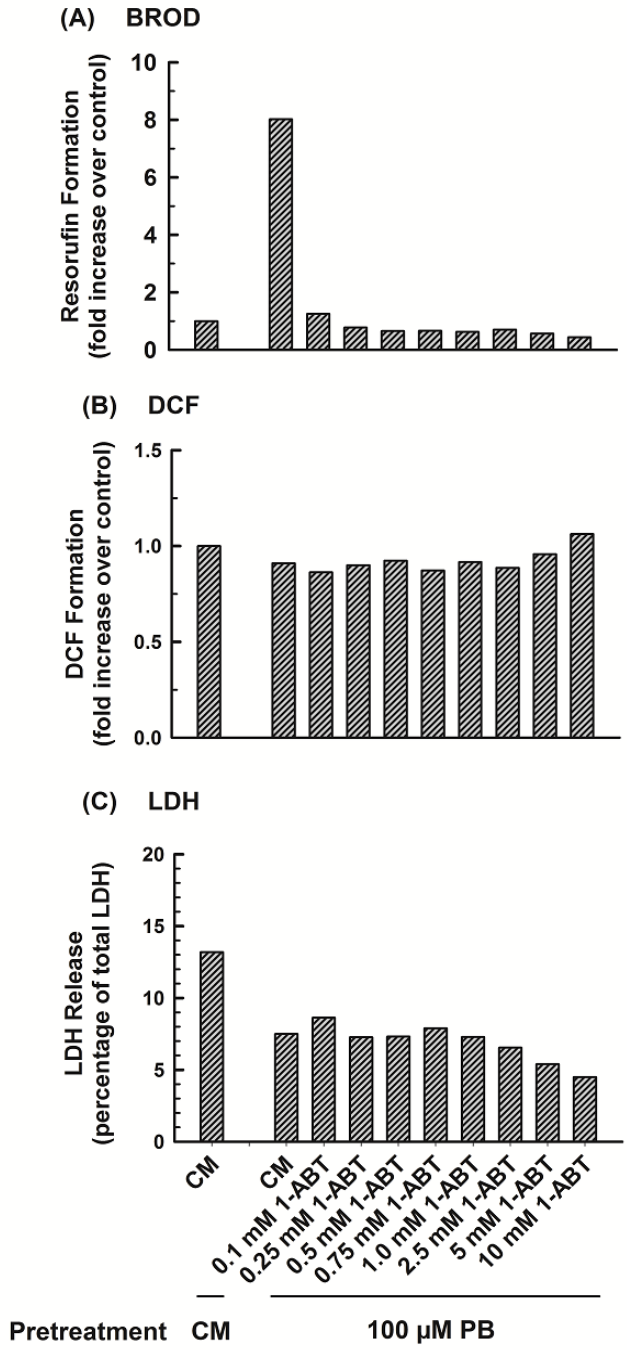

Figure A.2. Concentration-Response Effect of 1-ABT on (A) BROD Activity, (B) DCF Formation, and (C) LDH Release in Sandwich-Cultured Rat Hepatocytes.
At 48 h after plating, hepatocytes were pretreated with 100 µM PB or culture medium (CM; vehicle) once every 24 h for 72 h. At the end of the pretreatment period, hepatocytes were treated with 1-ABT (0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, or 10 mM) or culture medium (vehicle) for 24 h, and then subjected to BROD, DCF, and LDH assays. Data shown are from a single experiment (N = 1 rat).

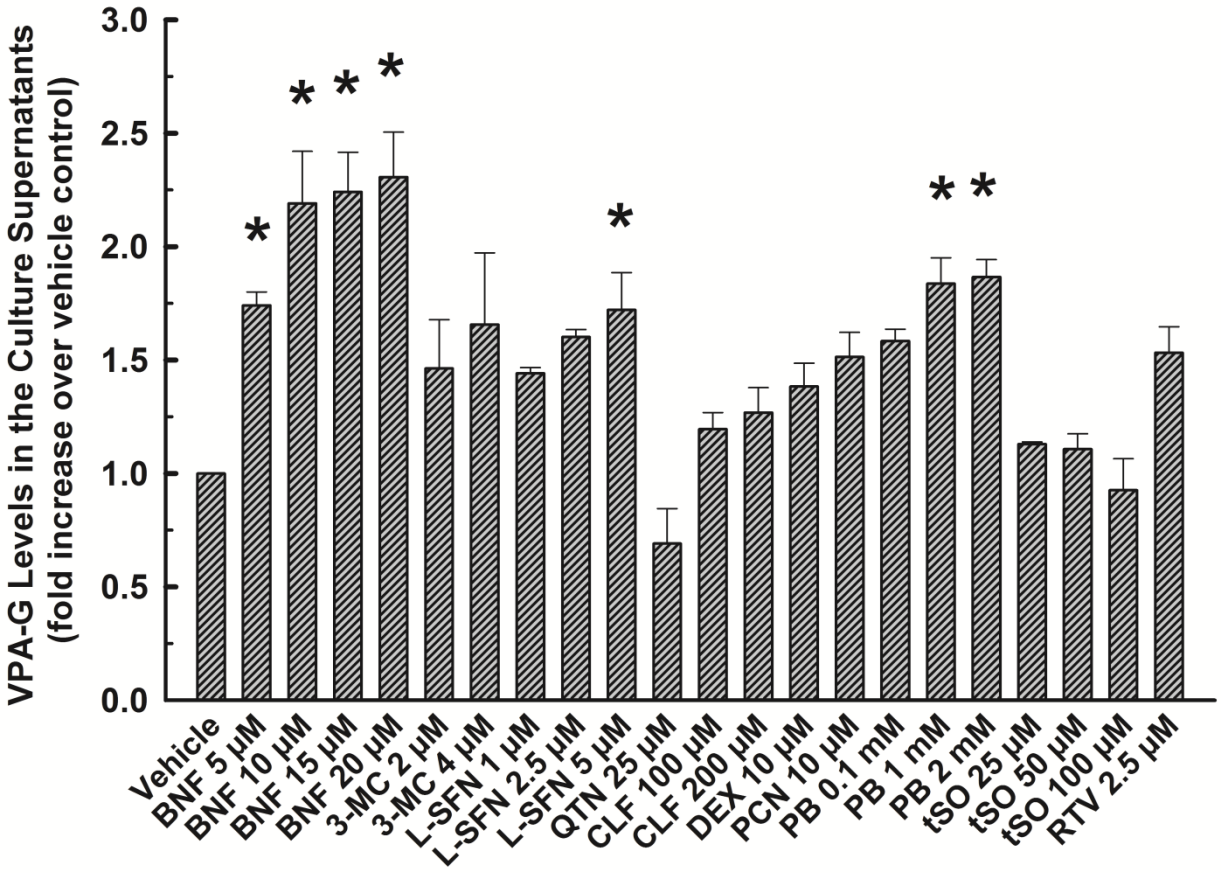

Figure A.3. Effect of Known Inducers of UGT Enzymes on VPA-G Levels in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

At 48 h after plating, hepatocytes were pretreated with *β*-naphthoflavone (BNF; 5, 10, 15, or 20 µM), 3-methylcholanthrene (3-MC; 2 or 4 µM), L-sulforaphane (L-SFN; 1, 2.5, or 5 µM), quercetin (QTN; 25 µM), clofibrate (CLF; 100 or 200 µM), dexamethasone (DEX; 10 µM), pregnenolone 16*α*-carbonitrile (PCN; 10 µM), phenobarbital (PB; 0.1, 1, or 2 mM), *trans*-stilbene oxide (*t*SO; 25, 50, or 100 µM), ritonavir (RTV; 2.5 µM), or vehicle (culture medium for PB; 0.1% DMSO for all other pretreatments) once every 24 h for 72 h. Subsequently, the hepatocytes were treated with 10 mM VPA for 24 h. Data are expressed as mean ± SEM (N = three rats). *Significantly different from the corresponding vehicle-pretreated control group, $P < 0.05$.

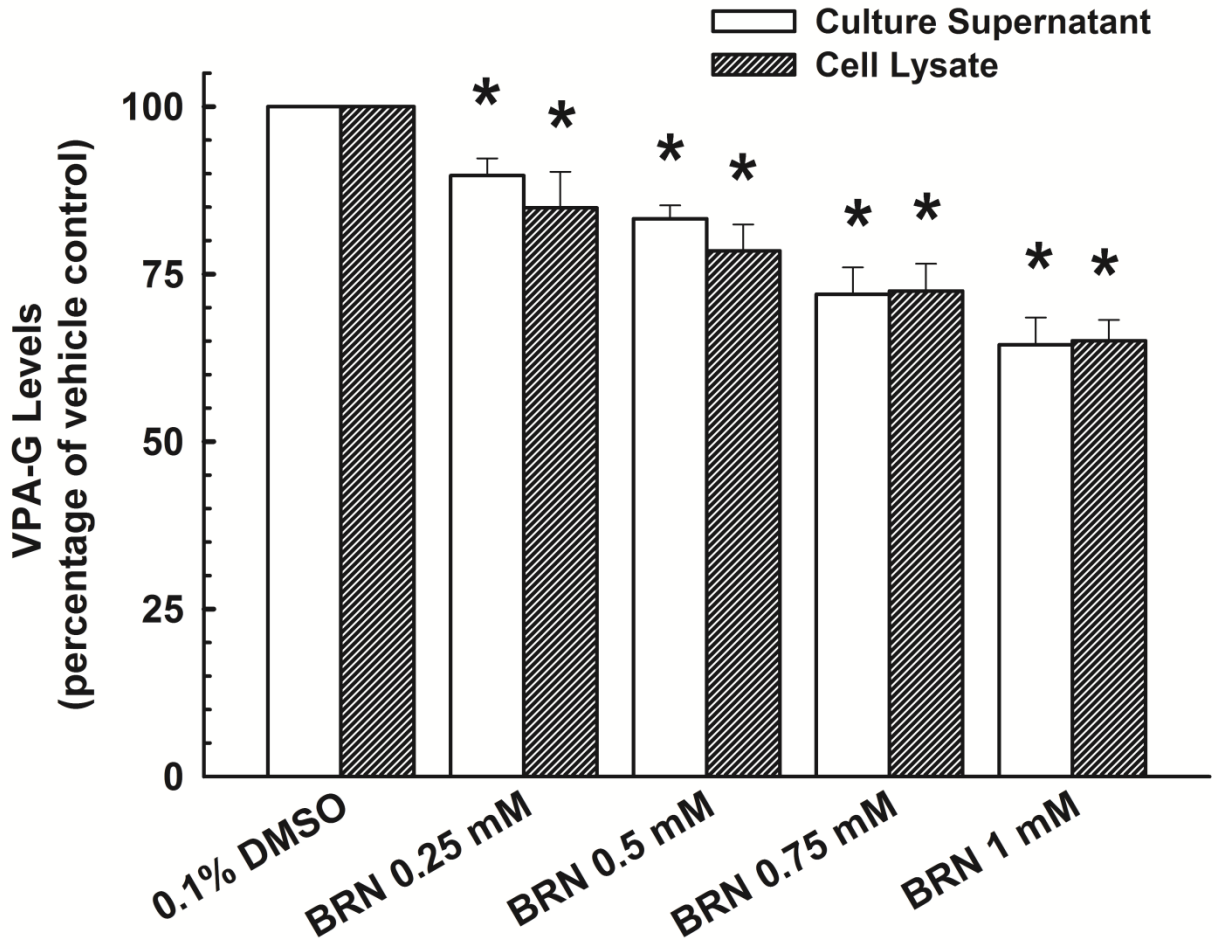

Figure A.4. Concentration-Response Effect of Borneol on *In Situ* Formation of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

At 48 h after plating, hepatocytes were pretreated with *β*-naphthoflavone (BNF; 5, 10, 15, or 20 μM), 3-methylcholanthrene (3-MC; 2 or 4 μM), L-sulforaphane (L-SFN; 1, 2.5, or 5 μM), quercetin (QTN; 25 μM), clofibrate (CLF; 100 or 200 μM), dexamethasone (DEX; 10 μM), pregnenolone 16*α*-carbonitrile (PCN; 10 μM), phenobarbital (PB; 0.1, 1, or 2 mM), *trans*-stilbene oxide (*t*SO; 25, 50, or 100 μM), ritonavir (RTV; 2.5 μM), or vehicle (culture medium for PB; 0.1% DMSO for all other pretreatments) once every 24 h for 72 h. Subsequently, the hepatocytes were treated with 10 mM VPA for 24 h. Data are expressed as mean ± SEM (N = three rats). *Significantly different from the corresponding vehicle-pretreated control group, $P < 0.05$.

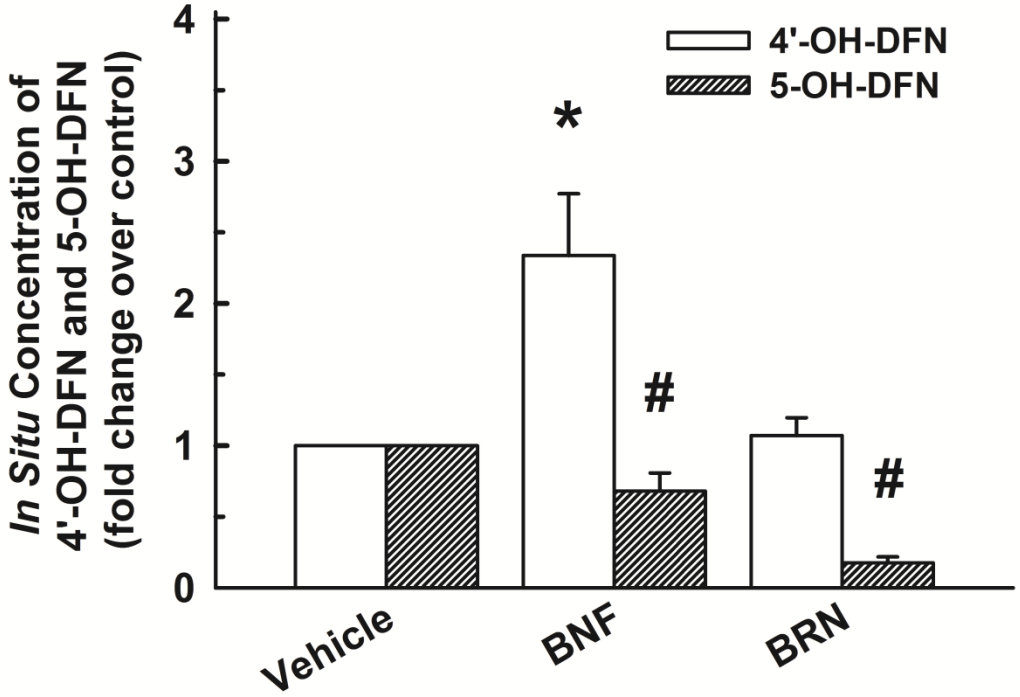

Figure A.5 Effect of β-Naphthoflavone and Borneol on *In Situ* Formation of 4'-OH-DFN and 5-OH-DFN in Sandwich-Cultured Rat Hepatocytes Treated with Diclofenac.
At 48 h after plating, hepatocytes were pretreated with β-naphthoflavone (BNF; 20 μM) or DMSO (vehicle; 0.1% v/v) once every 24 h for 72 h, followed by treatment with diclofenac (400 μM) or 0.1% DMSO (vehicle) for the next 24 h. In experiments involving borneol, hepatocytes after 120 h of culture were pretreated with borneol (BRN; 1 mM) or DMSO (0.1% v/v; vehicle) for 0.5 h, followed by treatment with diclofenac (400 μM) or 0.1% DMSO (vehicle) for the next 24 h in the presence of borneol or DMSO at the concentrations indicated above. Data are expressed as mean ± SEM (N = three rats). *Significantly different from 4'-OH-DFN concentration in the 0.1% DMSO pretreatment group, $P < 0.05$; #Significantly different from 5-OH-DFN concentration in the 0.1% DMSO pretreatment group, $P < 0.05$. The *in situ* concentrations (mean ± SEM) of 4'-OH-DFN and 5-OH-DFN in the 0.1% DMSO pretreatment group were 1.0 ± 0.2 and 0.5 ± 0.2 μM per 0.7 million cells, respectively.

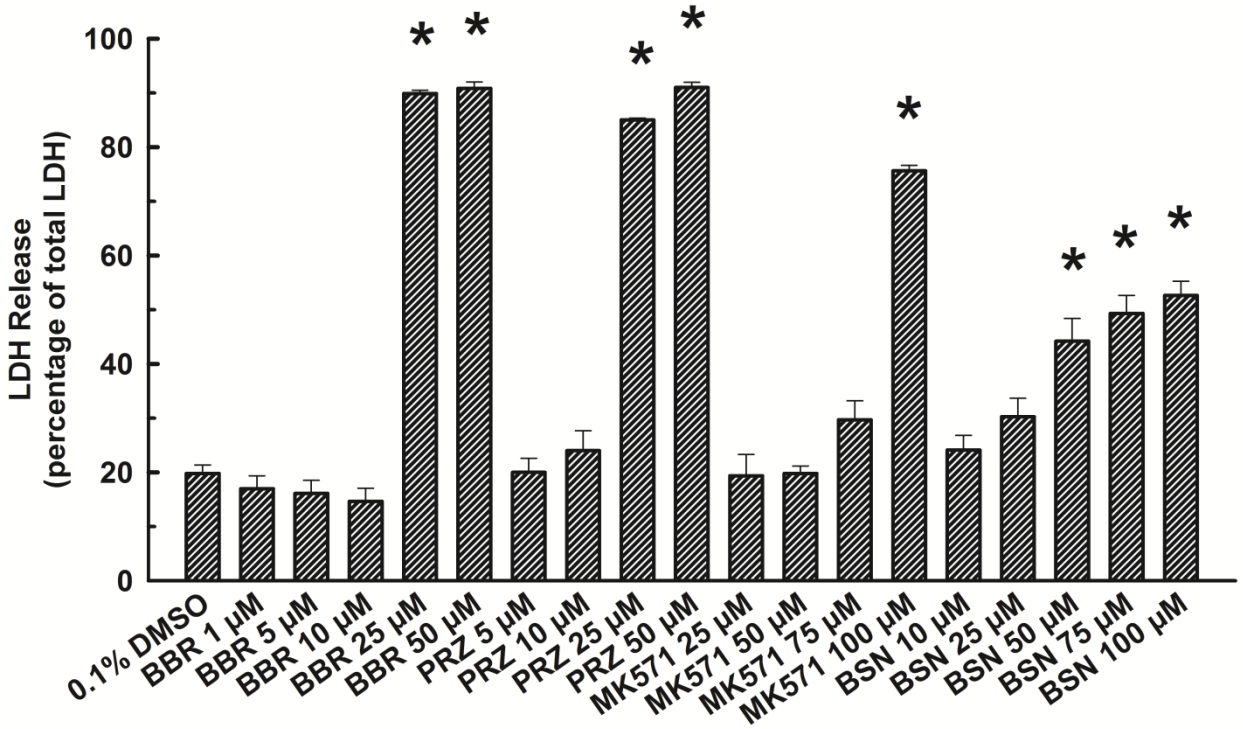

Figure A.6 Concentration-Response Effect of Benzbromarone, Prazosin, MK571, and Bromosulfalein on LDH Release in Sandwich-Cultured Rat Hepatocytes.
At 120 h after plating, hepatocytes were treated with benzbromarone (BBR; 1, 5, 10, 25, or 50 µM), prazosin (PRZ; 5, 10, 25, or 50 µM), MK571 (25, 50, 75, or 100 µM), bromosulfalein (BSN; 10, 25, 50, 75, or 100 µM), or vehicle (0.1% DMSO) for 24.5 h. Data are expressed as mean ± SEM (N = three rats). *Significantly different from vehicle-treated control group, $P < 0.05$.

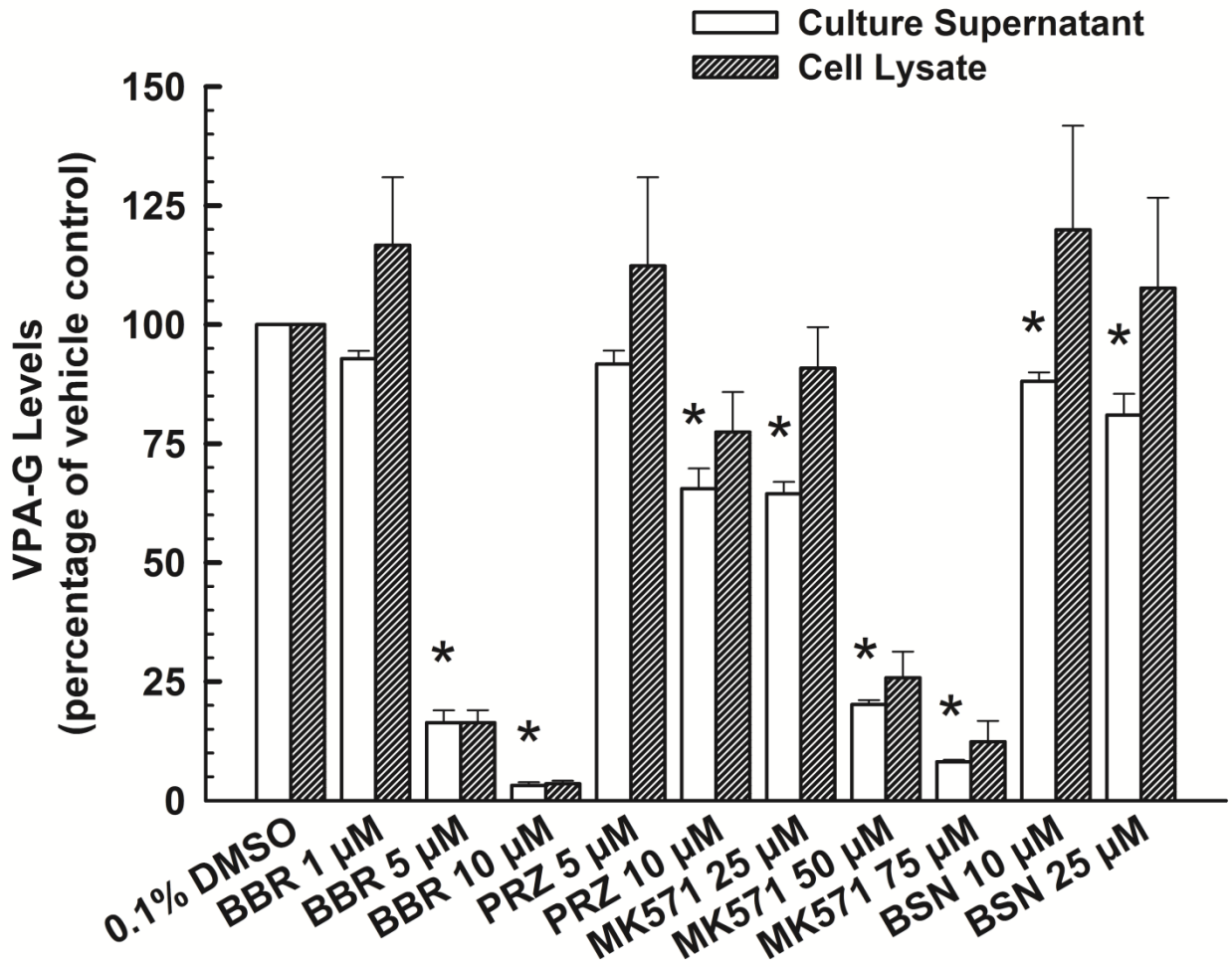

Figure A.7 Effect of Benzbromarone, Prazosin, MK571, and Bromosulfalein on Culture Supernatant and Cell Lysate Levels of VPA-G in Sandwich-Cultured Rat Hepatocytes Treated with VPA.

At 120 h after plating, hepatocytes were treated with benzbromarone (BBR; 1, 5, or 10 µM), prazosin (PRZ; 5 or 10 µM), MK571 (25, 50, or 75 µM), bromosulfalein (BSN; 10 or 25 µM), or DMSO (0.1% v/v; vehicle) for 0.5 h. Subsequently, the cells were treated with 10 mM VPA in the presence of BBR, PRZ, MK571, BSN, or DMSO at the concentrations indicated above. Data are expressed as mean ± SEM (N = three rats). *Significantly different from vehicle-treated control group, $P < 0.05$. Mean culture supernatant and cell lysate levels of VPA-G in vehicle-treated control group were 226 ± 22 µM (mean ± SEM; N = 3) and 2.5 ± 1.1 µM, respectively.