VALPROIC ACID: MECHANISMS OF HEPATOTOXICITY AND REACTION PHENOTYPING

by

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ABSTRACT

Valproic acid (VPA) therapy is associated with a rare but severe hepatotoxicity. The relationships between the various pathophysiological findings of VPA-induced hepatotoxicity and the role of VPA biotransformation in the induction of hepatotoxicity have not been systematically investigated. The present thesis compared the effects of VPA, synthesized VPA metabolites, and α-F-VPA on markers of mitochondrial dysfunction (WST-1), cytotoxicity (LDH), oxidative stress (DCF), and glutathione (GSH) depletion in a novel model of sandwich-cultured rat hepatocytes (SCRH). The contribution of the CYP- and UGT-mediated biotransformation of VPA in VPA-induced toxicity was also examined. Time-dependent effects of VPA on GSH depletion were characterized in relation to the effects of VPA on the WST-1, LDH, and DCF markers. The effects of glutathione supplementation on the attenuation of the markers for VPA-induced toxicities were investigated. Urine samples from children on VPA therapy were assayed to correlate levels of VPA metabolites with the lipid peroxidation marker, 15-F_{2t}-isoprostane. Lastly, the contributions of hepatic CYP-enzymes in the oxidative metabolism of VPA were characterized in human liver microsomes.

Our findings in SCRH indicated that (*E*)-2,4-diene-VPA was the only exogenously administered metabolite tested that was consistently more toxic than VPA. Consistent with this finding, α-F-VPA, which is resistant to bioactivation by several biotransformation pathways, was nontoxic. Chemical inhibition experiments indicated that the CYP- and UGT-mediated metabolism of VPA or the *in situ* generated VPA metabolites were unlikely involved in the observed VPA-induced toxicities in SCRH. Furthermore, VPA-associated GSH depletion appeared not to be a factor in the mitochondrial dysfunction, but may play a partial role in VPA-induced cytotoxicity. GSH may serve a protective role against VPA-induced oxidative stress in SCRH. In human subjects, the VPA-glucuronide or *N*-acetylcysteine metabolites were

extremely weak but statistically significant predictors of lipid peroxidation in the urine of children receiving VPA. From the reaction phenotyping experiments, CYP2C9 was the major catalyst for the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA in human liver microsomes, whereas CYP2A6 contributed partially to 3-OH-VPA formation. Overall, these findings add significant knowledge to the role of VPA and its metabolites in the induction of hepatotoxicity and how VPA is metabolized in humans.

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(*E*)-2,4-diene-VPA	(*E*)-2-propylpent-2,4-dienoic acid
(*E*)-2-ene-VPA	(*E*)-2-propyl-2-pentenoic acid
(*E,E*)-2,3′-diene-VPA	(*E,E*)-2-propyl-2,3′-pentadienoic acid
(*E,Z*)-2,3′-diene-VPA	(*E,Z*)- 2-propyl-2,3′-pentadienoic acid
1-ABT	1-Aminobenzotriazole
3-ene-VPA	(*E*)-2-propylpent-3-enoic acid
3-keto-VPA	2-propyl-3-oxopentanoic acid
3-OH-VPA	3-hydroxy-2-propylpentanoic acid
4-ene-VPA	2-propylpent-4-enoic acid
4-keto-VPA	4-oxo-2-propylpentanoic acid
4-OH-VPA	4-hydroxy-2-propylpentanoic acid
5-NAC-2-ene-VPA	2-propyl-5-(N-acetylcystein-S-yl) pent-2-enoic acid
5-NAC-3-ene-VPA	2-propyl-5-(N-acetylcystein-S-yl) pent-3-enoic acid
5-OH-VPA	5-hydroxy-2-propylpentanoic acid
ACN	Acetonitrile
AED	Antiepileptic drug
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BR	(-)-Borneol
cDNA	Complementary DNA
CoA	Co-enzyme A
CPTI	Carnitine palmitoyl transferase I
CYP	Cytochrome P450 enzymes
DCF	2′,7′-Dichlorofluorescein
DCFDA	Dichlorofluorescein diacetate
DCFH	Dichlorofluorescin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
GC/MS	Gas chromatography-mass spectrometry
GI	Gastrointestinal
GOT	Glutamate oxaloacetate transaminase
GPT	Glutamate pyruvate transaminase
GSH	Glutathione
GST	Glutathione-S-transferase
HPLC	High pressure liquid chromatography
Hy-Hel-9	Control monoclonal antibody against lysozyme
KTZ	Ketoconazole
LC/MS	Liquid chromatography-mass spectrometry
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate Dehydrogenase
MAb	Monoclonal antibodies
MAb-2A6	Monoclonal antibody against CYP2A6
MAb-2B6	Monoclonal antibody against CYP2B6
MAb-2C9	Monoclonal antibody against CYP2C9

MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MRM	Multiple reaction monitoring
NAC	*N*-acetylcysteine
NADPH	Nicotine adenine dinucleotide
PB	Phenobarbital
ROS	Reactive oxygen species
SCRH	Matrigel™ sandwich-cultured rat hepatocytes
thio-TEPA	Triethylenethiophosphoramide
TNB	5-thio-2-nitrobenzoic acid
UGT	*UDP* –glucuronosyltransferases
UPLC	Ultra Performance LC
VPA	Valproic acid, 2-n-propyl-pentanoic acid
VPA-G	VPA-1-*O*-acyl-glucuronide
α-F-4-ene-VPA	2-fluoro-2-propyl-4-pentenoic acid
α-F-VPA	2-fluoro-2-propylpentanoic acid

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DEDICATIONS

This thesis is dedicated to members of my family: Tina, Edward, James, Patty, and baby girl Charlotte

CO-AUTHORSHIP STATEMENT

The details of my contributions and that of the other co-authors in the individual manuscript chapters are stated.

Chapters 2 & 3. The synthesized VPA metabolites and α-F-VPA were synthesized and purified by Dr. Stoyan Karagiozov. Rat hepatocytes were isolated by Dr. Xiao Wei Teng. Mr. Jayakumar Surendradoss has also provided some assistance for a few biochemical assays. With guidance from Dr. Thomas Chang and Dr. Frank Abbott, I designed and conducted all of the experiments presented in these two chapters. I was responsible for collecting, processing, and analyzing the data. I also drafted the contents of Chapters 2 & 3, which will be further revised and submitted for publication.

Chapter 4. The patient urine samples and patient demographic data were provided by Dr. Kevin Farrell (British Columbia Children's Hospital) and Dr. Steve Leeder (University of Missouri). With guidance from Dr. Thomas Chang and Dr. Frank Abbott, I quantified the levels of VPA metabolites, 15-F_{2t}-isoprostane, and creatinine in the individual urine samples. I was also responsible for processing and analyzing the data. Dr. Xiao Wei Teng performed the majority of LC/MS analysis of VPA-glucuronide and the NAC metabolites of VPA. As well, I also drafted the contents of Chapter 4, which will be further revised and submitted for publication.

Chapter 5. A version of this chapter has already been published (Kiang TK, Ho PC, Anari MR, Tong V, Abbott FS, Chang TKH. *Toxicological Sciences*, 2006; 94: 261 – 271). Dr. P.C. Ho provided the data for Figure 5-1. Dr. M.R. Anari and Dr. V. Tong developed the GC/MS assay. With guidance from Dr. Thomas Chang and Dr. Frank Abbott, I was directly responsible for

collecting and analyzing the data that contributed Figures 5-2, 5-3, and 5-4. I was also responsible for preparing the initial draft of the manuscript, which was completely re-written by Dr. Thomas Chang for the final submission. The published version is presented as Chapter 5 in the present thesis.

1 Introduction

1.1 Valproic Acid History, Current Indications, and Pharmacology

The antiepileptic effects of valproic acid (2-n-propyl-pentanoic acid, VPA, Figure 1-1), initially reported by Meunier et al. (Meunier *et al.*, 1963), were serendipitously discovered by Pierre Eymard in France. Currently, VPA is indicated for primary generalized or partial seizures, including the absence, tonic-clonic, or myoclonic types (Johannessen and Johannessen, 2003). Aside from its anticonvulsant activity, VPA is also used for the management of bi-polar disorder, migraine headache, and nerve pain (Silberstein, 1996; Cutrer and Moskowitz, 1996; Bowden *et al.*, 1994). Recent reports also suggest the potential use of VPA in cancer therapy (Minucci and Pelicci, 2006) as well as the management of HIV infection (Lehrman *et al.*, 2005).

The anticonvulsant activities of VPA are believed to be mediated by its effects on the GABAnergic system. VPA elevates the levels of GABA in select regions of the brain by activating glutamic acid decarboxylase, an enzyme responsible for GABA synthesis (Loscher, 1989), or inhibiting succinic semialdehyde dehydrogenase or GABA transaminases, enzymes responsible for GABA degradation (Loscher *et al.*, 1985; van der laan *et al.*, 1979). Other pharmacological actions of VPA, such as the blockade of voltage-dependent sodium+ channels or the inhibition of N-methyl-D-aspartate (NMDA) receptors, may also contribute to its anticonvulsant effects (McLean *et al.*, 1986; Zeise *et al.*, 1991; Gean *et al.*, 1994). The exact mechanisms responsible for the anticonvulsant activities of VPA are not known, but the wide spectrum of pharmacological actions may explain why VPA is effective for various types of seizure disorders. The pharmacokinetic and pharmacodynamic properties of VPA have been extensively reviewed and details can be found in a number of published manuscripts or book

chapters (e.g. Davis *et al.*, 1994; Levy *et al.*, 1995; Zaccara *et al.*, 1988; Loscher, 1999b; Abbott and Anari, 1999; Loscher, 1999a; Shen, 1999).

1.2 VPA-Induced Hepatotoxicity

The administration of VPA, like any other drug, is associated with unwanted side effects. Frequent and minor side effects include gastrointestinal (GI) disorders, weight gain, or alopecia, which may be averted with the administration of GI-protective formulations or simply with continued usage (Egger *et al.*, 1981; Pylvanen *et al.*, 2006; Khan *et al.*, 1999; Mercke *et al.*, 2000; Marks *et al.*, 1988). The known rare side effects are pancreatitis (Batalden *et al.*, 1979; Camfield *et al.*, 1979; Asconape *et al.*, 1993), encephalopathy (Cuturic and Abramson, 2005; Rath *et al.*, 2005; Gerstner *et al.*, 2006a), coagulation disorder (Gerstner *et al.*, 2006b), teratogenicity (Bjerkedal *et al.*, 1982), and hepatotoxicity (Eadie *et al.*, 1988). The latter side effect is the focus of the present thesis.

VPA-induced hepatotoxicity is either dose-dependent or idiosyncratic. The dose-dependent toxicity is typically asymptomatic, characterized by mild elevations of liver transaminase enzymes, and often does not necessitate the discontinuation of therapy (Genton *et al.*, 2002). On the other hand, the idiosyncratic liver toxicity associated with VPA treatment is rare, irreversible, and often leads to fatalities (Zimmerman and Ishar, 1982; Scheffner *et al.*, 1988; Kuhara *et al.*, 1990; Konig *et al.*, 1994; Bryant and Dreifuss, 1996; Eadie, Hooper, and Dickinson, 1988). The onset of the idiosyncratic toxicity is usually seen within 3 months of initiating VPA therapy (Zimmerman and Ishak, 1982; Zafrani *et al.*, 1982; Jeavons, 1984; Scheffner *et al.,*1988), and the typical symptoms of anorexia, nausea, vomiting, fever, and jaundice do not correspond to a hypersensitivity reaction (Zimmerman and Ishak, 1982). According to retrospective investigations conducted between the 1980 – 90's, young age (< 2 years old), polytherapy, and congenital neurological disorders are associated with higher

incidences of VPA-induced hepatotoxicity (Dreifuss *et al.*, 1987; Dreifuss *et al.*, 1989; Bryant and Dreifuss, 1996). However, based on a recent report by Koenig *et al.* (2006), the majority of patients who had developed VPA-induced liver toxicity were greater than 5 years of age in their German population; thus the risk groups of VPA-induced hepatotoxicity could be more complex than previously thought.

The mechanisms responsible for VPA-induced hepatotoxicity remain unknown. The present thesis will discuss experiments to investigate the involvement of mitochondrial dysfunction, reactive metabolite formation, oxidative stress induction, and abnormal glutathione (GSH) homeostasis. The background in support of the thesis objectives is reviewed below.

1.3 VPA-Induced Mitochondrial Dysfunction

A common pathophysiological finding of VPA-induced hepatotoxicity in rats and humans is the presence of hepatic microvesicular steatosis, which is sometimes associated with necrosis (Jeavons, 1984; Zimmerman and Ishak, 1982). The observation that VPA-induced hepatotoxicity closely resembles that of Reye's syndrome, a disease of mitochondrial origin (Partin *et al.*, 1971; Mitchell *et al.*, 1980), suggests that mitochondrial dysfunction might be involved in VPA-induced liver injury. In support of this view, histopathology of rat and human liver in cases of VPA-induced liver toxicity often reveal abnormal mitochondrial ultrastructure characterized by densely packed mitochondria with enlarged matrix or abnormal cristae (Itoh *et al.*, 1982; Mathis *et al.*, 1979; Kesterson *et al.*, 1984; Lewis *et al.*, 1982; Sobaniec-Lotowska, 1997; Jezequel *et al.*, 1984). VPA-induced microvesicular steatosis is believed to be a consequence of impaired mitochondrial fatty acid ß-oxidation (Fromenty *et al.*, 1995; Pessayre *et al.*, 1999), which has been demonstrated in isolated rat mitochondrial preparations (Ponchaut *et al.*, 1992), rat hepatocytes (Silva *et al.*, 2001; Coude *et al.*, 1983; Turnbull *et al.*, 1983), or rat liver homogenate (Bjorge and Baillie, 1985). Elevations of urinary dicarboxylic acids (C6 –

C10), a consequence of reduced ß-oxidation, have also been reported in human subjects administered VPA (Mortensen, 1980). As a result of reduced fatty acid oxidation, hepatic lipid contents are also elevated, and ketogenesis markedly reduced, in rats or rat hepatocytes administered VPA (Jezequel *et al.,* 1984; Olson *et al.*, 1986; Turnbull *et al.,* 1983; Coude *et al.,* 1983).

The sequestration of ß-oxidation co-factors by VPA or VPA metabolites and/or the direct inhibition of ß-oxidation enzymes by VPA or VPA metabolites are the mechanisms(s) by which VPA inhibits the mitochondrial ß-oxidation. The data supporting these mechanisms are discussed in the sections below.

1.3.1 CoA or Carnitine Sequestration as a Mechanism of VPA-Associated Mitochondrial Dysfunction

Co-enzyme A (CoA) is an obligatory cofactor for the ß-oxidation of all fatty acids (Fromenty and Pessayre, 1995). The formation of VPA-CoA, which is somewhat resistant to hydrolysis, could result in the depletion of free CoA by VPA (Li *et al.*, 1991; Moore *et al.*, 1988). Evidence for the formation of VPA-CoA was initially reported by Becker and Harris (1983) in the liver extracts of rats administered VPA, and the effects of VPA on the depletion of free CoA have since been demonstrated *in vivo* in rat livers (Kesterson *et al.,* 1984) or *in vitro* in coupled rat liver mitochondria (Ponchaut *et al.,* 1992). Reduced levels of free CoA have also been observed in liver of rats administered (*E*)-2,4-diene-VPA, 4-ene-VPA, or 3-ene-VPA (Kesterson *et al.,* 1984), and in isolated rat liver mitochondria exposed to 4-ene-VPA or (*E*)-2,4-diene-VPA (Ponchaut *et al.,* 1992). Similar to VPA, the ability of the VPA metabolites to deplete free CoA appears to be associated with their ability to inhibit mitochondrial ß-oxidation of fatty acids (Kesterson *et al.,* 1984; Ponchaut *et al.,* 1992). Interestingly, a recent investigation using rat liver homogenate demonstrated that VPA-CoA could be generated in the cytosolic fraction

(Aires *et al.*, 2007), contrary to the conventional thinking that the formation of CoA esters is restricted to the mitochondrial compartment. However, the role of the non mitochondrial-generated VPA-CoA in the pathogenesis of VPA hepatotoxicity remains to be examined.

Like CoA, carnitine is an obligatory co-factor for mitochondrial β-oxidation because it is needed for the passage of long-chain fatty acids from the cytosol into the mitochondrial matrix (Hoppel, 2003). As a consequence of its effects on mitochondrial β-oxidation, low carnitine levels are often associated with decreased production of acetyl-CoA and inadequate generation of cellular energy (ATP) (Lheureux *et al.*, 2005). The effects of VPA treatment on the depletion of free or total carnitine have been demonstrated in the plasma or liver of human subjects (Coulter, 1984; Ohtani *et al.*, 1982; Laub *et al.*, 1986; Bohles *et al.*, 1982; Moreno *et al.*, 2005), although cases where VPA treatment did not affect carnitine levels have also been reported (Fung *et al.*, 2003). Similar findings are also evident in rats where chronic and high doses of VPA are able to deplete the serum-free or total carnitine (Sugimoto *et al.*, 1987b; Sugimoto *et al.*, 1987a), an effect inducible by the co-administration of phenobarbital (Sugimoto *et al.,* 1987b). Several mechanisms by which VPA depletes carnitine stores have been proposed: inhibition of hepatic carnitine synthesis, as demonstrated in rats (Farkas *et al.*, 1996); reduction of tubular reabsorption, as demonstrated in mice and humans (Camina *et al.*, 1991a; Camina *et al.*, 1991b; Matsuda *et al.*, 1986); or inhibition of carnitine transport into the cytosol, as demonstrated in human embryonic kidney cells transfected with organic cation carnitine transporters (Ohashi *et al.*, 1999).

In rats, L-carnitine supplementation reverses the effects of VPA on hepatic carnitine depletion and attenuates the adverse effects of VPA on hepatic mitochondrial morphology (Sugimoto *et al.,* 1987b, Nishida *et al.*, 1987). Heterozygous jvs +/- mice, an animal model with decreased carnitine stores, are also more susceptible to the hepatotoxic effects of VPA compared to the wild type controls (Knapp *et al.*, 2008). In humans, carnitine supplementation improves

the survival rates of individuals who have developed VPA-induced hepatotoxicity (Bohan *et al.*, 2001; DeVivo, 2002; Romero-Falcon *et al.*, 2003), although cases where L-carnitine supplementation proved ineffective have also been reported (Laub *et al.,* 1986). Overall, these data appear to support an association between carnitine deficiency and VPA-induced hepatotoxicity.

1.3.2 Inhibition of Enzymes Associated with Mitochondrial ß-Oxidation

In addition to the sequestration of ß-oxidation co-factors, VPA or its metabolites can directly or indirectly inhibit the enzymes associated with mitochondrial ß-oxidation. VPA-CoA is an inhibitor of purified human short and medium-chain acyl-CoA dehydrogenases (Ito *et al.*, 1990). (*E*)-2,4-diene-VPA is also able to bind to the mitochondrial trifunctional protein in a potent and reversible manner (Baldwin *et al.*, 1996), although it is not clear whether this type of binding would result in abnormal mitochondrial function. Furthermore, VPA-CoA appears to be a competitive inhibitor of carnitine palmitoyl transferase I (CPT I), as demonstrated in experiments using cultured human fibroblasts or recombinant human CPT I enzymes (Silva *et al.*, 2008). The inhibition of CPT I, an enzyme responsible for shuttling the Acyl-CoA esters from the cytosol to the mitochondrial matrix (Lheureux *et al.*, 2009), may lead to impaired ß-oxidation of long chain fatty acids.

Lastly, VPA or its metabolites may indirectly inhibit the mitochondrial ß-oxidation by affecting the process of oxidative phosphorylation and attenuating the production of energy (ATP). An example would be the ability of VPA-CoA or 4-ene-VPA-CoA to inhibit the uptake of pyruvate, a substrate of oxidative phosphorylation, by blocking the mitochondrial pyruvate/H+ symporter (Aires *et al.*, 2008). Reduced oxidative phosphorylation would result in decreased energy production, thereby inhibiting the energy-driven ß-oxidation of fatty acids in the mitochondria.

1.4 VPA Metabolism and the Production of Toxic Metabolites

Valproic acid is extensively metabolised in the liver, and its metabolites also undergo subsequent secondary metabolism (Abbott and Anari, 1999). The enzymes that are involved in the metabolism of VPA are *UDP* –glucuronosyltransferases (UGT), mitochondrial ß-oxidases, and cytochrome P450 (CYP) (Abbott and Anari, 1999). Select VPA metabolites also conjugate with GSH, and the GSH conjugates can undergo further metabolism via the mercapturic acid pathway to form the *N*-acetylcysteine metabolites. Figure 1-2 illustrates the common VPA metabolites produced from the various biotransformation pathways in the human liver.

1.4.1 Glucuronidation

Glucuronidation of VPA is quantitatively the most important biotransformation pathway in rats and humans (Dickinson *et al.*, 1979; Granneman *et al.*, 1984; Dickinson *et al.*, 1989). The reaction, which involves the cofactor D-glucuronic acid, takes place in the endoplasmic reticulum, and forms the VPA-*O*-acyl-ß-linked ester glucuronide. Human hepatic UGT1A3, UGT1A6, UGT1A9, and UGT2B7 are capable of forming the VPA glucuronide (Ethell *et al.*, 2003; Sakaguchi *et al.*, 2004), although their relative contributions in the liver toward the reaction remain unknown. Unlike some xenobiotic acyl glucuronides, VPA-glucuronide is relatively stable (Boelsterli, 2002) as it forms very little protein adducts (Bailey and Dickinson 1996). However, VPA-glucuronide can undergo structural rearrangements to generate the ß-glucuronidase-resistant isomers, which can be more reactive toward proteins (Williams *et al.*, 1992).

In support of the notion that the glucuronidation pathway maybe involved in the VPA-induced hepatotoxicity, general inhibitors of glucuronidation are able to attenuate VPA-induced lipid peroxidation in rats (Tong *et al.*, 2005c) or binding of ^{14}C-labelled 4-ene-VPA or VPA to isolated rat hepatocytes (Porubek *et al.*, 1989). Consistent with these findings, α-F-VPA or α-F-

4-ene-VPA, which forms very little glucuronide metabolites, are relatively ineffective in the induction of lipid peroxidation or hepatic steatosis in rats, respectively, in contrast to their non-fluorinated counterparts (Tong *et al.,* 2005c; Tang *et al.*, 1997). However, the selectivity of the glucuronidation inhibitors used in the study by Tong *et al.* (2005c) was not verified, and the α-fluorinated analogues are also resistant to the formation of ß-oxidation metabolites (Tang *et al.*, 1995) or acyl-CoA esters (Grillo *et al.*, 2001). Because of these limitations, the contribution of the glucuronidation pathway in the development of VPA-associated hepatotoxicity remains to be examined.

1.4.2 ß-Oxidation

ß-oxidation of VPA appears to take place primarily in the mitochondria (Matsumoto *et al.*, 1976; Draye and Vamecq, 1987). Studies using rat liver mitochondrial fractions or purified mitochondrial enzymes proved that VPA-CoA undergoes dehydrogenation to generate (*E*)-2-ene-VPA-CoA in a reaction catalyzed by the 2-methyl-branched-chain acyl-CoA dehydrogenase (Ito *et al.,* 1990). The subsequent production of 3-OH-VPA-CoA and 3-keto-VPA-CoA are catalyzed by enoyl-CoA hydratase and a membrane-bound NAD+ dependent dehydrogenase (Li *et al.,* 1991), respectively. Normally, 3-keto-acyl-CoA thiolase, the terminal mitochondrial ß-oxidation enzyme, would cleave the fatty acyl-CoA and release the acetyl-CoA, but 3-keto-VPA-CoA appears to be resistant to this reaction (Li *et al.,* 1991). This supports the hypothesis that VPA-associated inhibition of mitochondrial β-oxidation is partly due to the CoA sequestration by VPA metabolites that are incapable of completing the ß-oxidation cycle. Another β-oxidation metabolite is the 2,3-diene-VPA, which is generated from the ß-oxidation of 3-ene-VPA (Bjorge and Baillie, 1991). Using isolated mitochondrial preparations, 3-ene-VPA was shown to be interconverted from (*E*)-2-ene-VPA, thus both of these metabolites are capable of forming the 3-keto-VPA (Bjorge and Baillie, 1991). On the other hand, 3-OH-VPA, an intermediate in the ß-

oxidation of VPA, is also a product of the CYP-mediated oxidation of VPA (Rettenmeier *et al.*, 1987; Prickett and Baillie 1984). Another metabolite of duel origin is the (*E*)-2,4-diene-VPA, which can be generated from the β-oxidation of 4-ene-VPA (Tang *et al.,* 1995) or the CYP-mediated terminal desaturation of (*E*)-2-ene-VPA (Kassahun *et al.*, 1993b).

In support of the notion that the β-oxidation pathway of VPA maybe involved in the VPA-induced hepatotoxicity, general inhibitors of ß-oxidation are able to attenuate the covalent binding of ^{14}C-labelled 4-ene-VPA or VPA to isolated rat hepatocytes (Porubek *et al.,*1989). Although unproven, the extent of xenobiotic covalent binding to cellular proteins is generally assumed to be associated with the extent of cellular toxicity (Evans *et al.*, 2004). Consistent with these findings, α-F-4-ene-VPA or α-F-VPA, which could not form the ß-oxidation metabolites (Tang *et al.,* 1995; Tong *et al.,* 1997), are also less effective in the induction of hepatic steatosis or lipid peroxidation, respectively (Tong *et al.,* 2005c; Tang *et al.,*1997), when compared to their non-fluorinated counterparts in rats. However, one should interpret the findings obtained from the α-fluorinated analogues cautiously, since they are also resistant to the formation of other VPA metabolites, as discussed above.

Further support for a role of the ß-oxidation pathway in the development of VPA toxicity was illustrated by (*E*)-2,4-diene-VPA, a β-oxidation metabolite of 4-ene-VPA. (*E*)-2,4-diene-VPA is a potent inducer of hepatic steatosis and inhibitor of mitochondrial ß-oxidation in young rats (Kesterson *et al.,* 1984). The metabolite binds to the porcine mitochondrial tri-functional protein in a potent and reversible manner (Baldwin *et al.,* 1996). The CoA ester of (*E*)-2,4-diene-VPA is also reactive toward glutathione or cytosolic proteins in a reaction catalyzed by the glutathione-S-transferase enzymes (Tang *et al.*, 1996c). Based on this reaction, various thio-conjugates of (*E*)-2,4-diene-VPA have been identified in rats administered relatively high doses of 4-ene-VPA or (*E*)-2,4-diene-VPA, and in human subjects receiving therapeutic doses of VPA (Kassahun *et al.*, 1991; Kassahun *et al.*, 1993a; Kassahun *et al.*, 1994; Tang *et al.,* 1995; Tang

and Abbott 1996a; Tang and Abbott 1996b; Gopaul *et al.*, 2000b; Gopaul *et al.*, 2000a; Gopaul *et al.*, 2003). In support of an association between the formation of (*E*)-2,4-diene-VPA and the development of hepatotoxicity, urinary levels of thio-conjugated (*E*)-2,4-diene-VPA have been found elevated in individuals who had developed VPA-induced hepatotoxicity (Kassahun *et al.,* 1991), or in children who possessed the risk factors (i.e. age or polytherapy) of VPA-associated liver injury (Gopaul *et al.,* 2003).

As discussed above, the (*E*)-2,4-diene-VPA is also generated from CYP-mediated terminal desaturation of (*E*)-2-ene-VPA (Kassahun and Baillie, 1993b). However, the CYP-generated (*E*)-2,4-diene-VPA, being a free acid, is unlikely of toxicological significance, since it is not reactive toward GSH or proteins (Kassahun *et al.,* 1991; Tang and Abbott 1996b). The notion that the β-oxidation generated (*E*)-2,4-diene-VPA is more toxic than the CYP-generated equivalent was demonstrated in an experiment where equal molar doses of 4-ene-VPA proved to be more toxic, with respect to glutathione depletion and elevation of triglycerides, than (*E*)-2-ene-VPA in rats (Kassahun *et al.,* 1994). In the same study, the levels of thio-conjugated (*E*)-2,4-diene-VPA generated from 4-ene-VPA were also several-fold higher than that produced from (*E*)-2-ene-VPA (Kassahun *et al.,* 1994).

1.4.3 Cytochrome P450 (CYP)-Mediated Oxidation

Cytochrome-P450 (CYP)-mediated oxidative metabolism of VPA is a quantitatively minor biotransformation pathway, but significant from a toxicology point of view. Studies using isolated rat liver microsomes or purified rat enzymes indicated that VPA is hydroxylated at the 3, 4, and 5 positions (Rettie *et al.*, 1987; Prickett and Baillie, 1984), reactions that can be induced by phenobarbital (Prickett and Baillie, 1984). As discussed above, the 3-OH-VPA is also a product of the mitochondrial ß-oxidation pathway, thus the use of this metabolite as a marker for the CYP-mediated oxidative VPA metabolism should be cautioned. Furthermore, using isolated

rat liver microsomes, Rettie et al. (1987) showed that the terminal olefin, 4-ene-VPA, is also generated by the CYP enzymes. Like the hydroxylated metabolites, the formation of 4-ene-VPA can be induced by phenobarbital, as first shown in experiments using microsomes obtained from phenobarbital-treated rats, rabbits, or mice (Rettie *et al.*, 1988). Based on deuterium isotope effect experiments, the CYP-mediated dehydrogenation of VPA at the C4 position is a common step for the formation of either 4-ene-VPA or 4-OH-VPA, whereas the 5-OH-VPA appears to be generated from a separate CYP-hydroxylation reactions of VPA at the C5 position (Rettie *et al.,* 1988).

Little is known of the human hepatic CYP-enzymes responsible for the terminal desaturation or the hydroxylation of VPA. Studies with cDNA-expressed human CYP enzymes indicated that CYP2A6, CYP2B6, and CYP2C9 are capable of catalyzing the terminal desaturation of VPA, and that CYP2C9 is a catalyst of the ω and ω-1 hydroxylations of VPA (Ho *et al.*, 2003; Sadeque *et al.*, 1997; Anari *et al.*, 2000). The CYP enzymes responsible for the hydroxylation of VPA at the C3 position remains to be identified. Furthermore, even less is known of the relative contributions of the individual CYP enzymes in the oxidative metabolism of VPA in human hepatic microsomes. Modest contributions of CYP2A6 and CYP2C9, as determined by the extent of inhibition by coumarin or sulfaphenazole, respectively, to the terminal desaturation of VPA in human liver microsomes have been reported by Sadeque et al. (1997). However, that study only employed human liver microsomes obtained from two individuals, and the relative contributions of the other CYP enzymes were not systematically determined (Sadeque *et al.,* 1997). Unlike the terminal desaturation reaction, the relative contribution of CYP enzymes to the hydroxylation of VPA in human liver microsomes has not been reported.

With the availability of specific and effective inhibitory monoclonal antibodies (MAb) toward human CYP enzymes (Gelboin *et al.*, 2006), it is now possible to determine the relative

contributions of the individual CYP enzymes toward the oxidative metabolism of VPA. A reaction phenotyping study utilizing several complementary approaches, including the MAbs, to examine the CYP-mediated metabolism of VPA in human liver microsomes is presented in Chapter 5 of the present thesis.

4-ene-VPA, generated from the CYP-mediated oxidative metabolism of VPA, is thought to be involved in the development of hepatotoxicity. A significant amount of interest was placed on 4-ene-VPA due to its structural resemblance to known hepatotoxicants, hypoglycin and 4-pentenoic acid (Gerber *et al.*, 1979; Zimmerman and Ishak, 1982). 4-ene-VPA is a weak inhibitor of rat CYP enzymes and is capable of covalent binding to rat liver proteins (Prickett *et al.*, 1986; Porubek *et al.,* 1989). *In vivo* experiments with relatively high doses (100 mg/kg/day for 5 days) of 4-ene-VPA demonstrated that the metabolite is a potent inducer of hepatic steatosis and inhibitor of mitochondrial ß-oxidation in young rats weighting around 150 g (Kesterson *et al.,* 1984; Tang *et al.,* 1995). The inhibitory effects of 4-ene-VPA on mitochondrial ß-oxidation are also demonstrated in isolated rat hepatocytes (Silva *et al.,* 2001), rat mitochondrial preparations (Ponchaut *et al.,* 1992), or rat liver homogenate (Bjorge and Baillie, 1985).

The presence of thio-conjugated metabolites in rats exposed to 4-ene-VPA supports the reactivity of the terminal olefin (Kassahun *et al.,* 1991; Kassahun *et al.,* 1994; Tang *et al.,* 1995; Jurima-Romet *et al.*, 1996). Two independent pathways are known to generate the GSH conjugates of 4-ene-VPA. The CYP-mediated epoxidation of 4-ene-VPA produces the 4,5-epoxide-VPA, which is able to conjugate with GSH to form the 4-OH-5-GS-VPA-γ-lactone or 4-GS-5-OH-VPA (Kassahun *et al.,* 1994). Alternatively, 4-ene-VPA undergoes ß-oxidation to form the (*E*)-2,4-diene-VPA-CoA (Tang *et al.,* 1995), which readily conjugates with GSH in a reaction catalyzed by the GST enzymes (Tang *et al.,* 1996c). As discussed above, various thio-conjugates of (*E*)-2,4-diene-VPA have been identified in rats exposed to 4-ene-VPA or (*E*)-2,4-

diene-VPA. The formation of GSH conjugates constitutes about 20% of the 4-ene-VPA dose in rats, suggesting that a large dose of the metabolite is bioactivated to form reactive intermediates that may contribute to the development of hepatotoxicity (Kassahun *et al.,* 1994).

Conflicting findings on the toxicity of 4-ene-VPA have been reported in the literature. Loscher *et al.* (1993) did not observe the same extent of hepatic steatosis from 4-ene-VPA treatment in rats as reported by Kesterson *et al.* (1984) or Tang *et al.* (1995). Likewise, Jurima-Romet et al. (1996) were also not able to demonstrate any significant toxicity in cultured rat hepatocytes exposed to relatively high concentrations of 4-ene-VPA. In line with these observations, the formation of 4-ene-VPA was not associated with lipid peroxidation (Tong *et al.*, 2003) or hepatic steatosis (Loscher *et al.,* 1993) in rats. In humans, the majority of cases of VPA-induced hepatotoxicity also do not report abnormal 4-ene-VPA levels (Fisher *et al.*, 1992; Kondo *et al.*, 1992; McLaughlin *et al.*, 2000; Paganini *et al.*, 1987; Siemes *et al.*, 1993; Tennison *et al.*, 1988). Because of these conflicting findings on the toxicity of 4-ene-VPA, the role of this CYP-generated metabolite in VPA-induced hepatotoxicity remains to be confirmed.

1.5 Role of Oxidative Stress in VPA-Induced Hepatotoxicity

Oxidative stress results from increased generation of reactive oxygen species (ROS) and/or reduced antioxidant defence against reactive oxygen species (Halliwell *et al.*, 2004). The administration of VPA is associated with the induction of oxidative stress in various experimental models. Relatively high doses of VPA elevate the hepatic and plasma levels of 15-F_{2t}-isoprostane (Tong *et al.,* 2003) in the rat model, a sensitive marker of lipid peroxidation (Halliwell and Whiteman, 2004). Similar effects are also observed *in vitro* where VPA enhances the production of 15-F_{2t}-isoprostane and 2′,7′-dichlorofluorescein (DCF), an intracellular marker of oxidative stress (Halliwell and Whiteman, 2004), in cultured-rat hepatocytes (Tong *et*

al., 2005b). The effects of VPA on the elevation of 15-F_{2t}-isoprostane in rats are further enhanced by phenobarbital (Tong *et al.,* 2003; Tong *et al.,* 2005c) or attenuated by glucuronidation inhibitors (Tong *et al.,* 2005c), suggesting an apparent association between VPA biotransformation and the induction of oxidative stress.

Similar to its effects in animal models, the administration of VPA for therapeutic purposes is also associated with induction of oxidative stress in human subjects. This is evident in obese children receiving chronic VPA therapy where serum levels of antioxidants (vitamin E) are reduced and the levels of malondialdehyde, a marker of lipid peroxidation, are elevated compared to healthy, age-matched controls (Verrotti *et al.*, 2008). Serum levels of 8-hydoxy-2-deoxyguanosine, a marker of DNA oxidative damage, are also found to correlate with the levels of VPA in children receiving steady state VPA therapy (Schulpis *et al.*, 2006). Likewise, our research group reported an elevation of urinary levels of 15-F_{2t}-isoprostane in children receiving VPA therapy compared to age-matched controls or individuals taking carbamazepine or clobazam (Michoulas *et al.*, 2006). The results of Michoulas et al. (2006) are consistent with the findings in the animal model where VPA, at relatively high doses, also increases the hepatic or plasma 15-F_{2t}-isoprostane levels in the rat (Tong *et al.,* 2003). Unlike the rat model, however, the role of VPA-biotransformation in VPA-induced oxidative stress in humans has not been investigated.

An association appears to exist between the induction of oxidative stress and the VPA-induced hepatotoxicity. This is based on experiments where the supplementation of antioxidants (vitamin E and *N*,*N*′-diphenyl-*p*-phenylenediamine) attenuated the VPA-induced cytotoxicity in cultured rat hepatocytes (Buchi *et al.*, 1984), and that the elevation of 15-F_{2t}-isoprostane preceded the onset of hepatic steatosis or necrosis in rats chronically administered high doses of VPA (Tong *et al.*, 2005a). Buchi *et al.* (1984) did not use a marker of oxidative stress to correlate to cytotoxicity, and a cause-effect relationship between VPA-induced lipid

peroxidation and hepatotoxicity was not determined by Tong *et al.,* (2005a). Thus, the association between VPA-induced oxidative stress and hepatotoxicity has not been systematically studied.

1.6 Oxidative or Nitrosative Stress – General Concepts

Oxidative or nitrosative stress is defined as an "imbalance between production of reactive species and antioxidant defence" (Halliwell and Whiteman, 2004). In essence, oxidative or nitrosative stress can result from increased generation of reactive species and / or reduced detoxification of reactive species. Reactive oxygen or nitrogen species are non-specific terms which incorporate non-radicals such as hydrogen peroxide (H_2O_2) or peroxinitrite ($ONOO^-$), and free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals ($OH^\bullet$), hydroperoxal ($HO_2^\bullet$), or nitric oxide ($NO^\bullet$) (Halliwell and Whiteman, 2004). The free radicals are capable of causing direct damage to cellular macromolecules, whereas the non-radicals typically mediate damage indirectly by modulating cellular redox signalling pathways (Jones, 2008).

The administration of VPA is associated with the induction of oxidative stress (as reviewed by Chang and Abbott, 2006), but the identities of reactive oxygen species involved have not been determined. In an *in vitro* system consisting of phenobarbital-induced rabbit microsomes, the generation of hydroxyl radical ($OH^\bullet$) from the administration of VPA has been hypothesized to induce cytotoxicity in cultured-human lymphocytes (Tabatabaei *et al.*, 1999). In cultured-rat hepatocytes (Tong *et al.,* 2005), VPA treatment increases the production of 2′,7′-dichlorofluorescin (DCF) in cells exposed to 2′,7′-dichlorofluorescin diacetate (DCFDA), but the latter is a non-specific marker of stress in that it can be oxidized by a variety of reactive oxygen or nitrogen species such as H_2O_2 (in the presence of a peroxidase), $OH^\bullet$, or $ONOO^-$ (Lebel *et al.*, 1992; Myhre *et al.*, 2003). A direct approach, such as electron spin resonance, to

determine the identity of reactive species involved in VPA-induced oxidative or nitrosative stress may be warranted.

The sub-cellular origin(s) of VPA-associated oxidative or nitrosative stress have also not been determined. Reactive oxygen or nitrogen species are generated by multiple sources in the cell. For example, the superoxide anion is produced by NADH oxidases (NOX), xanthine oxidases, and enzyme complexes constituting the mitochondrial respiratory chain (Lambert and Brand, 2009; Glantzounis *et al.*, 2005; Sanders *et al.*, 1997; Badwey *et al.*, 1979); hydrogen peroxide can be generated by lipooxygenases or microsomal enzymes (O'Donnell and Azzi,1996; Rashba-Step and Cederbaum, 1994); whereas nitric oxide is a product of nitric oxide synthases (Wink and Mitchell, 1998). In any case, it is commonly believed that NOX and the mitochondrial respiratory chain are the major sources of reactive species production (Lambeth *et al.*, 2008; Chance *et al.*, 1979). The roles of these enzyme systems in VPA-associated oxidative or nitrosative stress have yet to be studied in a systematic manner.

In order to maintain a balanced redox state, several cellular enzymes are capable of detoxifying reactive oxygen or nitrogen species. With respect to reactive oxygen species, superoxide anion is converted to hydrogen peroxide by superoxide dismutase (Bannister *et al.*, 1987). Subsequently, the hydrogen peroxide is converted to H_2O and O_2 by glutathione peroxidase, a reaction that requires glutathione, or by catalase (Margis *et al.*, 2008; Percy, 1984; Fourquet *et al.*, 2008). Hydrogen peroxide, if not removed, can undergo iron-catalyzed conversion to form the hydroxyl radical (Ercal *et al.*, 2001; Starke *et al.*, 1985), which is capable of causing direct damage to macromolecules such as proteins, fatty acids, and DNA (Valko *et al.*, 2007). In addition to the antioxidant enzymes, molecules such as vitamin E and vitamin C also make up critical components of a cell's defence, as they disrupt the free radical chain reaction and are able to attenuate the free radical-associated oxidative damage (Jacob and Burri,1996; Sies *et al.*, 1992). Due to the abundance of detoxification enzymes and chain-breaking

antioxidant molecules, it is hypothesized that free radical species (e.g. $O_2^{\bullet -}$, $NO^{\bullet}$) are efficiently converted to non-radical species (e.g. H_2O_2, $ONOO^-$), and the latter is largely responsible for mediating the oxidative stress (Jones, 2008).

As it has been suggested that hydrogen peroxide is the predominant non-radical reactive species found in the cell (Jones, 2008), the following discussion will focus on the role of H_2O_2 – mediated oxidative stress and redox signalling. Specifically, redox systems that regulate H_2O_2-associated oxidative stress will be discussed and examples of H_2O_2-mediated redox signalling will be provided. With this in context, one should keep in mind that non-oxygen radicals (e.g. $NO^{\bullet}$) other than H_2O_2 are also involved in cellular stress-signalling (reviewed in Droge, 2002; Valko *et al.,* 2007; Biswas *et al.*, 2006).

1.6.1 H_2O_2-Induced Oxidative Stress and Redox Signalling

Hydrogen peroxide (H_2O_2) is considered a non-radical reactive species as the molecule does not contain an unpaired electron. It has been estimated that up to 4 % of oxygen is transformed to H_2O_2 by the mitochondria and the peroxisomes in a constitutive manner (Chance *et al.,*1979). Hydrogen peroxide is able to oxidize the thiol-moiety on cysteine or methionine residues resulting in the formation of sulfenic acid (protein-R-SOH). The unstable protein sulfenic acid either reacts with a free thiol to form a disulfide bond, a process termed S-glutathionylation, or is further oxidized by H_2O_2 to form the sulfinic acid R-S(O)OH and sulfonic acid R-$S(O)_2$-OH, in a consecutive manner (Bindoli *et al.*, 2008; Jones, 2008). In addition to oxidizing free thiol moieties, H_2O_2 also oxidizes disulfide bonds to form the thiosulfinate (R-S(O)-S-R), thiosulfonate ($RS(O)_2$-S-R, and disulfone (R-$S(O)_2$-$S(O)_2$-R) (Bindoli *et al.,* 2008).

The oxidation of protein thiols and / or the activation of redox sensor systems (to be discussed below) are the primary mechanisms by which H_2O_2 acts as a signal transducer.

Hydrogen peroxide-mediated disulfide bond formation or *S*-glutathionylation of functional cysteine residues in the active site of an enzyme could change the protein conformation and turn the enzyme "on" or "off" (Jones, 2008). On the other hand, oxidation of cysteine residues outside of the active site can also lead to alteration of enzyme function, in an allosteric manner (Jones, 2008; Valko *et al.,* 2007). An example of an enzyme that contains functional cysteine residues in the active site and that is sensitive to H_2O_2- associated redox control is glyceraldehyde 3-phosphate dehydrogenase (Mohr *et al.*, 1999). An example of an enzyme consisting of a functional cysteine residue that could lead to allosteric modulation is NADH dehydrogenase (Taylor *et al.*, 2003). Similar to hydrogen peroxide-mediated *S*-glutathionylation reaction, reactive nitrogen species such as nitric oxide are also capable of oxidizing protein thiols, in a process termed *S*-nitrosylation (Droge, 2002; Valko *et al.,* 2007; Biswas *et al.,* 2006).

Two distinct redox systems work in collaboration with H_2O_2 to mediate the transduction of oxidative stress-associated signals. Both the glutathione and thioredoxin systems are capable of detoxifying reactive species (e.g. H_2O_2) and acting as redox regulators (Holmgren *et al.*, 2005; Valko *et al.,* 2007). A difference between the two systems is that glutathione, a tripeptide, is found at a higher concentration in the cell compared to thioredoxin (Holmgren *et al.,* 2005). The glutathione system consists of glutathione, in reduced (GSH) or oxidized (GSSG) form; glutathione reductase, which catalyzes the reduction of oxidized glutathione; glutathione peroxidase, which detoxifies hydrogen peroxide; and glutaredoxins, which catalyze the reversible *S*-glutathionylation of proteins (Schafer and Buettner, 2001; Mieyal *et al.*, 2008; Bindoli *et al.,* 2008). In an oxidized environment, the glutathione peroxidase utilizes the reducing power of GSH to detoxify excess H_2O_2, resulting in an increase in the GSSG:GSH ratio. A high GSSG:GSH ratio signals the glutaredoxins to catalyze the *S*-glutathionylation of proteins, which, through mechanisms discussed above, controls enzyme function. The GSSG:GSH ratio can be restored by glutathione reductase, consuming NADPH in the process.

On the other hand, a low GSSG:GSH ratio in a reduced environment signals the glutaredoxins to catalyze the reduction of protein disulfides, resulting in the restoration of free protein thiols.

The thioredoxin system works in a similar fashion to that of glutathione. The system consists of thioredoxin, in reduced or oxidized forms; thioredoxin reductase, which catalyzes the reduction of oxidized thioredoxin; and peroxiredoxins, which are responsible for the detoxification of hydrogen peroxide (Holmgren, 1985; Arner and Holmgren, 2000). In an oxidized environment, the peroxiredoxins utilize the reducing power of thioredoxin to detoxify H_2O_2, resulting in an elevation of oxidized thioredoxin. The oxidized thioredoxin is reduced by thioredoxin reductase, consuming NADPH in the process. Thioredoxin acts as a signal transducer by two primary mechanisms: serving as a reducing agent or directly binding to proteins (Rhee *et al.*, 2005; Bindoli *et al.,* 2008; Jones, 2008). For example, thioredoxin reduces the redox factor-1 (REF1) and binds to the apoptosis signal-regulating kinase-1 (ASK-1), which are responsible for maintaining the transcription factors (e.g. NF-kB) in a state for optimal DNA binding and inhibiting cell death via apoptosis, respectively (Liu *et al.*, 2006; Matthews *et al.*, 1992).

1.7 Role of Hepatic Glutathione Homeostasis in VPA-Induced Hepatotoxicity

Glutathione (GSH) is an important cellular defence against reactive oxygen species or electrophilic xenobiotic intermediates (Meister, 1983; Meister, 1994). VPA depletes hepatic GSH *in vivo* in rats (Cotariu *et al.*, 1990; Seckin *et al.*, 1999) and *in vitro* in rat hepatocytes (Jurima-Romet *et al.,* 1996; Klee *et al.*, 2000). In humans, indirect proof that VPA has an effect on hepatic homeostasis is obtained from the identification of *N*-acetylcysteine (NAC) metabolites of VPA in the urine of human subjects administered VPA (Kassahun *et al.,* 1991; Gopaul *et al.,* 2000b; Gopaul *et al.,* 2000a; Gopaul *et al.,* 2003). The presence of these NAC

metabolites supports the notion that VPA is bioactivated to reactive metabolites that are capable of covalent binding to hepatic GSH.

The ability of VPA metabolites to conjugate with GSH was initially demonstrated by Kassahun et al. (1991) where 5-(glutathione-S-yl)-3-ene-VPA and 5-(*N*-acetylcystein-*S*-yl)-3-ene-VPA were found in the bile and urine, respectively, of rats administered 4-ene-VPA and (*E*)-2,4-diene-VPA. Subsequent experiments further identified other GSH conjugates of 4-ene-VPA (Kassahun *et al.,* 1994) or (*E*)-2,4-diene-VPA (Kassahun *et al.,* 1994; Tang and Abbott F.S., 1996a), as well as their corresponding NAC metabolites (Kassahun *et al.,* 1991; Kassahun *et al.,* 1994; Gopaul *et al.,* 2000b). Approximately 20% or 40% of the administered 4-ene-VPA or (*E*)-2,4-diene-VPA, respectively, is converted to GSH conjugated metabolites (Kassahun *et al.,* 1991; Kassahun *et al.,* 1994). Consistent with the notion that GSH conjugate formation can result in the depletion of hepatic GSH, 4-ene-VPA treatment was shown to reduce the levels of hepatic GSH in rats (Kassahun *et al.,* 1994) and in cultured rat hepatocytes (Jurima-Romet *et al.,* 1996). The effects of the other VPA metabolites on the depletion of hepatic GSH, however, remain unknown.

In support of an association between abnormal GSH homeostasis and VPA-induced hepatotoxicity, elevated VPA-NAC metabolite levels are reported in the urine of human subjects who have developed VPA-induced hepatotoxicity, and in children who possessed the risk factors of VPA-induced liver injury (Kassahun *et al.,* 1991; Gopaul *et al.,* 2003). An association between glutathione depletion and oxidative stress induction is also evident in an *in vitro* study where the effects of VPA on the production of 15-F_{2t}-isoprostane and 2′,7′-dichlorofluorescein (DCF) are enhanced in glutathione deficient rat hepatocytes (Tong *et al.,* 2005b). However, the relationships between the depletion of glutathione and the manifestation of various pathophysiological conditions associated with VPA-induced liver toxicity have not been systematically studied. To address this issue, further experiments were conducted to examine

the role of glutathione homeostasis in VPA-induced hepatotoxicity. The results of these experiments are presented in chapter 3 of the present thesis.

1.8 The Use of Cultured Hepatocytes to Investigate VPA-induced Hepatotoxicity

Rat hepatocytes in suspension are commonly used to investigate the metabolism of VPA (Aires *et al.,* 2007; Porubek *et al.*, 1988; Shirley and Baillie 1993; Porubek *et al.,* 1989) or the effects of VPA on the various metabolic processes of hepatocytes (Luis *et al.*, 2007; Silva *et al.,* 2001; Coude *et al.,* 1983b;Turnbull *et al.,* 1983; Bellringer *et al.*, 1988; Coude *et al.*, 1983a). However, the majority of the literature on the use of hepatocytes to investigate VPA-induced toxicity has been conducted in primary culture (Takeuchi *et al.*, 1988;Takeuchi *et al.,* 1988a; Vance *et al.*, 1994; Kingsley *et al.*, 1980; Kingsley *et al.*, 1983a; Buchi *et al.,* 1984; Costa *et al.*, 2004; Jurima-Romet *et al.,* 1996; Tong *et al.,* 2005b; Singh *et al.*, 1987b; Fujimura *et al.*, 2009; Rogiers *et al.*, 1992), and a summary of these studies is presented in Table 1-1. The toxicity data obtained were inconsistent between the different laboratories and it is not clear which hepatocyte culture conditions were optimal for the toxicity testing of VPA. The following short review will attempt to discuss the benefits and limitations of cultured hepatocytes for the toxicity testing of xenobiotics and identify the current "best practices" for maintaining hepatocytes in culture.

Primary cultures of parenchymal hepatocytes are suitable for the examination of metabolism-mediated, chemical-induced toxicity because they contain the complements of oxidative, conjugative, and transporter enzymes (Tuschl *et al.*, 2008; Dambach *et al.*, 2005; Farkas *et al.*, 2005; Hewitt *et al.*, 2007). When properly cultured, *in vitro* parenchymal hepatocytes maintain the differentiated characteristics and the morphologies of the native hepatocytes found *in vivo* in the liver; thus cultured-hepatocytes are suitable for the mechanistic investigations of drug-induced liver toxicity (Tuschl *et al.,* 2008; Dambach *et al.,* 2005; Farkas and Tannenbaum, 2005; Hewitt *et al.,* 2007). The use of cultured-hepatocytes also negates the

large amount of experimental subjects or quantity of drugs typically used in toxicology experiments *in vivo*. The potential reduction of experimental materials means that cultured-hepatocytes are suitable for high-throughput toxicity screening of large libraries of novel xenobiotics.

On the other hand, the intricacies of the whole organism could never be reproduced by cultured-hepatocytes, and the toxicity data derived from the *in vitro* model may not always correlate with the *in vivo* model. Unlike the *in vivo* situation, where the administered drug or the generated metabolites are promptly eliminated into the bile, blood, or urine, such clearance processes are not in place in *in vitro* cultures of hepatocytes. Furthermore, other cells in the liver, such as the non-parenchymal endothelial or Kupffer cells, may sometimes mediate the drug-induced hepatocyte toxicity (e.g. Edwards *et al.*, 1993; Nastevska *et al.*, 1999); therefore *in vitro* culture systems that do not contain these non-parenchymal cells may generate false negative toxicity findings in these instances. Hepatocytes in culture also tend to lose the differentiated characteristics, cell morphology, and viability during isolation or over extended culture time. With respect to the metabolism enzymes, the constitutive Phase I metabolic capacity is typically reduced during the isolation process, (Kocarek *et al.*, 1993; Richert *et al.*, 2002), whereas the Phase II conjugative enzymes are mostly unaffected or over expressed while the cells are in culture (Richert *et al.,* 2002; Kern *et al.*, 1997). This may lead to over expressed or false negative toxicity data of drugs that require these bioactivation or detoxification enzymes.

1.8.1 Culture Conditions that Preserve Hepatocyte Morphology, Viability, and Function

Although some of the limitations discussed above are inherent to any *in vitro* model, certain limitations, such as loss of metabolism capacity, liver specific function, or cell morphology can be overcome by appropriate culture conditions. The typical parameters that are

modulated to preserve the hepatocyte culture are the medium composition, plating density, and extracellular matrix composition (Hewitt *et al.,* 2007). The addition of fetal bovine serum to the culture medium facilitates the attachment of hepatocytes to the basement membrane (Williams *et al.*, 1977), whereas the omission of serum, epidermal growth factor or the addition of dexamethasone (e.g. 0.1 μM) in the subsequent culture medium help maintain hepatocyte morphology, viability, and metabolism capacity (Ching *et al.*, 1996; Greuet *et al.*, 1997; Yamada *et al.*, 1980; Laishes and Williams 1976; Grant *et al.*, 1985; Tuschl and Mueller 2006). Moreover, plating the hepatocytes at a high density (i.e. > 90 % confluency) and culturing hepatocytes between two layers of extracellular matrix also preserve the liver-like cell morphology and expression of metabolism enzymes (Dunn *et al.*, 1989; Dunn *et al.*, 1991; Dunn *et al.*, 1992; Tuschl and Mueller, 2006; Sidhu *et al.*, 1993; Ben-Ze'ev *et al.*, 1988; Oda *et al.*, 2008; Berthiaume *et al.*, 1996). The beneficial effects of the sandwich culture have been studied extensively, thus a review of the literature with respect to the rodent model, the animal species used in the present thesis, is provided.

1.8.2 The Use of Sandwich-Culture to Preserve Rat Hepatocyte Morphology, Viability, and Function

Rat hepatocytes cultured between two layers of extracellular matrix (i.e. the sandwich configuration) maintain the polarity and the differentiated characteristics of cells typically found in the native liver (Dunn *et al.,* 1989; Dunn *et al.,* 1991; Dunn *et al.,*1992; Sidhu *et al.,* 1993; LeCluyse *et al.*, 1999; LeCluyse *et al.*, 1994; Richert *et al.,* 2002; Musat *et al.*, 1993; Moghe *et al.*, 1996). The benefits of the sandwich culture were initially demonstrated by Dunn *et al.* (1989) where rat hepatocytes cultured between two layers of collagen retain the normal cell morphology and specific liver function, as determined by the secretion of albumin, for an extended period of time. In contrast, hepatocytes cultured on a single layer of collagen rapidly adapt to a flattened

morphology and stop producing albumin within a week after isolation (Dunn *et al.,* 1989). The benefit of the sandwich culture was further demonstrated when the ability of rat hepatocytes to secrete albumin, apparently diminished in the single layer culture, could be rescued by the application of the second collagen layer (Dunn *et al.,* 1989). Other benefits for culturing rat hepatocytes in the sandwich configuration are the formation of functional bile canalicular networks (Tuschl and Mueller, 2006; LeCluyse *et al.,* 1994), preservation of basal Phase I and II metabolism activities (Kern *et al.,* 1997; Mingoia *et al.*, 2007), and preservation of xenobiotic-mediated induction responses of biotransformation enzymes (Sidhu *et al.,* 1993; LeCluyse *et al.,* 1999; Mingoia *et al.,* 2007; Oda *et al.,* 2008).

Matrigel™, a basement membrane derived from the Engelbreth-Holm-Swarm mouse tumor, and type I collagen are the most widely used extracellular matrices for culturing hepatocytes (Farkas and Tannenbaum, 2005). Single layer Matrigel™ is superior to collagen with respect to the maintenance of normal cellular morphology or hepatocyte function (Oda *et al.,* 2008; Ben-Ze'ev *et al.,* 1988; Yoshida *et al.*, 1996; Oda *et al.*, 1995). However, only a few studies have compared the effects of Matrigel to that of collagen in the sandwich configuration. Supporting an advantage of Matrigel over that of collagen, Moghe et al. (1996) demonstrated that sandwich-cultured rat hepatocytes containing Matrigel are capable of expressing cellular proteins (connexion, EGF receptor, and F-actin) that are required for the polarization of hepatocytes, whereas such proteins are absent in rat hepatocytes cultured between two layers of collagen (Moghe *et al.,* 1996). Moreover, rat hepatocytes cultured between two layers of Matrigel also appear rounded, in contrast to the flattened appearance of cells in the collagen bi-layer (Moghe *et al.,* 1996). On the other hand, the rates of albumin secretion, a specific function of hepatocytes, are similar between rat hepatocytes sandwiched between Matrigel or collagen (Moghe *et al.,* 1996), and there is no advantage for culturing rat hepatocytes between Matrigel / collagen over that of collagen / collagen with respect to the responsiveness to phenobarbital

induction (LeCluyse *et al.,* 1999). Nevertheless, from the limited studies that have been published, it would appear that the Matrigel™ is better than collagen for the *in vitro* culture of rat hepatocytes.

Overall, hepatocytes cultured in various conditions have been used for the investigation of VPA-induced toxicity (Table 1-1), although it is not clearly evident based on these studies which culture conditions are optimal for the toxicity testing of VPA. As such, rat hepatocytes in the current thesis were cultured based on the parameters reviewed in Section 1.7 that are known to preserve hepatocyte function: Matrigel™ sandwich, high plating density, the addition of 10 % fetal bovine serum during attachment, and the omission of fetal bovine serum or epidermal growth factor during the subsequent culture period.

1.9 Research Rationale and Hypothesis

VPA is a broad-spectrum anticonvulsant that is also indicated for the management of various neurological disorders. VPA is relatively safe, although in very rare instances it may be associated with severe, life threatening hepatotoxicity. The mechanism(s) of VPA-induced hepatotoxicity remain unknown. Although the involvement of mitochondrial dysfunction, oxidative stress, or abnormal glutathione homeostasis have been suggested, the relationships between these events have not been systematically studied. VPA is also extensively metabolized, and the roles of biotransformation pathways or toxic metabolites in the VPA-induced hepatotoxicity have been the focus of research in our laboratory as well as many others for more than three decades. Despite the significant amounts of knowledge gained, no clear consensus has been reached on the contribution of putative toxic metabolites or metabolic pathways to VPA-induced hepatotoxicity.

The present thesis tested the hypotheses that select VPA metabolites are relatively more toxic than VPA, and the *in situ* generation of toxic or reactive VPA metabolites is associated

with altered markers of VPA-induced hepatotoxicity in cultured-rat hepatocytes and humans. As well, we hypothesized that cause-effect relationships exist between the various markers of VPA-induced hepatotoxicity. Specifically, the relationships between VPA-associated glutathione depletion and other markers of VPA-associated hepatotoxicity were characterized in a single experimental setting. Finally, with respect to the metabolism of VPA in humans, the hypothesis was that CYP2C9 was the principal enzyme responsible for the oxidative metabolism of VPA in human liver microsomes.

1.10 Research Objectives

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

Chapter 2:

i) To determine the relative effects of synthesized VPA metabolites on mitochondrial dysfunction, cytotoxicity, and oxidative stress in Matrigel™ sandwich-cultured rat hepatocytes (SCRH).

ii) To examine the contribution of biotransformation pathways or *in situ* generated VPA metabolites in VPA-induced hepatocyte toxicity.

Chapter 3:

i) To determine the relative effects of synthesized VPA metabolites on the depletion of total hepatic glutathione in SCRH.

ii) To characterize the relationships between the reduction of GSH levels, mitochondrial dysfunction, cytotoxicity, and oxidative stress associated with VPA treatment in SCRH.

iii) To examine the roles of biotransformation pathways or *in situ* generated VPA metabolites in the depletion of glutathione in SCRH treated with VPA.

Chapter 4:

i) To determine the association between specific VPA metabolites or VPA metabolism pathways and the elevation of lipid peroxidation in the urine of pediatric patients receiving therapeutic doses of VPA.

Chapter 5:

i) To determine the relative contributions of specific CYP enzymes to the oxidative metabolism of VPA in human liver microsomes

Table 1-1. A Summary of the Use of Cultured-Rat Hepatocytes for the Investigation of VPA-induced Toxicity

Rat Strain	Culture Conditions	Toxicity Findings	Reference
Male Sprague Dawley	**Cell density and ECM:** 1 million cells in 60 x 15 mm^2 plastic petri dishes. **Culture medium:** Eagle's Essential Medium. Additives not specified. **Treatment:** 24 hours of incubation followed by VPA treatment (0 – 1920 µg / mL) for up to 16 hours.	• VPA (320 µg / mL) increased LDH release in a time-dependent manner. • At 4 hours of treatment, VPA increased LDH release in a dose-dependent manner. • The co-administration of phenobarbital, phenytoin, primidone, or clonazepam did not enhance the VPA-induced LDH release.	(Kingsley *et al.*, 1980)
Male Sprague Dawley	**Cell density and ECM:** 1 million cells in 60 x 15 mm^2 plastic petri dishes. **Culture medium:** Eagle's Essential Medium. Additives not specified. **Treatment:** 24 hours of incubation followed by treatments of various concentrations of VPA, 4-OH-VPA, 4-ene-VPA, and 3-OH-VPA for 8 hours.	• Apparent concentration-dependent increase in LDH release was observed with 4-ene-VPA and VPA treatment. • The relative orders of LDH release from treatments of equal molar concentrations of VPA and metabolites were: VPA > 4-ene-VPA > 4-OH-VPA > 5-OH-VPA = 3-OH-VPA.	(Kingsley *et al.*, 1983)
Male Sprague Dawley	**Cell density and ECM:** 1 million cells in 60 x 15 mm^2 plastic petri dishes. **Culture medium:** Eagle's Essential Medium. Additives not specified. **Treatment:** 24 hours of incubation followed by VPA treatment (500 µg / mL) for 6 hours.	• Vitamin E or *N,N′*-diphenyl-*p*-phenylenediamine attenuated the VPA-induced LDH release in a concentration-dependent manner.	(Buchi *et al.*, 1984)

Rat Strain	Culture Conditions	Toxicity Findings	Reference
Male Sprague Dawley	**Cell density and ECM:** 2.5 million cells on collagen coated 75 cm^2 dishes. **Culture medium:** William's E Medium supplemented with 5 µM of dexamethasone. **Treatment:** 24 hours of culture followed by up to 72 hours of treatment with VPA (0.1 – 3 mM).	• VPA increased the activities of carnitine acetyltransferase, levels of total cellular glutathione, and the number of mitochondria in a concentration- and time-dependent manner. • VPA did not affect the levels of peroxisomal proteins.	(Singh *et al.*, 1987)
Male Wistar	**Cell density and ECM:** not specified **Culture medium:** not specified **Treatment:** 24 hours of culture followed by up to 24 hours and up to 5 mM of VPA treatment.	• VPA increased LDH, glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) activities in a time-dependent manner. VPA increased LDH release in a dose-dependent manner. • D,L-carnitine and albumin attenuated the effects of VPA on the LDH, GOT, and GPT markers.	(Takeuchi *et al.,* 1988)
Male Wistar	**Cell density and ECM:** not specified **Culture medium:** not specified **Treatment:** 24 hours of culture followed by treatments up to 24 hours with 0.1 or 1 mM VPA.	• VPA increased the ammonia concentration and decreased the ketone bodies in rat hepatocytes in a dose-dependent manner. • D,L-carnitine attenuated the effects of VPA on these toxicity markers.	(Takeuchi *et al.,* 1988a)

Rat Strain	Culture Conditions	Toxicity Findings	Reference
Male Wistar	**Cell density and ECM:** 1.5 x 10^6 hepatocytes co-cultured with 2x 10^6 epithelial cells per 25 cm^2 flask. **Culture medium:** Ham F12 medium supplemented with hydrocortisone hemisuccinate (3.5 x 10^{-6} M). The attachment medium contained the fetal bovine serum (% not specified). The basement membrane was not specified. **Treatment:** 3 hours of culture, followed by VPA treatment (25, 50, 100, and 200 µg / mL) up to 14 days.	• VPA increased the cytochrome P450 content and ECOD activity in a dose- and time- dependent manner • VPA decreased glutathione-*S*-transferase activity in a dose- and time- dependent manner.	(Rogiers *et al.*, 1992)
Male Sprague Dawley	**Cell density and ECM:** 1 million cells / mL, 2.5 mL plated onto 60 x 15 mm plastic petri dished**.** The basement membrane was not specified. **Culture medium:** Eagle's Minimum Essential Medium. No additives specified. **Treatment:** 24 hours of culture followed by 3, 6, and 18 hours of VPA treatment (0 – 19.2 mM).	• VPA increased LDH release and decreased oxidation of ^{14}C palmitate in a dose-dependent manner. • Glycine attenuated the effects of VPA on LDH release. • L-carnitine, L-alanine, L-cysteine did not attenuate the VPA-induced LDH release.	(Vance *et al.*, 1994)

Rat Strain	Culture Conditions	Toxicity Findings	Reference
Male Fisher 344	**Cell density and ECM:** 1 million viable hepatocytes per 25 cm^2 collagen-coated culture flask. **Culture medium:** William's E medium supplemented with 5 % fetal bovine serum during attachment. Other additives were not specified. **Treatment:** 2 hours of attachment period followed by 20 hours of treatment with VPA, 2-ene-VPA, 4-ene-VPA, or (*E,Z*)-2,3′-diene-VPA (up to 20 mM).	• VPA, 2-ene-VPA, and (*E,Z*)-2,3′-diene-VPA increased LDH release in a dose-dependent manner in normal, phenobarbital-induced, or pregnenolone-16-α-carbonitrile-induced hepatocytes. The 4-ene-VPA was not toxic (up to 20 mM) in these culture conditions. • The depletion of glutathione enhanced the effects of 2-ene-VPA, (*E,Z*)-2,3′-diene-VPA, and 4-ene-VPA on LDH release in all culture conditions. • VPA decreased the levels of total cellular glutathione in a dose-dependent manner. • Vitamin E and vitamin C attenuated the effects of 4-ene-VPA on the release of LDH in glutathione-depleted hepatocytes.	(Jurima-Romet *et al.*, 1996)
Male Sprague Dawley	**Cell density and ECM:** 0.75 million cells / mL in "multiwell"dishes. **Culture medium:** RPMI-1640 medium supplemented with 10% fetal bovine serum during attachment and 10 nM dexamethasone. **Treatment:** 30 minutes of culture, followed by 12 hours of VPA treatment (up to 300 µg/mL)	• VPA did not affect the MTT, thiobarbituric acid reactive substances, or aspartate transaminase markers up to 300 µg/mL. • VPA increased LDH release (slightly) at 150 and 300 µg/mL.	(Costa *et al.*, 2004)

Rat Strain	Culture Conditions	Toxicity Findings	Reference
Male Sprague Dawley	**Cell density and ECM:** 4 x 10^4 cells / well (96 well plate) or 2 x 10^5 cells / well (24 well plate). Single layer Matrigel™ basement membrane. **Culture medium:** Hepato-Stim media supplemented with 10% fetal bovine serum during attachment. Hepatozyme-SFM for the subsequent culture. No additives were specified. **Treatment:** 2 hours of attachment followed by various treatment durations (up to 8 hours) and concentrations of VPA (up to 1000 µg/mL).	• VPA increased DCF and 15-F_{2t}-isoprostane levels in a dose- and time- dependent manner. • VPA further increased the DCF and 15-F_{2t}-isoprostane levels in GSH-depleted rat hepatocytes. • VPA (up to 1000 µg/mL) had little effects on WST-1 product formation after 8 hours of treatment.	(Tong *et al.*, 2005)
Male Sprague Dawley	**Cell density and ECM:** 4 x 10^4 cells per mL on 24 well FIA glass bottom plate sandwiched between two layers of Matrigel™ (50 µg / mL). **Culture medium:** William's E medium supplemented with 10 % fetal bovine serum during attachment and 0.1 µM dexamethasone. **Treatment:** 48 hours of culture followed by 24 hrs of treatment with 3 mM VPA.	• VPA increased the fluorescence intensity of BODIPY558/568 C_{12}, a marker of hepatic steatosis.	(Fujimura *et al.*, 2009)

ECM (extracellular matrix); DCF (2′,7′-dichlorofluorescein); GOT (glutamate oxaloacetate transaminase), GPT (glutamate pyruvate transaminase); LDH (lactate dehydrogenase); and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).

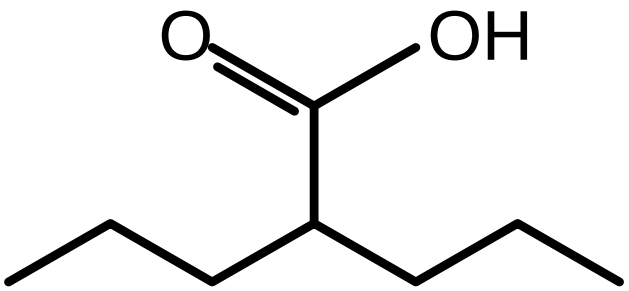

Figure 1-1.The chemical structure of valproic acid (VPA, 2-n-propyl-pentanoic acid), a branched, 8-carbon carboxylic acid.

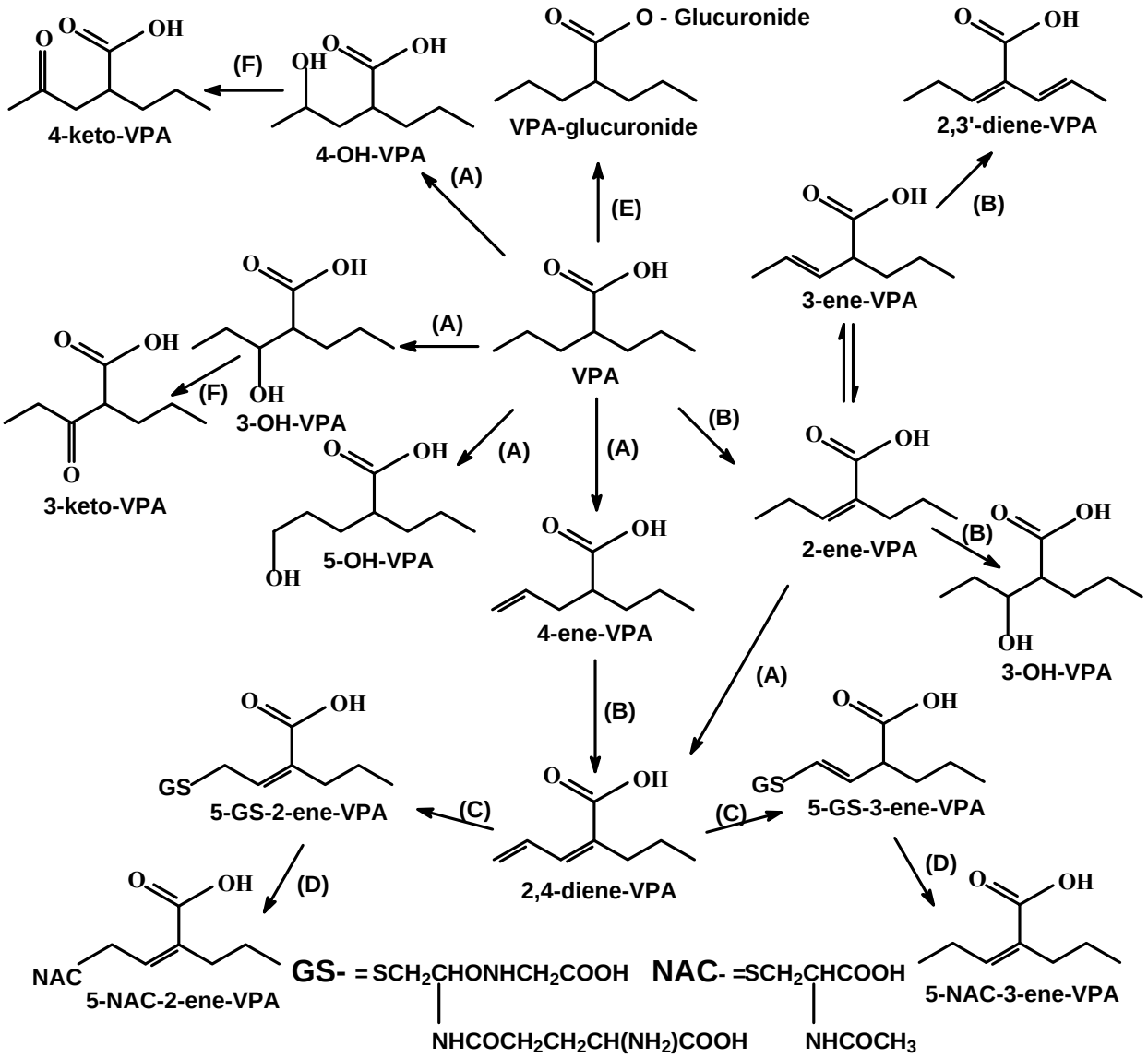

Figure 1-2. A summary of the common VPA metabolites and VPA biotransformation pathways in humans. (A) CYP oxidation, (B) ß-oxidation, (C) GSH conjugation, (D) mercapturic acid pathway, (D) glucuronidation, (F) alcohol dehydrogenation. (*E*)- and (*Z*)-isomers may exist for 2-ene-VPA, 3-ene-VPA, and 2,4-diene-VPA. (*E,E*)- and (*E,Z*)-isomers may exist for 2,3´-diene-VPA. Reproduced from Figure 1 in Chapter 4 in the present thesis.

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2 Role of Metabolism in Valproic Acid-Induced Mitochondrial Dysfunction, Cytotoxicity, and Oxidative Stress in Sandwich-Cultured Rat Hepatocytes[1]

2.1 Introduction

Valproic acid (VPA) is a broad spectrum anticonvulsant that is also indicated in the management of migraine headache, nerve pain, and bipolar disorder (Johannessen and Johannessen 2003). Despite its usefulness, the administration of VPA can be associated with a rare, but serious form of hepatotoxicity (Zimmerman and Ishak 1982; Kuhara *et al.*, 1990; Konig *et al.*, 1994; Bryant and Dreifuss 1996). The incidence of the idiosyncratic liver toxicity is highest in young children (< 2 years old) who are also taking concurrent enzyme-inducing anticonvulsants (Dreifuss *et al.*, 1989; Dreifuss *et al.*, 1987; Bryant and Dreifuss, 1996). The mechanism(s) behind VPA-induced hepatotoxicity remain unknown.

VPA is extensively metabolized, and it has been hypothesized that the hepatotoxicity associated with VPA treatment is mediated by one or more of its metabolites (Eadie *et al.*, 1988). Initial interests were placed on the cytochrome P450 (CYP)-generated metabolite, 4-ene-VPA, and its subsequent metabolite, (*E*)-2,4-diene-VPA. Research has been done to elucidate the effects of these VPA metabolites on hepatic steatosis (Tang *et al.*, 1995; Loscher *et al.*, 1993; Kesterson *et al.*, 1984), ß-oxidation inhibition (Bjorge and Baillie 1985; Ponchaut *et al.*, 1992; Silva *et al.*, 2001), CYP destruction (Prickett and Baillie 1986), covalent binding (Porubek *et al.*, 1989), and glutathione adduct formation (Tang and Abbott 1996a; Tang *et al.*, 1996c; Kassahun *et al.*, 1991; Kassahun and Abbott 1993; Kassahun *et al.*, 1994).

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More recently, the major phase II metabolite, VPA glucuronide, has generated some interest due to an apparent association between its formation and lipid peroxidation in the rat liver (Tong *et al.*, 2005b). Interestingly, the α-fluorinated analogues of 4-ene-VPA and VPA are resistant to the induction of hepatic steatosis or lipid peroxidation in rats, in contrast to their non-fluorinated counterparts (Tang *et al.,* 1995; Tong *et al.,* 2005b). In light of the fact that the fluorinated analogues could not form the (*E*)-2,4-diene-VPA or significant amounts of the glucuronide, it remains to be established which of these metabolites (i.e. 4-ene-VPA or (*E*)-2,4-diene-VPA vs. VPA-glucuronide) or pathways (i.e. CYP oxidation or β-oxidation vs. glucuronidation) are responsible for the VPA-induced hepatotoxicity.

A thorough review of the literature indicated that in the few reported studies of VPA metabolites, the hepatotoxicity data is far from consistent. Using 4-ene-VPA as an example, the degree of liver injury resulting from this metabolite was found to be not comparable by different investigators using similar experimental conditions (Loscher *et al.,* 1993; Tang *et al.,* 1995). Different studies also reported inconsistent rank orders of toxicity: VPA being more toxic (Jurima-Romet *et al.*, 1996), equally toxic (Kingsley *et al.*, 1983), or less toxic compared to 4-ene-VPA (Kesterson *et al.,* 1984), as a result of the different experimental models and toxicity markers employed. Given the incompleteness and inconsistencies of the literature, it was of interest to further examine the relative toxicities of the various VPA metabolites.

The majority of the studies examining the toxicity of VPA metabolites employed exogenously-added, synthesized metabolites at concentrations exceeding that which would normally be attained endogenously after VPA administration (e.g. Kesterson *et al.,* 1984; Tang *et al.,* 1995; Jurima-Romet *et al.,* 1996; Ponchaut *et al.,* 1992; Kassahun *et al.,* 1994; Loscher *et al.,* 1993). A synthesized metabolite may not exhibit the same pharmacokinetic and toxicity characteristics as the identical metabolite generated endogenously from the parent compound. Within this context, little is actually known whether the *in situ* production of a putative toxic

metabolite (e.g. 4-ene-VPA) is associated with VPA-induced hepatotoxicity. Various clinical studies have also reported abnormal metabolism patterns in individuals who have developed VPA-associated hepatotoxicity (Fisher *et al.*, 1992; Eadie *et al.*, 1990; Siemes *et al.*, 1993; Kuhara *et al.,* 1990; McLaughlin *et al.*, 2000), but it still remains to be established whether the process of biotransformation *per se* is responsible for the liver injury associated with VPA treatment.

Oxidative stress and mitochondrial dysfunction appear to be key markers associated with VPA-induced hepatotoxicity. At relatively high concentrations, VPA induces lipid peroxidation and depletes cellular glutathione in rat liver homogenate (Tong *et al.*, 2003; Cotariu *et al.*, 1990) and in rat hepatocytes (Tong *et al.*, 2005a; Jurima-Romet *et al.,* 1996). Evidence was also obtained in the human situation where therapeutic doses of VPA were associated with lipid peroxidation and oxidative DNA damage (Verrotti *et al.*, 2008; Michoulas *et al.*, 2006; Schulpis *et al.*, 2006). Moreover, VPA is reported to alter the ultrastructure of mitochondria, induce mitochondrial swelling, and to inhibit mitochondrial ß-oxidation (Sobaniec-Lotowska, 1997; Trost *et al.*, 1996; Becker and Harris 1983; Coude *et al.*, 1983; Turnbull *et al.*, 1986). Still, the effects of individual VPA metabolites and the role of VPA biotransformation on the induction of oxidative stress or mitochondrial dysfunction remain to be examined. It is also unknown if the extent of oxidative stress or mitochondrial dysfunction are related to the severity of the hepatotoxicity.

The present study investigated the dose-dependent effects of VPA on markers of oxidative stress (2′,7′-dichlorofluorescein), mitochondrial function (WST-1), and cytotoxicity (lactate dehydrogenase) in rat hepatocytes cultured in a sandwich configuration between two layers of Matrigel™. The effects of synthesized VPA metabolites, 4-ene-VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 4-keto-VPA, and VPA-glucuronide, or α-F-VPA on the selected toxicity markers were examined in comparison to VPA.

Furthermore, correlations between the extent of oxidative stress, mitochondrial dysfunction, and cytotoxicity were established for the first time using the data obtained from the treatment of rat hepatocytes with VPA and VPA metabolites. Finally, to study the role of VPA biotransformation in the induction of hepatocyte toxicity, chemical inhibition experiments were designed to attenuate the CYP-mediated oxidation and/or UGT-mediated glucuronidation of VPA. These two metabolic pathways of VPA are associated with either production of putative toxic metabolites (i.e. 4-ene-VPA and (*E*)-2,4-diene-VPA) and/or induction of lipid peroxidation (Tong *et al.,* 2005b).

2.2 Materials and Methods

2.2.1 Chemicals

Amiodarone hydrochloride, 1-aminobenzotriazole, [(1s)-endo]-(-)-borneol (99 %), octanoic acid (caprylic acid, ≥ 99%), fetal bovine serum, dimethyl sulfoxide (≥ 99.5%, GC grade), D-saccharic acid 1,4-lactone (D-saccharolactone), ketoconazole, sodium valproate, *t*-octylphenoxypolyethoxyethanol (Triton X–100), and *tert*-butylhydroperoxide were obtained from Sigma-Aldrich Ltd. (Oakville, ON, Canada). Matrigel™ basement membrane and Hepato-Stim media were obtained from BD Biosciences (Mississauga, ON, Canada). Dichlorofluorescein diacetate (DCFDA), Hepatozyme-serum free media, Penicillin-Streptomycin (10,000 units / mL), and L-glutamine (200 mM) were purchased from Invitrogen Corporation (Burlington, On, Canada). The WST-1 reagent and the LDH Cytotoxicity Detection Kit were obtained from Roche Applied Sciences (Mississauga, On, Canada). Ammonium acetate (HPLC grade), anhydrous ethyl ether (certified A.C.S. grade), ethylenediaminetetracetic acid disodium salt dehydrate (EDTA, ≥ 99 %), *o*-phosphoric acid (85 %), *n*-hexanes (65 %, GC resolve grade), methanol (HPLC grade), and sodium hydroxide (certified A.C.S. grade) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Acetonitrile (HPLC grade) was purchased

from EMD™ chemicals (Darmstadt, Germany). Analogues and metabolites of VPA (Figure 2-1) were synthesized and purified by Dr. Stoyan Karagiozov in our laboratories: VPA-1-*O*-acyl-glucuronide (VPA-G) and [$^2H_6$]-VPA-glucuronide (Tong *et al.,* 2005b); 2-propylpent-4-enoic acid (4-ene-VPA) and (*E*)-2-propylpent-2,4-dienoic acid ((*E*)-2,4-diene-VPA) (Kassahun *et al.,* 1991); 2-fluoro-2-propylpentanoic acid (α-F-VPA) (Tang *et al.*, 1997); (*E*)-2-propylpent-2-enoic acid ((*E*)-2-ene-VPA), (*E*)-2-propylpent-3-enoic acid (3-ene-VPA), 3-hydroxy-2-propylpentanoic acid (3-OH-VPA), 4-hydroxy-2-propylpentanoic acid (4-OH-VPA), 5-hydroxy-2-propylpentanoic acid (5-OH-VPA), and 4-oxo-2-propylpentanoic acid (4-keto-VPA) (Zheng, 1993). Chemicals were deemed suitable for testing only if the purity exceeded 99 %, as determined by liquid chromatography and mass-spectrometry. Briefly, a 10 µL sample (1 µg / mL) solubilized in methanol was injected into a Waters Acquity™ Ultra Performace LC fitted with an Acquity UPLC™ BEH C18 column (100 x 2.1 mm, I.D., 1.7 µm). The mobile phase, delivered at a flow rate of 0.2 mL/min, consisted of water and acetonitrile, containing 0.1 % formic acid. The percentage of ACN was increased linearly from 8 % to 98 % over 15 minutes, then sharply reduced to and held at 2 % for 8 minutes prior to the next injection. Analyte detection was achieved by a Waters Quattro Premier XE Micromass Tandem Mass Spectrometer (Micromass Ltd., Montreal, Canada) under positive and negative MS scan mode between m/z 50 to 600. The capillary (3 KV), cone (20V), and extractor (5V) voltages were optimized for this purpose. Purity was determined by expressing the absolute area count of the analyte as a percentage of the area counts of all detected peaks (i.e. the sum of the analyte peak and the impurity peaks).

2.2.2 Animal Care

Adult male Sprague-Dawley rats were obtained from the Charles River Laboratory and housed in a temperature controlled (22 ºC) room with alternate 12 hour dark and light cycles.

The rat diet (Labdiet 5001 rodent diet, PMI Feeds Inc., Richmond IN) and water were provided *ad libitum*. Rats were anesthetized with sodium pentobarbital (60 mg/kg, ip) prior to the hepatocyte isolation procedure (Tong *et al.,* 2005a) and terminated by the removal of the heart. All animal experiments were approved by the UBC Animal Care Committee.

2.2.3 Hepatocyte Isolation and Culture

Hepatocytes were isolated from male Sprague-Dawley rats (250 – 350 g), according to a previously published protocol (Tong *et al.,* 2005a), and suspended in a concentration of 0.7 million cells / mL in Hepato-Stim media (supplemented with 100 U / mL of penicillin, 100 μg / mL of streptomycin, 2 mM of L-glutamine, and 10 % heat inactivated fetal bovine serum). Hepatocytes (0.56 million cells / well) were seeded onto Matrigel™-coated 12-well culture plates (Costar® 3513, Corning Incorporated) and allowed to attach in the incubator (37 ºC, 5 % CO_2) for 4 hours. The attachment media was subsequently aspirated and replaced with 0.8 mL of Hepatozyme-serum free media (supplemented with 100 U / mL of penicillin, 100 μg / mL of streptomycin, 2 mM of L-glutamine, and 250 μg / mL of Matrigel™). The culture plate was then placed in the incubator (37 ºC, 5% CO_2) for 20 hours to allow the formation of the sandwich culture in a procedure similar to that described by Sidhu et al. (1993).

2.2.4 Drug Treatment

Drug treatments were initiated after the formation of the sandwich culture, 24 hours after the initial plating of the hepatocytes. The medium was aspirated and replaced with drug solutions prepared in the culture medium (Hepatozyme-serum free media supplemented with 100 U / mL of penicillin, 100 μg / mL of streptomycin, 2 mM L-glutamine). The concentrations of the test chemicals, their vehicles, and the exposure periods are indicated in each figure legend.

2.2.5 Chemical Inhibition Experiments

To inhibit the metabolism of VPA, hepatocytes were exposed to VPA with or without 1-aminobenzotriazole (1-ABT, 0.5 mM), ketoconazole (KTZ, 25 μM), or (-)-borneol (BR, 125 μM). Hepatocytes were pre-incubated with 1-ABT (30 minutes), KTZ or BR (for 60 minutes), or the respective vehicle controls. 1-ABT was dissolved in the culture medium, KTZ in methanol (0.5 % v/v), and BR in DMSO (0.5 % v/v). Subsequently, a chemical inhibitor or its vehicle was co-incubated with VPA or the vehicle for 24 hours. Initial experiments determined a non-toxic concentration for each chemical inhibitor for the inhibition of P450 oxidation (1-ABT, KTZ) and glucuronidation (KTZ, BR).

2.2.6 WST-1 Assay

The overall metabolic function of mitochondria was assessed by the WST-1 reagent, which has been employed for this purpose in cultured rat hepatocytes (Kikkawa *et al.*, 2005; Tong *et al.,* 2005a). After drug exposure, hepatocytes were washed with warm (37 ºC) and sterile phosphate buffered saline (pH 7.4) and incubated at 37 ºC (5 % CO_2) with 1 mL of the culture medium containing 50 μL of the stock WST-1 solution. The reduction of the WST-1 reagent to its formazan product was monitored at regular intervals at an absorption wavelength of 450 nm on a Labsystems Multiskan Ascent® Multiwell plate reader (Thermo Electron Corp., Burlington, ON, Canada). To measure mitochondrial function, the rates of the WST-1 formazan product formation were calculated and expressed as percentages of the vehicle-treated control. The blank consisted of the culture medium and an equal amount of the formazan dye. To determine the responsiveness of the WST-1 assay in the current culture model, positive control experiments were conducted with amiodarone, a known mitochondrial toxin (Kikkawa *et al.,* 2005; Wang *et al.*, 2002; de Longueville *et al.*, 2003). Negative control experiments were conducted with octanoic acid, a straight chain, 8-carbon carboxylic acid.

2.2.7 2´,7´-Dichlorofluorescein (DCF) Assay

Oxidative stress was measured by the fluorescence emission of 2´,7´-dichlorofluorescein (DCF), which has been used for this purpose in various cell types, as previously reviewed (Tarpey *et al.*, 2004; Halliwell and Whiteman 2004; Bartosz, 2006). The dichlorofluorescin diacetate (DCFDA) penetrates the cellular membrane and is converted by cellular esterases to form dichlorofluorescin (DCFH). Reactive free radicals (e.g. $OH^{\bullet}$, ONOO-), if present, further convert the non-fluorescent DCFH to the highly fluorescent DCF. After drug exposure, hepatocytes were washed with sterile phosphate buffered saline (pH 7.4) and incubated at 37 ºC (5 % CO_2) with 1 mL of the culture medium containing DCFDA. Preliminary experiments indicated the optimum concentration of DCFDA was 2 µM (Appendix, Figure A-1). The fluorescence emission of DCF was monitored at regular intervals with a Cytofluor Series 4000 multi-well fluorescence plate reader (Applied Biosystems, Bedford CA) at an excitation wave length of 485 nm (slit width of 20 nm) and an emission wavelength of 530 nm (slit width of 25 nm). To measure oxidative stress, the rate of DCF formation was calculated and expressed as a percentage of the vehicle-treated control. The blank consisted of the culture medium in the absence of cells. The amount of DCF (µmole) generated was calculated based on calibration curves contructed using authentic DCF standards (PolySciences Inc, Warrington, PA) To determine the responsiveness of the DCF assay in our culture model, positive control experiments were conducted with *tert*-butylhydroperoxide, an agent known to cause significant oxidative stress in cultured rat hepatocytes (Gomez-Lechon *et al.*, 2003; Lautraite *et al.*, 2003). Negative control experiments were conducted with octanoic acid.

2.2.8 Lactate Dehydrogenase (LDH) Assay

Cytotoxicity was measured by the cellular release of lactate dehydrogenase (Jauregui *et al.*, 1981) with a commercial kit (Roche Applied Sciences, Mississauga, On, Canada). After drug

exposure, an aliquot of the culture supernatant was immediately stored on ice. The hepatocytes were then washed with warm phosphate buffered saline (pH 7.4) and exposed to 1.25 mL of the lysis buffer (2 % Triton X–100 and 20 mM EDTA in phosphate buffered saline, pH 7.4). Cells were covered with the lysis buffer for 2 hours at room temperature prior to being harvested. After scraping, the cell lysate was vortexed and the unwanted debris pelleted by centrifugation (13,000 rpm, 4 ºC, 10 minutes). Subsequently, a 5 µL aliquot of the culture supernatant or the processed cell lysate was added to 95 µL of phosphate buffered saline (pH 7.4, 25 ˚C), and the enzymatic reaction was started by the addition of 100 µL of the reaction mixture (provided by the Cytotoxicity Detection Kit, Roche Diagnostics, Mississauga, On, Canada). The generation of the reaction product was monitored at regular intervals at an absorption wavelength of 492 nm on a Labsystems Multiskan Ascent® Multiwell plate reader (Thermo Electron Corp., Burlington, ON, Canada). The blank consisted of the culture medium and the lysis buffer, for the culture supernatant or the processed cell lysate, respectively. To quantify cytotoxicity, the LDH activity in the culture supernatant was expressed as a percentage of the total LDH activity (i.e. the combination of the LDH activities in the culture supernatant and the cell lysate). The LDH activities were determined at an incubation time where the reaction (i.e. the formation of the formazan product) was still linear. To determine the responsiveness of the LDH assay in our culture model, positive control experiments were conducted with *tert*-butylhydroperoxide, an agent known to induce the cellular release of LDH in rat hepatocytes (Haidara *et al.*, 2002). Octanoic acid was employed as the negative control.

2.2.9 Determination of VPA Metabolite Levels

Levels of VPA metabolites generated *in situ* in the hepatocytes were determined by gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). After drug exposure, an aliquot of the culture supernatant was

added to D-saccharolactone (final concentration = 5 mM), immediately frozen in liquid nitrogen, and stored in the freezer (- 80 ºC) prior to the assays. Levels of (*E,E*)-2,3′-diene-VPA, (*E,Z*)-2,3′-diene-VPA, 4-ene-VPA, 3-ene-VPA, (*E*)-2-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA were determined by a GC/MS assay as described by Kiang *et al.* (2006). Levels of VPA-glucuronide were determined by an LC/MS/MS assay similar to that described by Tong et al. (2005b). Briefly, an assay mixture (1000 µL) containing the culture supernatant, D-saccharolactone, and distilled water was spiked with 40 µL of the internal standard ([$^2H_6$]-VPA-glucuronide, 0.1 mg/mL). The mixture was acidified with 20 µL of o-phosphoric acid and subjected to a single liquid-liquid extraction with a 1:1 mixture of ethyl acetate (4 mL) and anhydrous ethyl ether (4 mL). The organic layer was evaporated (0.5 psi at 25 ˚C, Zymark Turbo Vap LV Evaporator, Zymark, Hopkinton, MA) in the presence of 600 µL of distilled water containing 10 mM ammonium acetate. Subsequently, the remaining aqueous layer was mixed with 200 µL of acetonitrile (ACN) containing 10 mM ammonium acetate. A 10 µL volume of the mixture was then injected into a Waters Acquity™ Ultra Performance LC fitted with an Acquity UPLC™ BEH C_{18} column (100 x 2.1 mm, I.D., 1.7 µm). The mobile phase, delivered at a flow rate of 0.2 mL/min, consisted of a mixture of ACN and water, containing 10 mM ammonium acetate. The percentage of ACN was increased linearly from 10% to 90% over 5 minutes, then sharply reduced to and held at 10% for 3 minutes prior to the next injection. Analyte detection was achieved by a Waters Quattro Premier XE Micromass® Tandem Mass Spectrometer (Micromass Ltd., Montreal, Canada) under the negative electrospray mode. VPA-glucuronide was quantified under multiple reaction monitoring (MRM) of the following transitions (m/z 315.0 → 112.8; 315.0 → 139.1; and 315.0 → 175.1). [$^2H_6$]-VPA-glucuronide was monitored by the following transitions (m/z 325.2 → 112.9; 325.2 → 149.2; and 325.2 → 175.1). The dwell time (0.050s), cone voltage (20V), and collision energy (10 eV) were all optimized for this assay.

2.2.10 Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) (SigmaStat for Windows, Version 3.5, Systat Software, Inc.). In cases where assumptions for a parametric test were violated, the Kruskal-Wallis test was used in place of one-way ANOVA. The appropriate *host hoc* analyses are indicated in each figure legend. The EC_{50} values were generated from sigmoidal curve fitting using the Hill-4 parameters equation; $y = yo + [ax^b / (c^b + x^b)]$, (SigmaPlot 2001 for Windows, version 7.0, SPSS, Inc.).

2.3 Results

2.3.1 Concentration-Dependent Effects of VPA on Markers of Mitochondrial Function (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF) in Sandwich-Cultured Rat Hepatocytes.

To determine the concentration responses of VPA on the individual markers of toxicity, hepatocytes were exposed to VPA at various concentrations for a period of 24 hours. VPA decreased WST-1 product formation (a marker of mitochondrial function, Figure 2-2), increased the release of lactate dehydrogenase (LDH, a marker of cytotoxicity), and increased the production of 2′,7′-dichlorofluorescein (DCF, a marker of oxidative stress) in a concentration-dependent manner. The obtained EC_{50} values for these markers were 1.1 ± 0.4 mM (mean ± SEM, n = 4), 12.2 ± 1.4 mM, and 12.3 ± 1.9 mM, respectively. The effects of VPA became statistically significant at concentrations ≥ 0.75 mM, 6 mM, and 3 mM for the WST-1, LDH, and DCF markers, respectively, when compared to the vehicle control. Positive control experiments with amiodarone (100 μM) decreased WST-1 product formation in the hepatocytes by 97 ± 1 % (mean ± SEM, n = 4, $p < 0.05$ vs. the vehicle control). Treating hepatocytes with *tert*-butylhydroperoxide (5 mM) increased LDH release by 106 ± 6 % (n = 3, $p < 0.05$) and DCF production by 117 ± 6 % (n = 3, $p < 0.05$) compared to the vehicle control. On the other hand,

octanoic acid (negative control) did not decrease WST-1 product formation at 1 mM, enhance LDH release at 12 mM, or increase DCF production at 12 mM in this model (Appendix, Figure A-2). The concentrations of octanoic acid were based on the EC_{50} values of VPA for the individual assays.

2.3.2 Effects of VPA Metabolites on Markers of Mitochondrial Function (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF) Compared to Valproic Acid

To determine the relative toxicity of the VPA metabolites, the effects of 4-ene-VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 4-keto-VPA, and VPA-glucuronide on the WST-1, LDH, and DCF markers were compared to that of the parent compound. The concentrations of exposure were based on the EC_{50} values of VPA (i.e. 1 mM for the WST-1 marker and 12 mM for the DCF and LDH markers), after 24 hours of exposure (Figure 2-2). Of the metabolites tested, (*E*)-2-ene-VPA and (*E*)-2,4-diene-VPA were more effective than VPA in decreasing WST-1 product formation, whereas the effects of 4-ene-VPA were similar to VPA (Figure 2-3). On the other hand, the effects of 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 4-keto-VPA, and VPA-glucuronide on the WST-1 marker were weaker than VPA, hence these metabolites are considered less toxic with respect to this assay (Figure 2-3).

Similar to the effects on the WST-1 marker, (*E*)-2-ene-VPA and (*E*)-2,4-diene-VPA generated more DCF compared to VPA (Figure 2-3). The effects of 4-ene-VPA, 3-OH-VPA, and VPA-glucuronide on the formation of DCF were similar to VPA, whereas that of 4-OH-VPA, 5-OH-VPA, and 4-keto-VPA were less effective than VPA (Figure 2-3). For the LDH marker, 4-ene-VPA, (*E*)-2-ene-VPA, 4-keto-VPA, 3-OH-VPA, and VPA-glucuronide were relatively less cytotoxic than VPA, whereas 4-OH-VPA and 5-OH-VPA were equally toxic compared to VPA. Consistent with the other two markers, (*E*)-2,4-diene-VPA induced more LDH release when compared to the parent compound (Figure 2-3).

2.3.3 Rank Correlations of the Extent of Mitochondrial Dysfunction (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF)

To further investigate the relationships between the induction of mitochondrial dysfunction (WST-1), oxidative stress (DCF), and cytotoxicity (LDH), quantitative correlations between the individual toxicity markers in SCRH treated with VPA or VPA metabolites (n = 9) were determined. A strong correlation ($r^2 = 0.77$, $p < 0.05$) was obtained between DCF fluorescence and WST-1 product formation (Figure 2-4), whereas a weaker correlation was observed between DCF fluorescence and LDH release ($r^2 = 0.53$, $p < 0.05$). A significant correlation was not obtained between WST-1 product formation and LDH release ($r^2 = 0.27$, $p = 0.12$).

2.3.4 Effects of 1-Aminobenzotriazole on VPA-Associated Mitochondrial Dysfunction (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF)

To determine if the cytochrome P450 (CYP)-mediated oxidation of VPA was associated with the induction of toxicity, hepatocytes were exposed to VPA in the presence or absence of 1-aminobenzotriazole (1-ABT), a broad-spectrum, mechanism-based inhibitor of the CYP enzymes (Ortiz de Montellano and Mathews 1981). Preliminary experiments determined a non-toxic concentration of 1-ABT which maximally inhibited the oxidative metabolism of VPA (Appendix, Figure A-3). Hepatocytes were exposed to the vehicle (culture medium), 1-ABT, VPA, or the combination of 1-ABT and VPA as described in the Experimental Section. As evident in Figure 2-5, 1-ABT did not attenuate the effects of VPA on the WST-1, LDH, or DCF markers. Furthermore, 1-ABT alone enhanced WST-1 product formation by 12 ± 4 % (mean ± SEM, n = 4, $p < 0.05$) when compared to the vehicle control, suggesting a slight protective effect against mitochondrial dysfunction (Figure 2-5).

To verify the inhibitory effects of 1-ABT, levels of VPA metabolites were determined in

the culture supernatant after drug treatment. 1-ABT decreased the levels of known CYP-generated metabolites (i.e. 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA) by 99 ± 1 % (mean ± SEM, n = 6, $p < 0.05$), 85 ± 4 %, and 82 ± 12 %, respectively, when compared to the 12 mM VPA control (Figure 2-6). Similar effects were observed when hepatocytes were exposed to 1 mM of VPA, as 1-ABT reduced the levels of these same metabolites by 82 ± 1 % (n = 3, $p < 0.05$), 94 ± 0 %, and 95 ± 0 %, respectively. On the other hand, 1-ABT had very little or no effects on the levels of (*E,Z*)-2,3´-diene-VPA, (*E,E*)-2,3´-diene-VPA, 3-ene-VPA, and (*E*)-2-ene-VPA, which are metabolites from the mitochondrial ß-oxidation pathway (Abbott *et al.*, 1999). Likewise, the levels of the major Phase II metabolite, VPA-glucuronide, were not affected to a great extent by 1-ABT treatment (Figure 2-6 A & B).

Interestingly, 1-ABT attenuated the levels of 3-OH-VPA by 48 ± 6 % (n = 3 - 6, $p < 0.05$) and 32 ± 5 % when compared to VPA, at 12 mM and 1 mM, respectively. The lack of complete inhibition of this metabolite might be explained by the fact that 3-OH-VPA is produced from both the CYP- and ß-oxidation- mediated pathways of VPA metabolism (Prickett and Baillie 1984). Furthermore, the levels of (*E*)-2,4-diene-VPA were below the limit of quantitation (1 ng / mL) and attempts to quantify its glucuronide or glutathione conjugates (as surrogates for the diene) after VPA treatment were also unsuccessful. The absolute metabolite levels from the treatments are presented in Table 2-1.

2.3.5 Effects of Ketoconazole on VPA-Associated Mitochondrial Dysfunction (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF)

To further examine the role of biotransformation in VPA-induced toxicity, rat hepatocytes were exposed to VPA in the presence or absence of ketoconazole, an inhibitor of CYP and *UDP*-glucuronosyltransferase (UGT) enzymes (Eagling *et al.*, 1998; Takeda *et al.*, 2006). Initial experiments determined a non-toxic concentration of ketoconazole (i.e. 25 µM)

that was capable of attenuating the formation of CYP-generated metabolites (i.e. 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA) and VPA-glucuronide in this culture model (Appendix, Figure A-4). Hepatocytes were exposed to the vehicle (culture medium containing 0.5 % MeOH v/v), ketoconazole (KTZ), VPA, or the combination of KTZ and VPA as described in the Experimental Section. As evident in Figure 2-7, KTZ did not attenuate the effects of VPA on WST-1, LDH, or DCF markers. On the other hand, KTZ further enhanced the VPA-induced DCF fluorescence by 182 ± 55 % (mean ± SEM, n = 7, $p < 0.05$) when compared to the VPA control.

To verify the inhibitory effects of KTZ toward VPA metabolism, levels of VPA metabolites were determined in the culture supernatant after drug treatment. KTZ decreased the levels of known CYP metabolites (i.e. 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA) by 89 ± 4 % (mean ± SEM, n = 11, $p < 0.05$), 88 ± 1 %, and 73 ± 2 %, respectively, when compared to the 12 mM VPA control (Figure 2-8). Similar effects were observed when hepatocytes were exposed to 1 mM of VPA, as KTZ reduced the levels of these same metabolites by 92 ± 1 % (n = 6, $p < 0.05$), 89 ± 1 %, and 81 ± 3 %, respectively. As discussed for 1-ABT, KTZ also inhibited the formation of 3-OH-VPA by 71 ± 4 % (VPA at 12 mM) and 66 ± 2 % (VPA at 1 mM). Comparable to the lack of effects of 1-ABT on the ß-oxidation metabolites, KTZ treatment did not attenuate the levels of (*E,Z*)-2,3´-diene-VPA, (*E,E*)-2,3´-diene-VPA, or (*E*)-2-ene-VPA, and slightly reduced the levels of 3-ene-VPA only at a VPA concentration of 1 mM (Figure 2-8). In contrast to the effects of 1-ABT, KTZ treatment attenuated the levels of VPA-glucuronide by 65 ± 6 % (mean ± SEM, n = 11, $p < 0.05$ vs. the VPA control) and 51 ± 11 % (n = 6, $p < 0.05$) at VPA concentrations of 12 and 1 mM, respectively. Maximal inhibition of VPA-glucuronide formation was not obtained with 25 µM of ketoconazole, as higher concentrations of the chemical inhibitor proved toxic to the SCRH with respect to the WST-1, LDH, and DCF markers. The absolute VPA metabolite levels from the KTZ treatments are presented in Table

2-2.

2.3.6 Effects of (-)-Borneol on VPA-Associated Mitochondrial Dysfunction (WST-1) and Cytotoxicity (LDH)

To further investigate the potential role of VPA-glucuronide formation in the VPA-induced hepatotoxicity, rat hepatocytes were exposed to VPA in the presence or absence of (-)-borneol, which is a broad-spectrum glucuronidation inhibitor (Watkins and Klaassen, 1982). The regimens of (-)-borneol used in this study were limited by its inherent toxicity as observed on the individual toxicity markers. (-)-borneol itself was toxic to SCRH at concentrations ≥ 50 and 125 μM with respect to the WST-1 and the LDH markers, respectively. On the other hand, all concentrations of (-)-borneol tested in the preliminary experiment (i.e. 50 μM, 75 μM, 125 μM, and 150 μM) increased basal DCF formation (Appendix, Figure A-5), thus the results of BR on this marker were not reported. Based on these considerations, hepatocytes were exposed to the vehicle (culture medium containing 0.5 % DMSO v/v), (-)-borneol (BR), VPA, or the combination of BR and VPA as described in the Experimental Section. As evident in Figure 2-9, BR (50 μM) further enhanced the effects of VPA on the WST-1 marker, whereas BR (125 μM) did not affect LDH release when compared to the VPA control.

To verify the inhibitory effects of BR toward VPA glucuronidation, levels of VPA metabolites were determined in the culture supernatant after drug treatment. BR (125 μM) selectively attenuated the levels of VPA-glucuronide by 47 ± 4 % (mean ± SEM, $n = 8$, $p < 0.05$, vs. VPA control) at a VPA concentration of 12 mM (Figure 2-10). For the 1 mM VPA, BR (50 μM) reduced the levels of both VPA-glucuronide and 3-OH-VPA by 24 ± 4 % ($n = 7$, $p < 0.05$) and 22 ± 5 % ($n = 7$, $p < 0.05$), respectively (Figure 2-10). Maximal inhibition of VPA-glucuronide formation was not obtained with (-)-borneol, as higher concentrations of the chemical inhibitor proved toxic to the SCRH with respect to the WST-1, LDH, and DCF

markers. The absolute VPA metabolite levels in the various treatment groups are presented in Table 2-3.

2.3.7 Concentration-Dependent Effects of (*E*)-2,4-diene-VPA on Markers of Mitochondrial Function (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF)

Based on Figure 2-3, (*E*)-2,4-diene-VPA was the only metabolite tested in SCRH that was consistently more toxic than VPA with respect to the WST-1, LDH, and DCF markers. In order to determine the potency of (*E*)-2,4-diene-VPA on these markers, SCRH were also exposed to different concentrations of the metabolite (0 – 12 mM) for 24 hours. As evident in Figure 2-11, (*E*)-2,4-diene-VPA decreased WST-1 product formation, increased LDH release, and increased DCF fluorescence in a concentration-dependent manner. The effects of (*E*)-2,4-diene-VPA became significant at concentrations ≥ 0.3 mM, 1 mM, and 0.3 mM, for the WST-1, LDH, and DCF markers, respectively, when compared to the vehicle control. This corresponds to a 2.5-fold, 6-fold, and 10-fold increase in potency, respectively, when compared to the effects of VPA on these same markers.

2.3.8 Effects of α-F-VPA on Markers of Mitochondrial Function (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF)

To further investigate the role of bioactivation in the VPA-induced hepatotoxicity, the effects of α-F-VPA on the individual toxicity markers were also determined in SCRH. α-F-VPA can not generate the (*E*)-2,4-diene- metabolite and is resistant to the formation of the glucuronide (Tang *et al.,* 1995; Tang *et al.,* 1997) or CoA esters (Grillo *et al.,* 2001). Hepatocytes were exposed to the vehicle (culture medium), VPA, or α-F-VPA for 24 hours. The concentrations of exposure were based on the EC_{50} values of VPA (i.e. 1 mM for the WST-1 marker and 12 mM for the DCF and LDH markers). As evident in Figure 2-12, α-F-VPA was less toxic than VPA

with respect to the WST-1, LDH, and DCF markers. Except for a slight decrease in WST-1 product formation, the effects of α-F-VPA were comparable to the vehicle control in SCRH.

2.4 Discussion

In the present study, rat hepatocytes were cultured in the sandwich configuration between two layers of Matrigel™ in a method similar to that described by Sidhu et al. (1993). This is one of the the first instances where sandwich-cultured rat hepatocytes (SCRH) were used to investigate the toxicity associated with VPA. The sandwich configuration is ideal for *in vitro* toxicology studies since the morphology and functions (e.g. phase I and phase II biotransformation activity) of cultured hepatocytes are better preserved (e.g. Berthiaume *et al.*, 1996). A discussion on the use of cultured hepatocytes in the investigation of VPA-induced hepatotoxicity is provided in the Introduction Chapter (section 1.7). Specifically, section 1.7.2 discusses the benefits of using the Matrigel™ system and provides a comparison to another model (i.e. collagen system) that is commonly used today. The selection of the individual toxicity markers for our experiments was based on the established toxicology of VPA. VPA is known to adversely affect the functions or structures of mitochondria in rats (Sobaniec-Lotowska, 1997; Trost and Lemasters, 1996; Becker and Harris, 1983; Coude *et al.,* 1983; Turnbull *et al.,* 1986), hence the WST-1 marker, which measures mitochondrial respiratory function (Berridge *et al.*, 2005), was employed. The use of the LDH marker for the assessment of necrotic cell death has been well established for VPA in cultured rat hepatocytes (Kingsley *et al.,* 1983; Jurima-Romet *et al.,* 1996; Takeuchi *et al.*, 1988; Buchi *et al.*, 1984), thus the same marker was used for the assessment of cytotoxicity in our experiments. The administration of VPA is also associated with induction of oxidative stress (as reviewed by Chang and Abbott 2006), thus the DCF marker was used in our SCRH model as a sensitive indicator of reactive oxygen species formation. Several novel and intriguing findings are discussed below.

We are the first to comprehensively characterize the effects of several synthesized VPA metabolites on markers of mitochondrial function, cytotoxicity, and oxidative stress in a single *in vitro* model. Of the metabolites investigated in the current study, only (*E*)-2,4-diene-VPA was consistently more toxic than VPA when tested on an equal molar basis (Figure 2-3). Supporting the adverse effects of (*E*)-2,4-diene-VPA on mitochondrial function in SCRH, (*E*)-2,4-diene-VPA is more potent than VPA on the induction of hepatic steatosis and the inhibition of mitochondrial ß-oxidation when dosed to rats (Kesterson *et al.,* 1984). As well, (*E*)-2,4-diene-VPA was reported to interact with the mitochondrial trifunctional protein in a potent (IC_{50} value of 42 μM) and reversible manner (Baldwin *et al.,* 1996), and such an interaction may be associated with mitochondrial dysfunction.

Furthermore, we demonstrated for the first time that (*E*)-2,4-diene-VPA was much more toxic than VPA with respect to the LDH and DCF markers in SCRH. The reported ability for the activated (*E*)-2,4-diene-VPA to bind to key cellular proteins or GSH (Tang *et al.,* 1996c) could possibly explain its effects on cytotoxicity or oxidative stress, respectively. As well, further support for the reactivity of (*E*)-2,4-diene-VPA as a potential factor in VPA induced-toxicity was suggested when elevated levels of thio-conjugated (*E*)-2,4-diene-VPA were reported in the urine of patients who developed hepatotoxicity or in patients who possessed the risk factors of VPA-associated hepatotoxicity (Kassahun *et al.,* 1991; Gopaul *et al.*, 2003). Having shown (*E*)-2,4-diene-VPA to be more toxic than VPA in the SCRH model, attempts were made to determine whether the VPA-induced toxicities observed in the hepatocytes were associated with the *in situ* generation of (*E*)-2,4-diene-VPA. The results are discussed below.

4-ene-VPA was not more toxic than VPA in the SCRH model (Figure 2-3). These results are consistent with published reports where the effects of exogenously added 4-ene-VPA on mitochondrial function or cytotoxicity were similar to or less than VPA in various *in vitro* preparations (Ponchaut *et al.,* 1992; Jurima-Romet *et al.,* 1996; Kingsley *et al.,* 1983). Likewise,

a correlation was not observed between the plasma levels of 4-ene-VPA and the extent of hepatic steatosis in rats (Loscher *et al.,* 1993). In humans, the majority of the clinical literature also have not reported abnormal urinary or plasma 4-ene-VPA levels in cases of VPA-associated hepatotoxicity (McLaughlin *et al.,* 2000; Kondo *et al.*, 1992; Fisher *et al.,* 1992; Tennison *et al.*, 1988; Kuhara *et al.,* 1990; Paganini *et al.*, 1987; Siemes *et al.,* 1993; Eadie *et al.,* 1990). Collectively, these findings do not support 4-ene-VPA as a toxic metabolite.

On the contrary, the exogenously administered 4-ene-VPA is a potent inducer of hepatic steatosis and inhibitor of mitochondrial β-oxidation when compared to VPA in rats (Kesterson *et al.,* 1984; Loscher *et al.,* 1993; Tang *et al.,* 1995), thus an obvious discrepancy in the toxicity of 4-ene-VPA is evident between *in vitro* and *in vivo* experimental models. The reasons behind the discrepancy are not readily apparent, but maybe explained by the fact that young (~ 150 g) and fasted rats were employed in these *in vivo* studies. Young rats are more resistant to the development of VPA-induced hepatic steatosis (Kesterson *et al.,* 1984), and fasting is known to deplete hepatic GSH (Vogt and Richie 1993), as shown in a mouse model. At least in cultured rat hepatocytes, the toxicity of 4-ene-VPA, but not VPA, is markedly enhanced when total GSH is depleted (Jurima-Romet *et al.,* 1996). Obviously further studies are needed to determine the apparent discrepancy in the toxicity associated with the exogenously added 4-ene-VPA *in vivo* from that seen using the different *in vitro* experimental models.

Furthermore, we also demonstrated the novel effects of 4-ene-VPA on the induction of oxidative stress in SCRH. It is reported in the rat that approximately 20 % of the 4-ene-VPA dose is converted to GSH-conjugated metabolites (Kassahun *et al.,* 1994), and correspondingly, hepatic and mitochondrial GSH are depleted by 4-ene-VPA exposure (Tang *et al.,* 1995; Kassahun *et al.,* 1994). Based on these *in vivo* observations, one might postulate that the depletion of GSH in SCRH could be responsible for the oxidative stress observed with 4-ene-VPA. On the other hand, 4-ene-VPA is also known to destroy the CYP enzymes in a suicidal

manner (Prickett and Baillie, 1986) and disrupt the oxidative phosphorylation of the mitochondria (Aires et al., 2008). These mechanisms, in addition to its ability to deplete hepatic GSH, may also contribute to the effects of 4-ene-VPA on the induction of oxidative stress. Further mechanistic studies are needed to probe these individual hypotheses in SCRH.

Overall, based on the inconsistent findings on the observed toxicity of 4-ene-VPA in the literature, the role of this metabolite in VPA-induced hepatotoxicity remains inconclusive. In order to further understand the toxicity associated with 4-ene-VPA, experiments were also conducted to determine whether the VPA-induced toxicities observed in SCRH were associated with *in situ* generation of 4-ene-VPA.

A proposed mechanism for the toxicity of 4-ene-VPA in the rat model is the further bioactivation of 4-ene-VPA in the formation of (*E*)-2,4-diene-VPA (Tang *et al.,* 1995). This is based on the observation that substituting a fluorine atom on the α position of 4-ene-VPA completely prevented the formation of (*E*)-2,4-diene-VPA and the induction of hepatic steatosis in rats (Tang *et al.,* 1995). However, care should be taken when interpreting the data obtained from α-F-4-ene-VPA, as subsequent observations indicated that the α-fluorinated analogues of VPA were also resistant to the formation of the glucuronide (Tang *et al.,* 1997) or Co-A esters (Grillo *et al.*, 2001). To address the non-selectivity of these α-fluorinated analogues of VPA, a VPA analogue that is only resistant to the formation of (*E*)-2,4-diene-VPA, yet still able to form the glucuronide or CoA esters, is warranted. Such analogues, already being developed and tested in our laboratory, would allow one to further investigate the role of bioactivation in VPA-induced hepatotoxicity.

In contrast to 4-ene-VPA, (*E*)-2-ene-VPA tended to be equally or slightly more toxic than VPA in SCRH (Figure 2-3). Consistent with our observation of (*E*)-2-ene-VPA on mitochondrial dysfunction in SCRH, the effects of (*E*)-2-ene-VPA on the inhibition of mitochondrial β-oxidation are reported to be similar to or slightly more potent than VPA in

various *in vitro* preparations (Ponchaut *et al.,* 1992; Silva *et al.,* 2001). However, (*E*)-2-ene-VPA was significantly less toxic compared to VPA in our model with respect to the LDH marker, which is consistent with the lack of effect of (*E*)-2-ene-VPA treatment on the induction of hepatic steatosis in rats (Kesterson *et al.,* 1984; Loscher *et al.,* 1993). However, our finding on the LDH assay is in contrast to the results reported by Jurima-Romet et al. (1996), where the (*E*)-2-ene-VPA appeared equally cytotoxic as VPA in their *in vitro* hepatocyte model. The reasons for the discrepancy between the two *in vitro* studies are not clear, but differences in rat strains or culture models may be proposed as the possible contributing factors. Furthermore, a novel observation of (*E*)-2-ene-VPA in our experiment was the effect on oxidative stress. A major metabolite of (*E*)-2-ene-VPA is the microsomal produced metabolite, (*E*)-2,4-diene-VPA (Loscher *et al.*, 1992; Loscher *et al.,* 1993), and one might postulate that the effects of (*E*)-2-ene-VPA to produce oxidative stress in SCRH could be mediated by the (*E*)-2,4-diene-VPA metabolite, as discussed previously. Further experiments are needed to test this hypothesis.

With respect to the oxygenated metabolites (5-OH-VPA, 4-OH-VPA, 3-OH-VPA, and 4-keto-VPA) tested in the present study, our results showing relatively little toxicity when compared to VPA in SCRH are in agreement with the data from Kingsley et al. (1983) in another *in vitro* rat hepatocyte model. To our knowledge, no reactive forms arising from the oxygenated metabolites of VPA have been identified, and this may explain their lack of toxicity in SCRH. Likewise, the synthesized VPA-glucuronide was also less toxic than VPA in SCRH. Control experiments verified that the exogenously added VPA-glucuronide was able to gain entry into the hepatocytes (Appendix, Figure A-6), thus mitigating the concern that the lack of toxicity was the result of limited hepatocyte exposure. Given the intriguing finding from Tong *et al.* (2005b) where the administration of glucuronidation inhibitors apparently attenuated the VPA-induced lipid peroxidation in the rat model, attempts were also made to determine whether the VPA-

induced toxicities observed in SCRH were associated with the *in situ* generation of VPA-glucuronide.

Overall, our toxicity data on the synthesized metabolites of VPA in SCRH are, in most cases, in agreement with what has been reported in the literature in various experimental models. This suggests that SCRH maybe a useful model for the rapid and accurate assessment of the hepatotoxic potential of novel second generation VPA analogues, and such studies are currently being conducted in our laboratory. Another purpose for our testing of VPA metabolites in SCRH was to investigate the possible associations between mitochondrial dysfunction, oxidative stress, and cytotoxicity. As evident in Figure 2-4, a strong correlation was obtained between mitochondrial dysfunction (WST-1 marker) and oxidative stress (DCF marker) in hepatocytes exposed to VPA or its metabolites. It is safe to say, from the various studies that have been published, that VPA or its metabolites adversely affect the function or structure of the mitochondria (Trost and Lemasters, 1996; Becker and Harris, 1983; Coude *et al.,* 1983; Turnbull *et al.,* 1986; Ponchaut *et al.,* 1992; Silva *et al.,* 2001). The mitochondria are also a major source of oxidative stress in hepatocytes (Pessayre *et al.*, 1999), thus the possibility of VPA-induced oxidative stress having a mitochondrial origin should be further examined. Moreover, we also report a strong correlation between oxidative stress and cytotoxicity in hepatocytes exposed to VPA or its metabolites (Figure 2-4). In support of this observed association, exogenously added antioxidants (i.e. vitamin E or *N*,*N*′-diphenyl-p-phenylenediamine) were able to attenuate the VPA-induced LDH release in cultured rat hepatocytes (Buchi *et al.,* 1984), although the effects of the same antioxidants on a marker of oxidative stress was not done (Buchi *et al.,* 1984). Based on these observations, further mechanistic studies are warranted to establish the cause-effect relationships between VPA-induced oxidative stress and hepatotoxicity.

Hepatocytes in the current study were exposed to VPA metabolites at concentrations equal to the EC_{50} values of VPA for the individual toxicity markers (i.e. 1 mM for the WST-1

assay and 12 mM for the LDH and DCF assays). Virtually all toxicity studies on VPA metabolites in the literature also use high concentrations of the synthesized metabolites (Kingsley *et al.,* 1983; Kesterson *et al.,* 1984; Ponchaut *et al.,* 1992; Tang *et al.,* 1995; Loscher *et al.,* 1992; Loscher *et al.,* 1993; Jurima-Romet *et al.,* 1996; Silva *et al.,* 2001; Kassahun *et al.,* 1994). Although such comparative studies are valid in delineating the relative orders of toxicity, it is important to note that these metabolite concentrations would not be obtained after the administration of VPA. Moreover, studies with synthesized metabolites may not clarify the true contribution of VPA metabolites in VPA-induced toxicity, because the kinetic characteristics of exogenously added metabolites likely differ from that of the *in situ* generated metabolites. For example, the partitioning into the hepatocytes may be limited as a result of the hydrophilicity of the synthesized drug metabolites. As well, some metabolites of VPA may only exert their effects if formed at the site of toxicity. An example would be the mechanism-based inactivation of CYP enzymes by the generation of reactive intermediates from the oxidative metabolism of 4-ene-VPA (Prickett and Baillie, 1986). To attempt to address the limitations with synthesized metabolites, we investigated the role of putative toxic metabolites that are formed *in situ* from the administration of VPA in VPA-induced toxicity. As well, it maybe the process of metabolism itself, rather than the presence of toxic metabolites, that is responsible for the VPA-induced hepatotoxicity. For example, VPA-induced toxicity to human lymphocytes *in vitro* was thought to be the result of microsomal-dependent generation of hydrogen peroxide and not the production of the putative toxic metabolite, 4-ene-VPA (Tabatabaei *et al.*, 1999).

To examine the role of biotransformation in VPA-induced hepatotoxicity, primary interest must be given to the CYP pathway, which is responsible for the production of the putative hepatotoxins, 4-ene-VPA and (*E*)-2,4-diene-VPA (Abbott and Anari, 1999). To determine the contribution of CYP-mediated biotransformation and/or the *in situ* formation of CYP metabolites in VPA-induced toxicity, the broad-spectrum CYP inhibitor 1-

aminobenzotriazole (1-ABT) was used in SCRH. The 1-ABT, which inhibits VPA oxidative metabolism in rats (Tong *et al.,* 2003), selectivity inhibited the CYP-generated metabolites (i.e. 4-OH-VPA, 5-OH-VPA, and 4-ene-VPA) in SCRH without significantly affecting the β-oxidation or glucuronidation of VPA (Figure 2-6). Interestingly, 1-ABT did not attenuate the toxicity of VPA as measured with respect to all the markers (Figure 2-5), despite completely blocking the formation of the CYP-generated metabolites (Figure 2-6). This finding is the first clear evidence in rat hepatocytes demonstrating a lack of contribution of CYP-mediated oxidative metabolism or the formation of the putative toxic 4-ene-VPA in relation to VPA-induced changes in mitochondrial function, cytotoxicity, or oxidative stress. A similar finding was reported on a single marker of lipid peroxidation by Tong *et al.* (2003), where 1-ABT (100 mg/kg) reduced the levels of CYP-generated VPA metabolites without attenuating the effects of VPA on lipid peroxidation in the rat. However, in that study, the selectivity of 1-ABT toward the CYP-mediated oxidative metabolism of VPA was not verified (Tong *et al.,* 2003).

A metabolite of toxicological interest is (*E*)-2,4-diene-VPA, as it was the only metabolite tested in the current study that consistently generated more toxicity than VPA (Figure 2-3). Unfortunately, levels of (*E*)-2,4-diene-VPA in our SCRH model were below the limit of quantitation (~ 7.2×10^{-6} mM) following the administration of VPA. As discussed previously, there is substantial evidence that activated forms of (*E*)-2,4-diene-VPA are able to conjugate with GSH or cellular proteins (Tang *et al.,* 1996c; Kassahun *et al.,* 1991; Gopaul *et al.,* 2003), thus one possibility for the lack of detection of (*E*)-2,4-diene-VPA following VPA administration is that the metabolite underwent covalent binding or rapid metabolism as soon as it is generated. However, attempts to measure the GSH- or glucuronide-conjugates of (*E*)-2,4-diene-VPA were unsuccessful, and the extent of (*E*)-2,4-diene-VPA protein binding in SCRH is unknown. Further studies with a radio-labelled derivative are needed to determine the kinetics of (*E*)-2,4-diene-VPA binding to cellular proteins in rat hepatocytes. Despite the lack of

detection of (*E*)-2,4-diene-VPA in our *in vitro* model, it is very unlikely that (*E*)-2,4-diene-VPA would have played a role in VPA-induced toxicity observed in SCRH. The minimum concentrations of (*E*)-2,4-diene-VPA required to produce toxic responses in the SCRH model were 0.3, 1, and 0.3 mM, for the WST-1, LDH, and DCF markers, respectively (Figure 2-11). However, the trace amounts of (*E*)-2,4-diene-VPA generated in SCRH from the administration of VPA (i.e. below the LOQ of the assay) were many fold lower than the determined minimum toxic concentrations of (*E*)-2,4-diene-VPA. Furthermore, (*E*)-2,4-diene-VPA is directly (2-ene-VPA) or indirectly (4-ene-VPA) generated by the actions of CYP enzymes (Abbott and Anari, 1999). Thus, our use of 1-ABT or KTZ, which are broad spectrum CYP inhibitors, should have further inhibited the formation of (*E*)-2,4-diene-VPA in our experiments. If (*E*)-2,4-diene-VPA were indeed *the* toxic metabolite modulating the hepatotoxic response of VPA, then 1-ABT or KTZ treatment, which completely inhibited CYP-mediated VPA oxidative metabolism (Figure 2-6; Figure 2-8), would have attenuated the toxicities associated with VPA. None of the altered markers due to VPA were affected and this would lead one to conclude that (*E*)-2,4-diene-VPA was not relevant to VPA-induced hepatotoxicity in SCRH.

In an *in vivo* study in rats, VPA-induced lipid peroxidation was attenuated by the administration of (-)-borneol, which inhibited the glucuronidation of VPA (Tong *et al.,* 2005b), suggesting an association between VPA glucuronide and oxidative stress. To take this intriguing finding further, we tested the hypothesis that VPA-glucuronide formation is associated with the observed toxicity of VPA in the SCRH model. Valproic acid is a substrate of various human UGT enzymes (Ethell *et al.*, 2003; Sakaguchi *et al.*, 2004), but the rat UGT enzymes responsible for the glucuronidation of VPA remain unknown. In our hands, initial experiments with salicylamide, a broad spectrum UGT-inhibitor (Howell *et al.*, 1986), were unsuccessful because the chemical itself appeared to be toxic to the hepatocytes with respect to LDH release (Appendix, Figure A-7). The subsequent selection of ketoconazole (KTZ) for our experiments

was based on the assumption that inhibitors of VPA-glucuronidation in humans may possibly inhibit the same reaction in rats. It is known that VPA is a substrate of human UGT2B7, of which KTZ is a potent inhibitor (Ethell *et al.*, 2003; Sakaguchi *et al.*, 2004; Takeda *et al.,* 2006). Moreover, KTZ inhibits the glucuronidation of zidovudine in rat liver microsomes, a reaction known to be catalyzed by the human UGT2B7 enzyme (Mano *et al.*, 2007). The regimen of KTZ used in the SCRH model only partially attenuated the glucuronidation of VPA (Figure 2-8), as higher concentrations of the inhibitor proved toxic to the hepatocytes (Appendix, Figure A-4). It was also determined that KTZ inhibited the CYP-mediated oxidative metabolism of VPA at a concentration required for UGT inhibition, but the effects of KTZ on ß-oxidation were minimal, as determined by measuring metabolites of VPA from the ß-oxidation pathway (Figure 2-8). In the SCRH model, treatment of KTZ did not attenuate the effects of VPA on the WST-1 and LDH toxicity markers, whereas KTZ further enhanced the effects of VPA on DCF formation (Figure 2-7). These observations with KTZ support the conclusion from the 1-ABT experiment that the CYP-mediated oxidation of VPA is unlikely a factor in VPA-induced toxicity. The fact that KTZ inhibited glucuronide formation and enhanced VPA-induced DCF formation would suggest that the glucuronidation of VPA is indeed a detoxification reaction. Consistent with these findings, the exogenously administered VPA-glucuronide, at relatively high concentrations, was not as toxic as VPA with respect to the induction of mitochondrial dysfunction or oxidative stress in SCRH (Figure 2-3).

To support the observations obtained from the KTZ experiment, we also used (-)-borneol (BR) as an inhibitor of VPA-glucuronidation (Watkins and Klaassen 1982). Similar to KTZ, the use of BR was limited by its inherent toxicity at higher concentrations and its inability to completely inhibit the formation of VPA glucuronide in SCRH (Figure 2-10). BR itself induced DCF formation at all concentrations tested (Appendix, Figure A-5), thus its effects on VPA-induced oxidative stress could not be assessed in our *in vitro* model. On the other hand, the

advantage of BR was that it was somewhat selective toward the glucuronidation of VPA, as demonstrated by measuring VPA metabolites generated from all biotransformation pathways of VPA (Figure 2-10). Similar to KTZ, BR did not attenuate the effects of VPA on the WST-1 and the LDH markers (Figure 2-9). However, the effects of BR on VPA-G formation were minimal, and one may argue that the residual VPA-G formed in the presence of BR may have contributed to the toxicity associated with VPA.

These novel findings on VPA-glucuronide in SCRH are in contrast to the report by Tong *et al.* (2005b), where the results of (-)-borneol experiments suggested that the formation of VPA-glucuronide was associated with the induction of lipid peroxidation in the rat. The association between VPA-glucuronide formation and lipid peroxidation *in vivo* in rats maybe attributed to the enterohepatic recycling of VPA-glucuronide and the subsequent release of glucuronic acid (Tong *et al.,* 2005b). If this were indeed *the* mechanism leading to the toxicity associated with the formation of VPA-glucuronide, we would not have been able to reproduce the same phenomenon, given the physical limitations of an *in vitro* model. Furthermore, another limitation in our approach is that selective and complete inhibition of VPA-glucuronide formation could not be obtained with the chemical inhibitors used in the present experiment. Thus, future experiments with potent and selective inhibitors are warranted to help clarify the role of VPA-glucuronide formation in the VPA-induced hepatotoxicity.

Finally, the α-F-VPA was used as another approach to determine the role of biotransformation or reactive metabolite formation in the VPA-induced toxicity in SCRH. Our finding that α-F-VPA was significantly less toxic compared to VPA (Figure 2-12) is consistent with the literature, whereby treatments of α-F-VPA or α-F-4-ene-VPA *in vivo* in rats are not associated with lipid peroxidation or hepatic steatosis, respectively, in contrast to their non-fluorinated counterparts (Tang *et al.,* 1995; Tong *et al.,* 2005b). The lack of toxicity observed with the α-fluorinated VPA analogues was originally attributed to their inability to form the toxic

(*E*)-2,4-diene- metabolite (Tang *et al.,* 1995), but subsequent studies indicated that they are also resistant to the formation of the glucuronide (Tang *et al.,* 1997) or the CoA esters (Grillo *et al.,* 2001). Based on our experiments with the CYP or the glucuronidation inhibitors, it is unlikely that the *in situ* production of (*E*)-2,4-diene-VPA or VPA-glucuronide was associated with the toxicities of VPA in SCRH. These findings, in conjunction with the characteristics of α-F-VPA, would seem to suggest that VPA-CoA is the putative toxic metabolite. Indeed, the CoA esters of carboxylic acids are electrophilic reactive species (Boelsterli, 2002), as demonstrated by the reactivity of (*E*)-2,4-diene-VPA-CoA toward GSH or proteins (Tang and Abbott, 1996b). As well, the depletion of CoA from VPA exposure has also been associated with impaired mitochondrial β-oxidation or reduced oxidative phosphorylation in various experimental models (Eadie *et al.,* 1988; Luis *et al.*, 2007). Thus, further mechanistic studies are warranted to test the hypothesis that the formation of VPA-CoA maybe associated with the pathogenesis of VPA-induced hepatotoxicity.

In conclusion, our SCRH model is useful for the assessment of the hepatotoxic potential of novel VPA structural analogues. From the experiments with the synthesized metabolites, we report the novel finding that exogenously added (*E*)-2,4-diene-VPA is relatively more potent and toxic than VPA with respect to markers of mitochondrial dysfunction, cytotoxicity, and oxidative stress in SCRH. As well, our results appear to demonstrate that the *in situ* CYP-mediated oxidative metabolism of VPA to 4-ene-VPA or (*E*)-2,4-diene-VPA or UGT-mediated glucuronidation of VPA to VPA-glucuronide are not associated with VPA-induced toxicity in our SCRH model.

Table 2-1.VPA Metabolite Levels in the Supernatant of Sandwich-Cultured Rat Hepatocytes Exposed to VPA or the Combination of VPA and 1-Aminobenzotriazole (1-ABT)

Levels of VPA metabolites (μM)

	VPA 12 mM (N = 6)	**VPA + 1-ABT (N = 6)**	**VPA 1 mM (N = 3)**	**VPA + 1-ABT (N = 3)**
(E,Z)-2,3-diene-	0.38 ± 0.09	0.42 ± 0.08	0.30 ± 0.04	0.33 ± 0.04*
(E,E)-2,3-diene-	3.65 ± 1.23	3.72 ± 1.21	1.83 ± 0.34	2.17 ± 0.27
4-ene-	0.11 ± 0.04	0.00 ± 0.00*	0.20 ± 0.03	0.04 ± 0.01*
3-ene-	0.82 ± 0.23	0.80 ± 0.22	1.28 ± 0.35	1.58 ± 0.44*
2-ene-	2.76 ± 0.46	3.03 ± 0.44	2.36 ± 0.32	2.53 ± 0.29
4-OH-	3.78 ± 0.69	0.45 ± 0.11*	1.41 ± 0.18	0.08 ± 0.01*
3-OH-	0.32 ± 0.05	0.15 ± 0.02*	0.15 ± 0.03	0.10 ± 0.01*
5-OH-	3.09 ± 0.64	0.31 ± 0.14*	2.58 ± 0.29	0.11 ± 0.01*
VPA-glucuronide	94.0 ± 26.0	98.0 ± 27.0	107 ± 6	91 ± 2*

Note: Hepatocytes were exposed to the vehicle (culture medium), VPA (1 or 12 mM), 1-ABT (0.5 mM), or VPA + 1-ABT as described in the Experimental Section. Shown are the mean ± SEM of the number of experiments as indicated in the table, with the exception that the levels of VPA-G in the 12 mM VPA-treated cells were obtained from 4 individual experiments. *$p < 0.05$ vs. the VPA control as assessed by one-way ANOVA followed by the Student-Newman-Keuls *post hoc* analysis.

Table 2-2. VPA Metabolite Levels in the Supernatant of Sandwich-Cultured Rat Hepatocytes Exposed to VPA or the Combination of VPA and Ketoconazole (KTZ)

Levels of VPA metabolites (μM)

	VPA 12 mM (N = 11)	**VPA + KTZ (N = 11)**	**VPA 1 mM (N = 6)**	**VPA + KTZ (N = 6)**
(E,Z)-2,3-diene-	0.37 ± 0.04	0.37 ± 0.05	0.39 ± 0.03	0.49 ± 0.04*
(E,E)-2,3-diene-	2.76 ± 0.30	2.20 ± 0.33	3.33 ± 0.14	3.38 ± 0.26
4-ene-	0.23 ± 0.04	0.03 ± 0.01*	0.13 ± 0.03	0.02 ± 0.00*
3-ene-	2.31 ± 0.34	1.90 ± 0.27	3.40 ± 0.05	3.21 ± 0.35*
2-ene-	2.98 ± 0.31	2.33 ± 0.31	2.51 ± 0.17	2.39 ± 0.26
4-OH-	3.03 ± 0.43	0.50 ± 0.05*	0.96 ± 0.17	0.11 ± 0.02*
3-OH-	0.38 ± 0.05	0.09 ± 0.01*	0.18 ± 0.01	0.07 ± 0.00*
5-OH-	2.03 ± 0.19	0.54 ± 0.06*	1.93 ± 0.33	0.41 ± 0.08*
VPA-glucuronide	157 ± 15	58 ± 14*	201 ± 17	105 ± 25*

Note: Hepatocytes were exposed to the vehicle (culture medium containing 0.5 % MeOH, v/v), VPA (1 or 12 mM), KTZ (25 μM), or VPA + KTZ as described in the Experimental Section. Shown are the mean ± SEM of the number of experiments as indicated in the table, with the exception that the levels of VPA-G in the 12 mM and 1 mM VPA-treated cells were obtained from 10 and 7 individual experiments, respectively. *$p < 0.05$ vs. the VPA control as assessed by one-way ANOVA followed by the Student-Newman-Keuls *post hoc* analysis.

Table 2-3. VPA Metabolite Levels in the Supernatant of Sandwich-Cultured Rat Hepatocytes Exposed to VPA or the Combination of VPA and (-)-Borneol (BR)

Levels of VPA metabolites (μM)

	VPA 12 mM (N = 8)	**VPA + BR[1] (N = 8)**	**VPA 1 mM (N = 7)**	**VPA + BR[2] (N = 7)**
(E,Z)-2,3-diene-	0.42 ± 0.05	0.42 ± 0.06	0.36 ± 0.05	0.34 ± 0.05
(E,E)-2,3-diene-	3.18 ± 0.27	2.79 ± 0.30	3.67 ± 0.29	3.21 ± 0.34
4-ene-	0.21 ± 0.03	0.21 ± 0.03	0.16 ± 0.03	0.15 ± 0.03
3-ene-	2.91 ± 0.20	2.79 ± 0.27	4.10 ± 0.25	3.59 ± 0.47
2-ene-	3.13 ± 0.26	2.55 ± 0.19	2.92 ± 0.25	2.57 ± 0.25
4-OH-	2.88 ± 0.40	2.73 ± 0.32	1.17 ± 0.26	1.02 ± 0.24
3-OH-	0.31 ± 0.03	0.32 ± 0.03	0.25 ± 0.04	0.20 ± 0.03*
5-OH-	2.30 ± 0.20	1.94 ± 0.18	3.44 ± 0.58	2.89 ± 0.52
VPA-glucuronide	240 ± 17	132 ± 15*	260 ± 16	159 ± 18*

Note: Hepatocytes were exposed to the vehicle (culture medium containing 0.5 % DMSO, v/v), VPA (1 or 12 mM), BR (125[1] or 50[2] μM), or VPA + BR as described in the Experimental Section. Shown are the mean ± SEM of the number of experiments as indicated in the table. *p < 0.05 vs. the VPA control as assessed by one-way ANOVA followed by the Student-Newman-Keuls *post hoc* analysis.

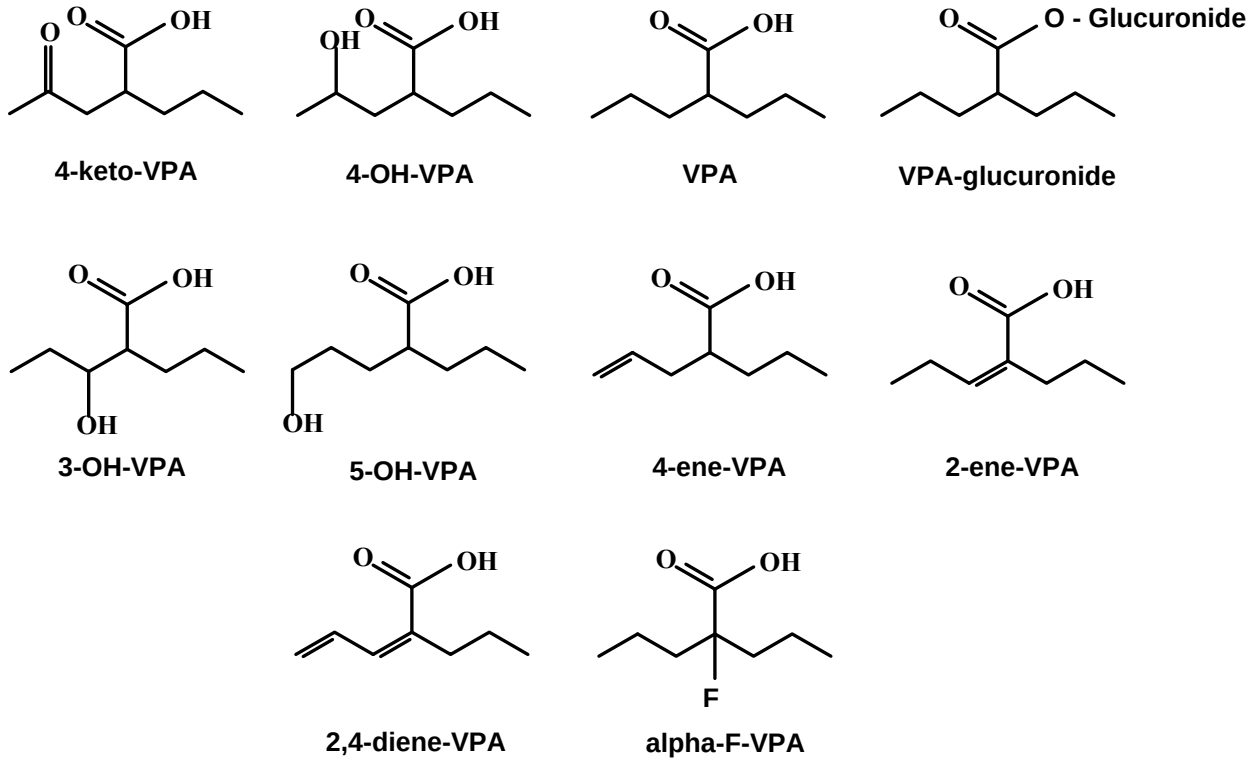

Figure 2-1. The chemical structures of VPA, VPA metabolites, and α-F-VPA tested in the current study. The metabolites and α-F-VPA were synthesized and purified in our laboratory as described in the Experimental Section.

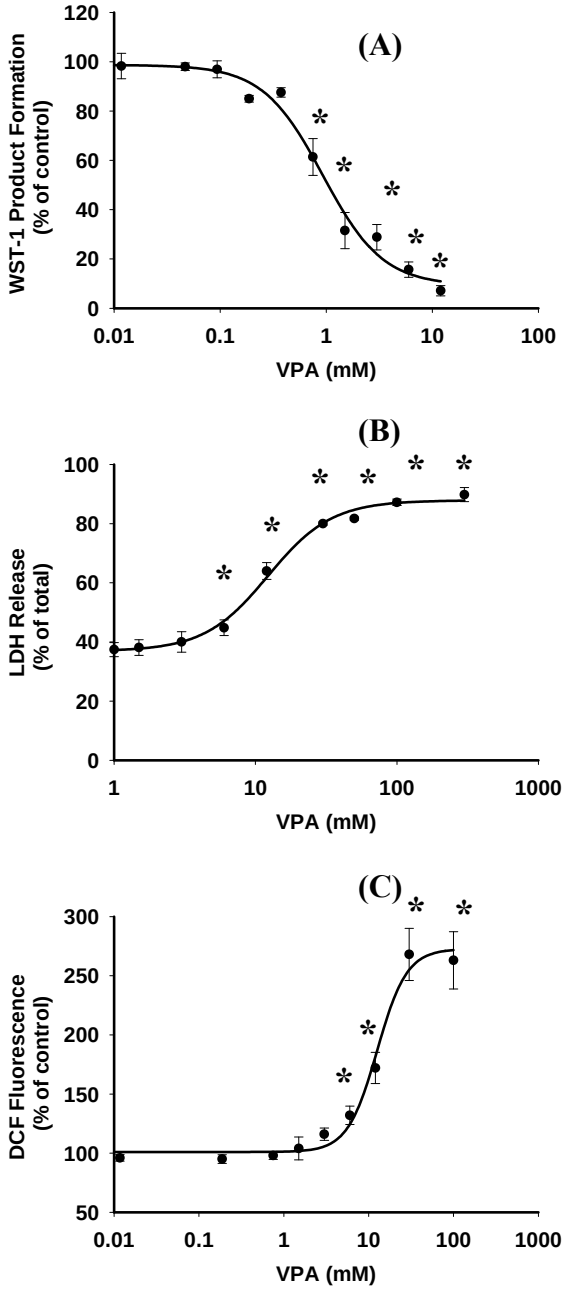

Figure 2-2.Concentration-dependent effects of VPA on (A) WST-1 product formation, (B) LDH release, and (C) DCF fluorescence in sandwich-cultured rat hepatocytes. Hepatocytes were exposed to the vehicle (culture medium) or various concentrations of VPA (0 – 300 mM) for 24 hours. The individual assays were conducted as described in the Experimental Section. The EC_{50} values were 1.1 ± 0.4 mM (mean ± SEM, n = 4), 12.2 ± 1.4 mM, and 12.3 ± 1.9 mM, respectively. The effects of VPA became significant at concentrations ≥ 0.75 mM, 6 mM, and 3 mM for the WST-1, LDH, and DCF markers, respectively. *p<0.05 vs. the vehicle control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $2.2 \times 10^{-3} \pm 0.6 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; 0.042 ± 0.008 (supernatant activity) and 0.120 ± 0.026 (total activity) absorption unit / min for the LDH marker; and $1.57 \times 10^{-3} \pm 0.73 \times 10^{-3}$ µmole DCF / min for the DCF marker.

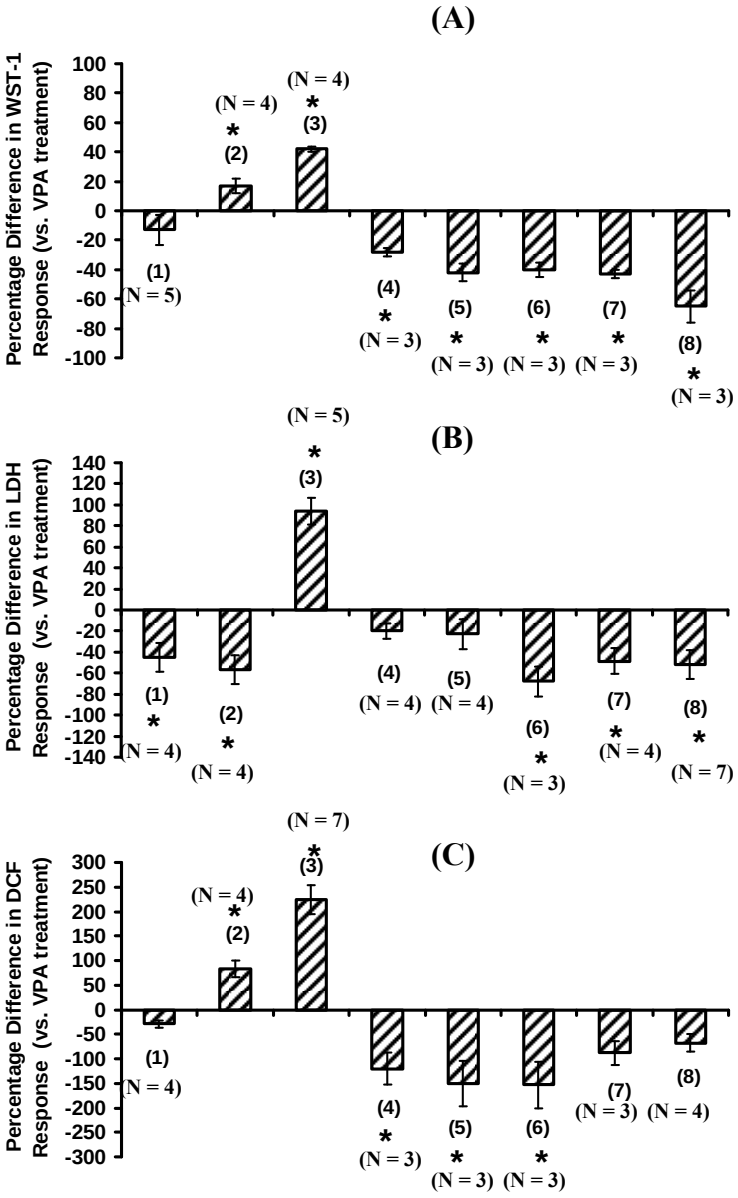

Figure 2-3. Effects of (1) 4-ene-VPA, (2) (*E*)-2-ene-VPA, (3) (*E*)-2,4-diene-VPA, (4) 5-OH-VPA, (5) 4-OH-VPA, (6) 4-keto-VPA, (7) 3-OH-VPA, and (8) VPA-glucuronide on the (A) WST-1, (B) LDH, and (C) DCF markers relative to the parent compound in SCRH. Rat hepatocytes were exposed to the vehicle (culture medium), each metabolite, or VPA for 24 hours. The concentrations of exposure were 1 mM (WST-1 assay) and 12 mM (DCF and LDH assays), and the individual assays were conducted as described in the Experimental Section. Data are expressed as the difference (in percentage points) of the effects of each metabolite compared to that of VPA (mean ± SEM). The sample sizes are indicated in brackets abvoe. Positive values indicate enhanced toxicity (vs. VPA). Negative values indicate reduced toxicity relative to VPA. *p < 0.05 vs. the baseline control (i.e. VPA treatment), based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle and VPA control values for the WST-1 marker were 3.7 x 10^{-3} ± 0.4 x 10^{-3} (mean ± SEM) and 2.0 x 10^{-3} ± 0.3 x 10^{-3} absorbance units / min, respectively. The vehicle and VPA control values for the LDH marker were 31 ± 2 % (mean ± SEM) and 54 ± 3 % of total LDH activity, respectively. The vehicle and VPA control values for the DCF marker were 1.4 x 10^{-3} ± 0.2 x 10^{-3} (mean ± SEM) and 2.7 x 10^{-3}± 0.5 x 10^{-3} µmole DCF / min, respectively. See Appendix Figure A-8 for values in reference to the vehicle control.

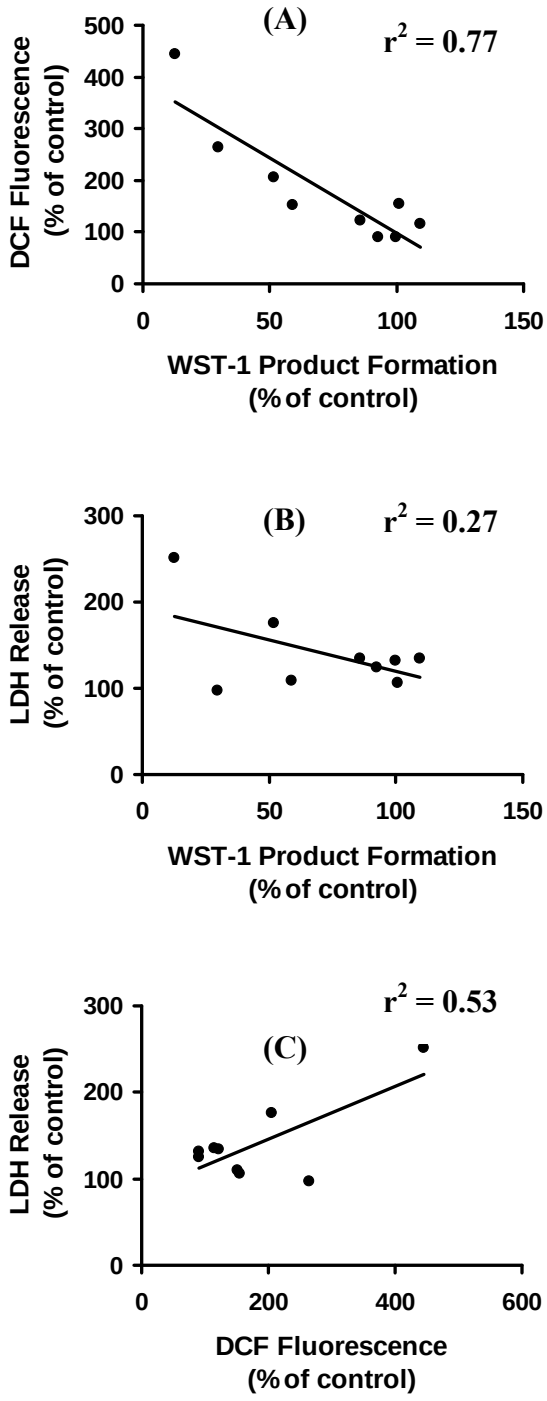

Figure 2-4. Rank correlations of WST-1 product formation, DCF fluorescence, and LDH release in SCRH treated with VPA or VPA metabolites. Rat hepatocytes were exposed to the vehicle (culture medium), VPA or VPA metabolites for 24 hours. The concentrations of exposure were 1 mM (WST-1 assay) and 12 mM (DCF and LDH assays). The individual assays were conducted as described in the Experimental Section. (A) Correlation between the effects of VPA and metabolites on WST-1 product formation and DCF fluorescence (n = 9, $r^2 = 0.77$, $p < 0.05$). (B) Lack of correlation between the effects on the WST-1 marker and LDH release (n = 9, $r^2 = 0.27$, $p > 0.05$). (C) Correlation between the effects on the DCF marker and LDH release (n = 9, $r^2 = 0.53$, $p < 0.05$).

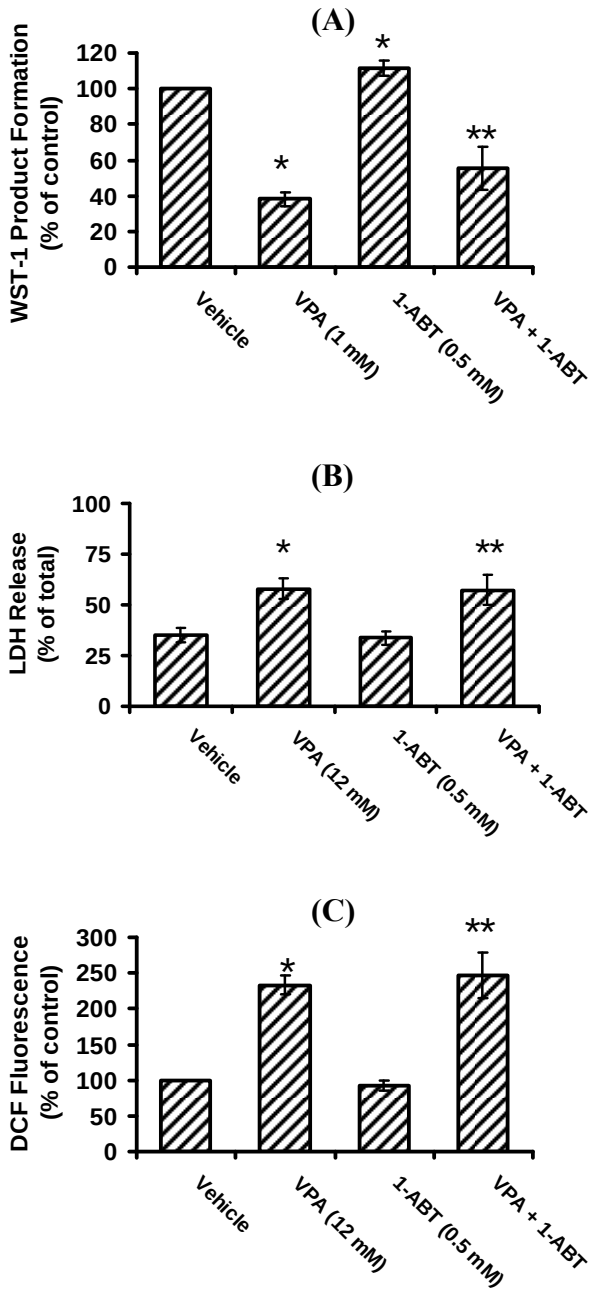

Figure 2-5. Effects of 1-aminobenzotriazole (1-ABT) on VPA-associated (A) WST-1 product formation (N = 4), (B) LDH release (N = 7), and (C) DCF production (N = 5) in SCRH. Rat hepatocytes were pre-treated with the vehicle (culture medium) or 1-ABT (0.5 mM) for 30 minutes prior to treatments with the vehicle, VPA, 1-ABT, or the combination of VPA and 1-ABT for 24 hours. The concentrations of VPA were 1 mM (WST-1 assay) and 12 mM (DCF and LDH assays). The individual assays were conducted as described in the Experimental. Data are presented as the mean ± SEM from the number of individual experiments indicated above. *$p < 0.05$ vs. the vehicle control, **$p < 0.05$ vs. the 1-ABT and vehicle control; based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $3.1 \times 10^{-3} \pm 1.3 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; 0.034 ± 0.003 (supernatant activity) and 0.104 ± 0.011 (total activity) absorption unit / min for the LDH marker; and $1.8 \times 10^{-3} \pm 0.4 \times 10^{-3}$ µmole DCF / min for the DCF marker.

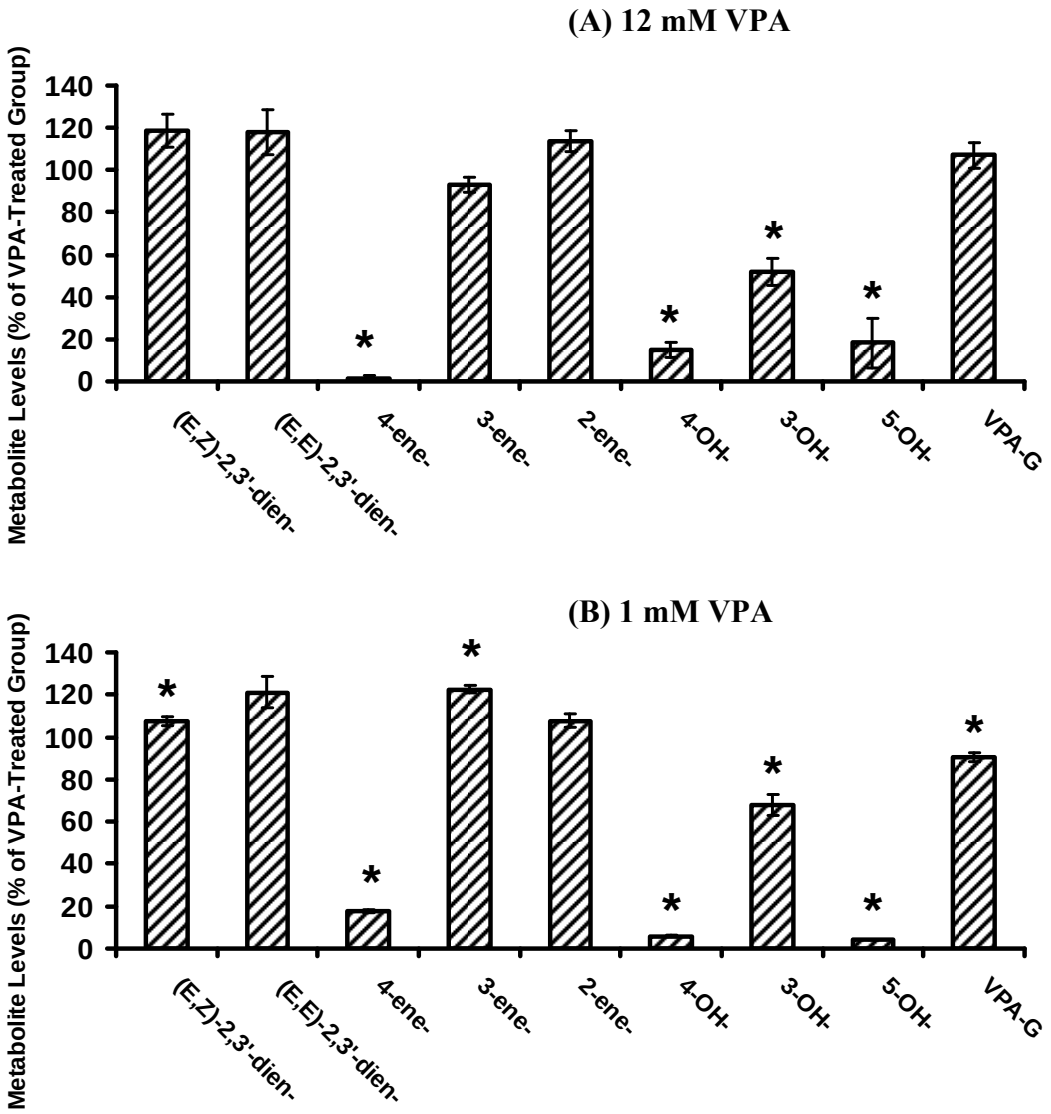

Figure 2-6. Effects of 1-aminobenzotriazole (1-ABT) on levels of VPA metabolites in the supernatant of SCRH. Rat hepatocytes were pre-treated with the vehicle (culture medium) or 1-ABT (0.5 mM) for 30 minutes prior to treatments with the vehicle, VPA, 1-ABT, or the combination of VPA and 1-ABT for 24 hours. The VPA substrate concentrations were (A) 12 mM (N = 6, or N = 4 for VPA-G) and (B) 1 mM (N = 3), which were based on its EC_{50} values for the WST-1, LDH, and DCF assays (Figure 2-2). Levels of VPA-glucuronide (VPA-G) were determined by an LC/MS/MS assay, and of the other metabolites by a GC/MS assay, as described in the Experimental Section. Levels of VPA metabolites from 1-ABT treatment are expressed as percentages of the VPA control and presented as the mean ± SEM from multiple experiments. The control metabolite values for (*E,Z*)-2,3´-diene-, (*E,E*)-2,3´-diene-, 4-ene-, 3-ene-, 2-ene-, 4-OH-, 3-OH-, 5-OH-, and VPA-G were 0.38 ± 0.09 (mean ± SEM), 3.65 ± 1.23, 0.11 ± 0.04, 0.82 ± 0.23, 2.76 ± 0.46, 3.78 ± 0.69, 0.32 ± 0.05, 3.09 ± 0.64, and 94.0 ± 26.0 µM, respectively for the 12 mM VPA; and 0.30 ± 0.04, 1.83 ± 0.34, 0.20 ± 0.03, 1.28 ± 0.35, 2.36 ± 0.32, 1.41 ± 0.18, 0.15 ± 0.03, 2.58 ± 0.29, and 107 ± 6 µM respectively for the 1 mM VPA. See Table 2-1 for the absolute metabolite levels. *p < 0.05 vs. the VPA control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis.

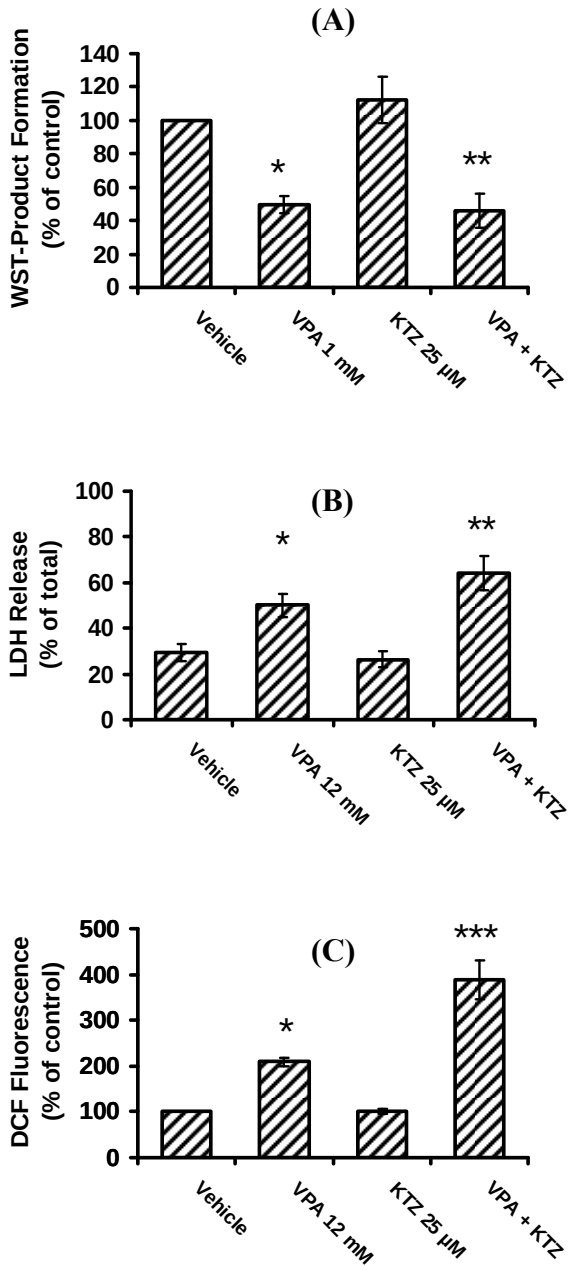

Figure 2-7. Effects of ketoconazole (KTZ) on VPA-associated (A) WST-1 product formation (N = 6), (B) LDH release (N = 10), and (C) DCF production (N = 10) in SCRH. Rat hepatocytes were pre-treated with the vehicle (culture medium containing 0.5 % MeOH v/v) or KTZ (25 µM) for 60 minutes prior to treatments with the vehicle, VPA, KTZ, or the combination of VPA and KTZ for 24 hours. The concentrations of VPA were 1 mM (WST-1 assay) and 12 mM (DCF and LDH assays). The individual assays were conducted as described in the Experimental Section. Data are presented as the mean ± SEM. *p < 0.05 vs. the vehicle control, **p < 0.05 vs. the KTZ and vehicle controls; ***p < 0.05 vs. the KTZ, VPA, and vehicle controls; based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $4.9 \times 10^{-3} \pm 0.9 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; 0.036 ± 0.003 (supernatant activity) and 0.122 ± 0.008 (total activity) absorption unit / min for the LDH marker; and $1.5 \times 10^{-3} \pm 0.2 \times 10^{-3}$ µmole DCF / min for the DCF marker.

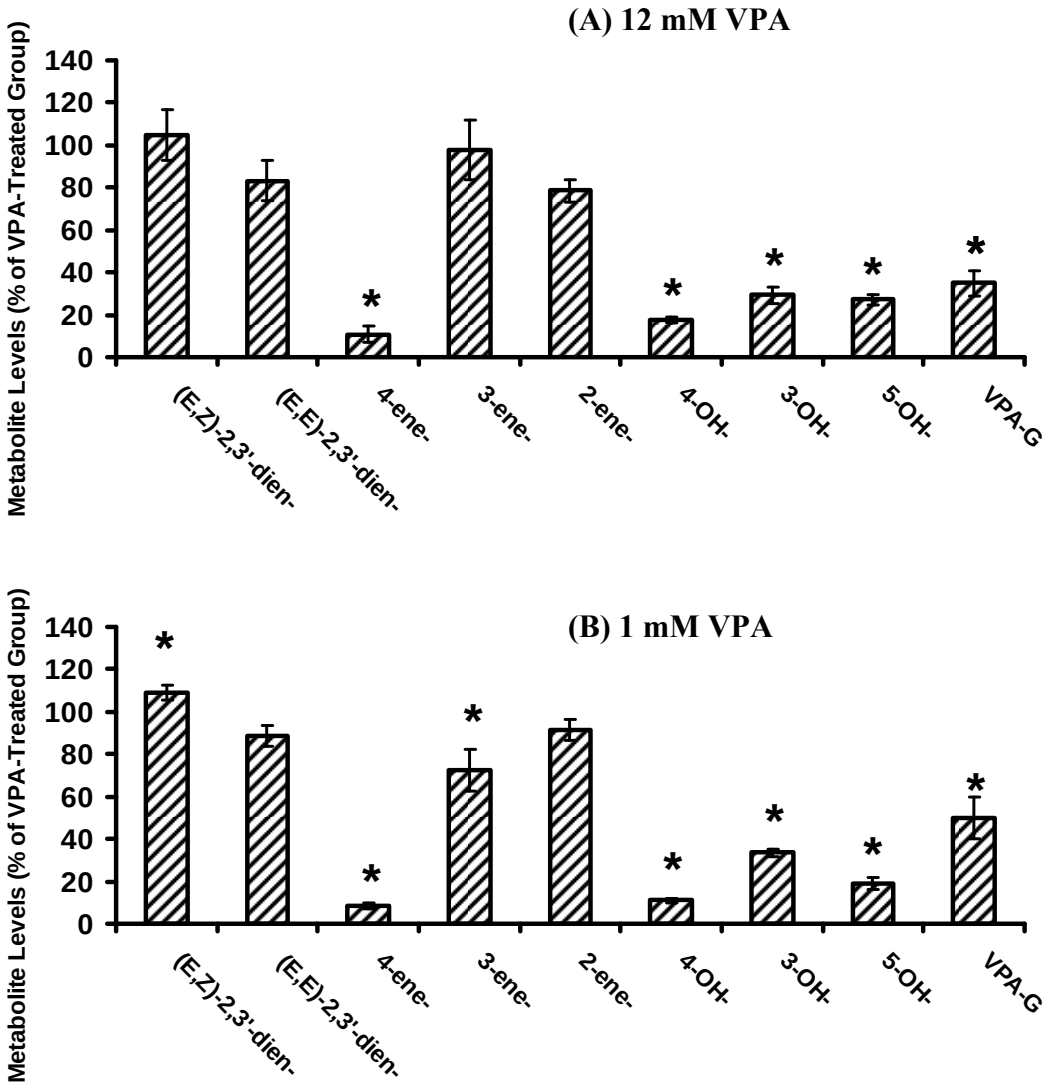

Figure 2-8. Effects of ketoconazole (KTZ) on levels of VPA metabolites in the supernatant of SCRH. Rat hepatocytes were pre-treated with the vehicle (culture medium containing 0.5 % MeOH v/v) or KTZ (25 µM) for 60 minutes prior to treatment with the vehicle, VPA, KTZ, or the combination of VPA and KTZ for 24 hours. The VPA substrate concentrations were (A) 12 mM (N = 11, or N = 10 for VPA-G), and (B) 1 mM (N = 6, or N = 7 for VPA-G), which were based on the EC_{50} values for the WST-1, LDH, and DCF assays (Figure 2-2). Levels of VPA-glucuronide (VPA-G) were determined by an LC/MS/MS assay, and of the other metabolites by a GC/MS assay, as described in the Experimental Section. Levels of VPA metabolites from KTZ treatment are expressed as percentages of the VPA control and presented as the mean ± SEM. The control metabolite values for (*E,Z*)-2,3´-diene-, (*E,E*)-2,3´-diene-, 4-ene-, 3-ene-, 2-ene-, 4-OH-, 3-OH-, 5-OH-, and VPA-G were 0.37 ± 0.04 (mean ± SEM), 2.76 ± 0.30, 0.23 ± 0.04, 2.31 ± 0.34, 2.98 ± 0.31, 3.03 ± 0.43, 0.38 ± 0.05, 2.03 ± 0.19, and 157 ± 15 µM, respectively for the 12 mM VPA; and 0.39 ± 0.03, 3.33 ± 0.14, 0.13 ± 0.03, 3.40 ± 0.05, 2.51 ± 0.17, 0.96 ± 0.17, 0.18 ± 0.01, 1.93 ± 0.33, and 201 ± 17 µM respectively for the 1 mM VPA. See Table 2-2 for the absolute metabolite levels. *p < 0.05 vs. the VPA control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis.

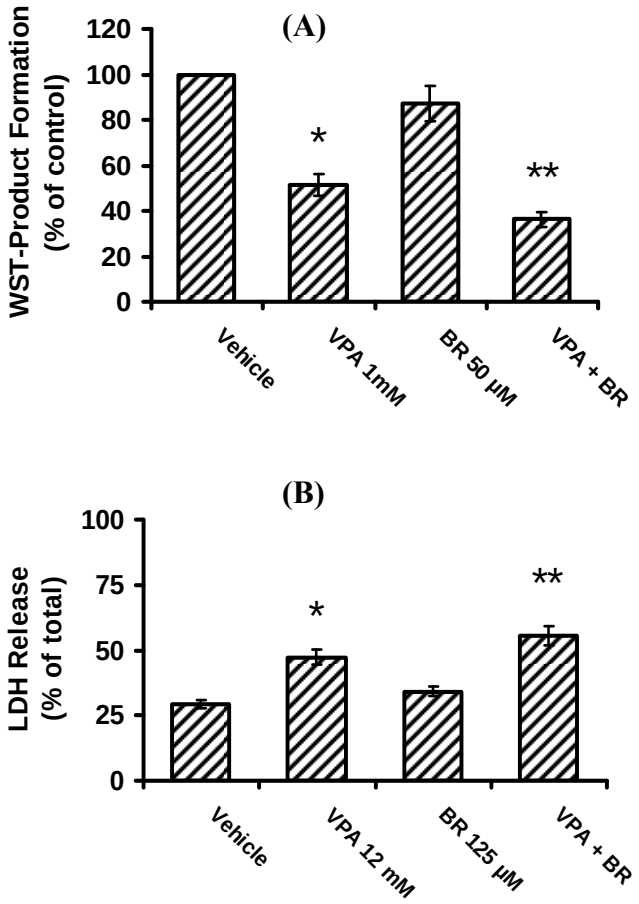

Figure 2-9. Effects (-)-borneol (BR) on VPA-associated (A) WST-1 product formation (N = 5) and (B) LDH release (N = 8) in SCRH. Rat hepatocytes were pre-treated with the vehicle (culture medium containing 0.5 % DMSO v/v) or BR (50 or 125 µM) for 60 minutes prior to treatments with the vehicle, VPA (1 or 12 mM), BR, or the combination of VPA and BR for 24 hours. The individual assays were conducted as described in the Experimental Section. Data are presented as the mean ± SEM from 5 or 8 individual experiments. *p < 0.05 vs. the vehicle control, **p < 0.05 vs. the BR and vehicle; based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $3.2 \times 10^{-3} \pm 0.7 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; and 0.035 ± 0.003 (supernatant activity) and 0.119 ± 0.009 (total activity) absorption unit / min for the LDH marker.

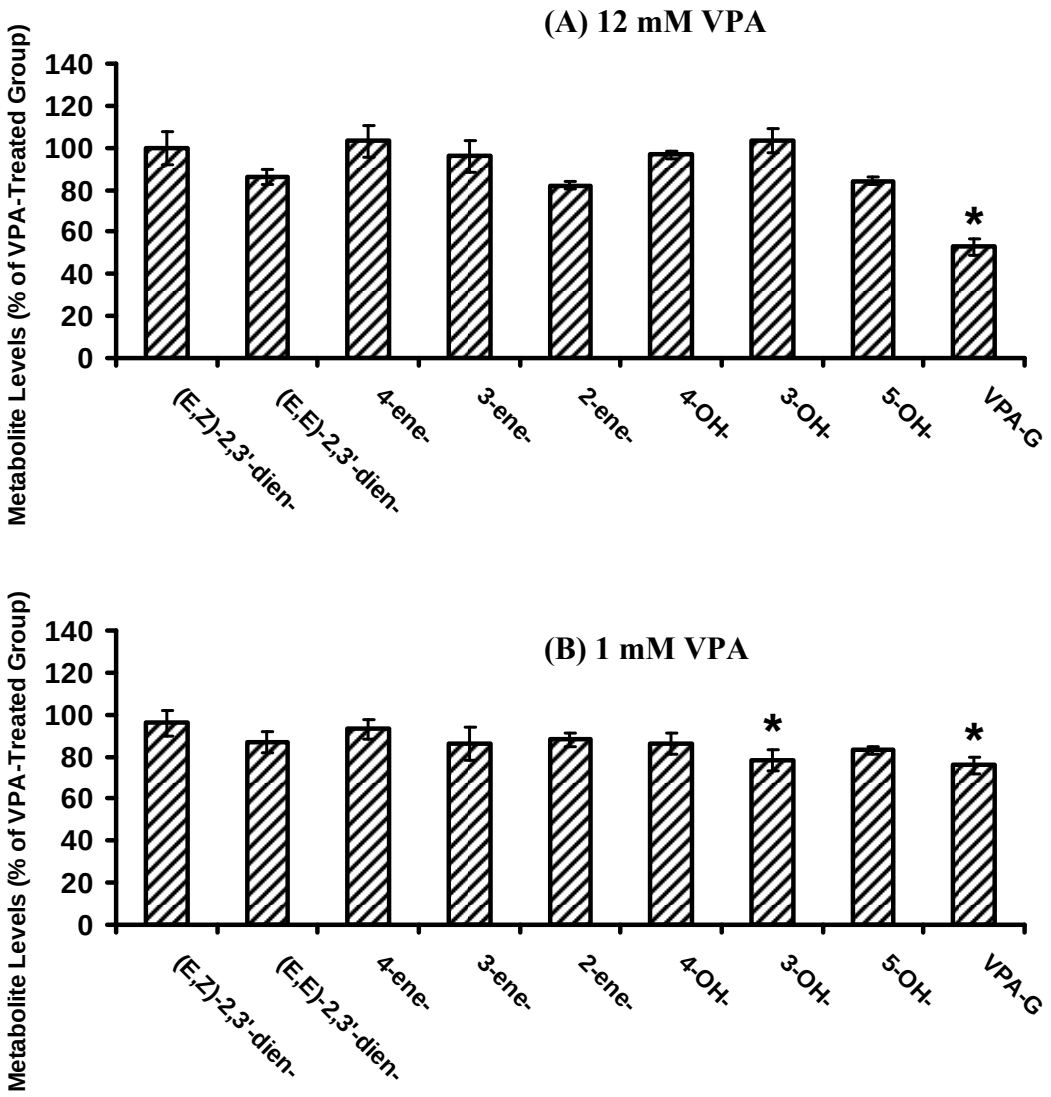

Figure 2-10. Effects of (-)-borneol (BR) on levels of VPA metabolites in the supernatant of SCRH. Rat hepatocytes were pre-treated with the vehicle (culture medium containing 0.5 % DMSO v/v) or BR for 60 minutes prior to treatments with the vehicle, VPA, BR, or the combination of VPA and BR for 24 hours. The VPA and (-)-borneol concentrations were (A) 12 mM and 125 µM (N = 8) and (B) 1 mM and 50 µM (N = 7), respectively. Levels of VPA-glucuronide (VPA-G) were determined by an LC/MS/MS assay, and of the other metabolites by a GC/MS assay, as described in the Experimental Section. Levels of VPA metabolites from BR treatment are expressed as percentages of the VPA control and presented as the mean ± SEM from 7 or 8 individual experiments. The control metabolite values for (*E,Z*)-2,3´-diene-, (*E,E*)-2,3´-diene-, 4-ene-, 3-ene-, 2-ene-, 4-OH-, 3-OH-, 5-OH-, and VPA-G were 0.42 ± 0.05 (mean ± SEM), 3.18 ± 0.27, 0.21 ± 0.03, 2.91 ± 0.20, 3.13 ± 0.26, 2.88 ± 0.40, 0.31 ± 0.03, 2.30 ± 0.20, and 240 ± 17 µM, respectively for the 12 mM VPA; and 0.36 ± 0.05, 3.67 ± 0.29, 0.16 ± 0.03, 4.10 ± 0.25, 2.92 ± 0.25, 1.17 ± 0.26, 0.25 ± 0.04, 3.44 ± 0.58, and 260 ± 16 µM respectively for the 1 mM VPA. See Table 2-3 for the absolute metabolite levels. *p < 0.05 vs. the VPA control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis.

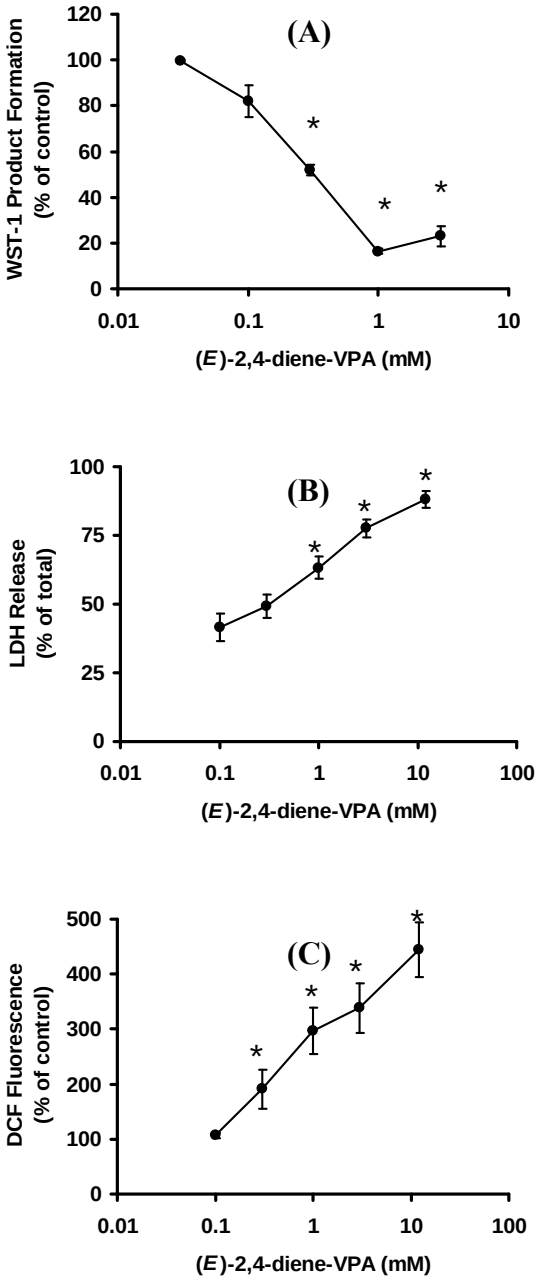

Figure 2-11. Concentration-dependent effects of (*E*)-2,4-diene-VPA on (A) WST-1 product formation, (B) LDH release, and (C) DCF fluorescence in SCRH. Hepatocytes were exposed to the vehicle (culture medium) or various concentrations of (*E*)-2,4-diene-VPA (0 – 12 mM) for 24 hours. The individual assays were conducted as described in the Experimental Section. The data are expressed as the mean ± SEM of 3 individual experiments. The minimum concentrations of (*E*)-2,4-diene-VPA required to induce an effect on the WST-1, LDH, and DCF assays were 0.3 mM, 1 mM, and 0.3 mM, respectively. *p<0.05 vs. the vehicle control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $5.9 \times 10^{-3} \pm 1.4 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; 0.049 ± 0.006 (supernatant activity) and 0.133 ± 0.010 (total activity) absorption unit / min for the LDH marker; and $1.0 \times 10^{-3} \pm 0.1 \times 10^{-3}$ µmole DCF / min for the DCF marker.

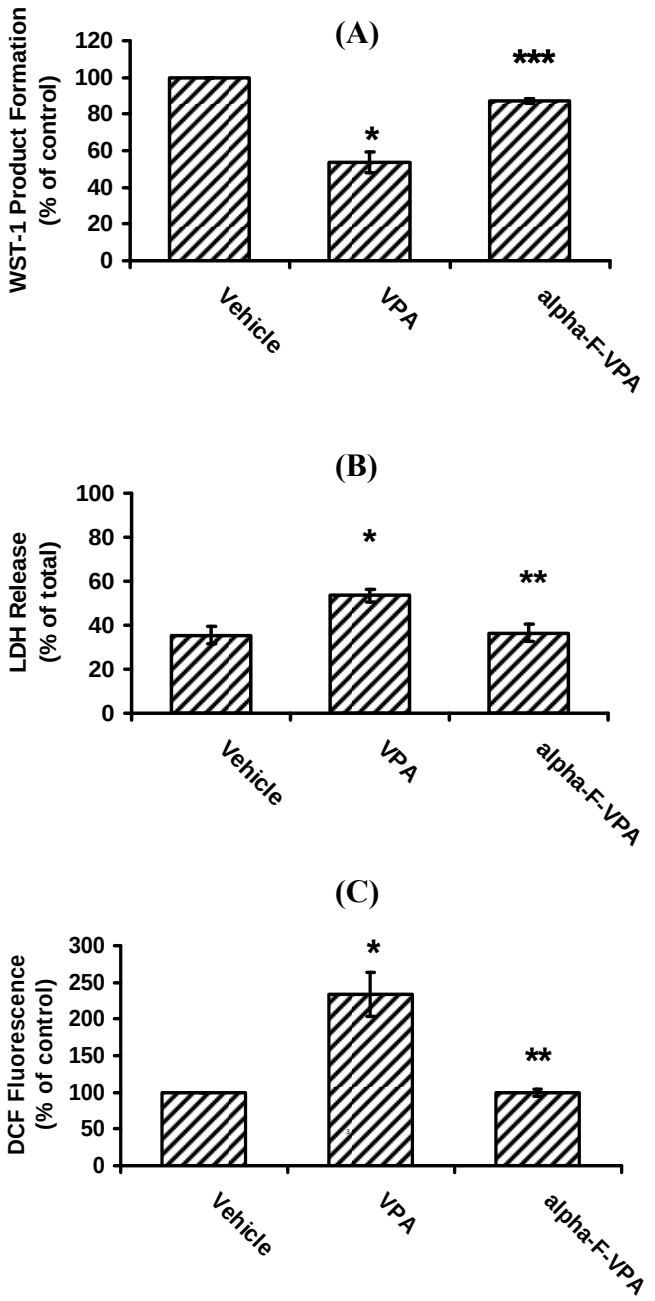

Figure 2-12. Effects of α-F-VPA on the (A) WST-1 (N = 3), (B) LDH (N = 4), and (C) DCF (N = 4) markers in SCRH. Rat hepatocytes were exposed to the vehicle (culture medium), α-F-VPA, or VPA for 24 hours. The concentrations of exposure were 1 mM (WST-1 assay) and 12 mM (DCF and LDH assays). The individual assays were conducted as described in the Experimental Section. The data are presented as the mean ± SEM from 3 or 4 individual experiments. *p < 0.05 vs. the vehicle control, **p < 0.05 vs. VPA, ***p < 0.05 vs. the vehicle and VPA; based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $3.4 \times 10^{-3} \pm 0.3 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; 0.040 ± 0.004 (supernatant activity) and 0.118 ± 0.008 (total activity) absorption unit / min for the LDH marker; and $1.6 \times 10^{-3} \pm 0.4 \times 10^{-3}$ μmole DCF / min for the DCF marker.

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3 Mechanisms of Valproic Acid-Associated Glutathione Depletion and Its Role in the Induction of Mitochondrial Dysfunction, Cytotoxicity, and Oxidative Stress in Sandwich-Cultured Rat Hepatocytes[2]

3.1 Introduction

Valproic acid (VPA) is commonly used in the clinic for the management of various types of epilepsies and neurologic disorders (Johannessen and Johannessen 2003). It is relatively safe in most patients, but in rare occasions may be associated with severe liver injury (Eadie *et al.*, 1988). The mechanism(s) of VPA-induced hepatotoxicity remain unknown, although pathophysiological findings in rat (Sobaniec-Lotowska, 1997; Trost and Lemasters 1996; Coude *et al.*, 1983; Tong *et al.*, 2003; Tong *et al.*, 2005b) or human (Turnbull *et al.*, 1986; Zimmerman and Ishak 1982; Michoulas *et al.*, 2006; Schulpis *et al.*, 2006; Verrotti *et al.*, 2008) models suggest that mitochondrial dysfunction or oxidative stress maybe involved.

VPA is known to affect glutathione (GSH) homeostasis in various experimental models. The acute administration of VPA in rats decreases hepatic GSH in a dose- (Cotariu *et al.*, 1990) and time- (Seckin *et al.*, 1999) dependent manner. Similar effects are observed *in vitro* where VPA reduces the levels of GSH in cultured- (Jurima-Romet *et al.*, 1996) or suspended- (Klee *et al.*, 2000) rat hepatocytes. The *N*-acetylcysteine (NAC) metabolites of VPA, which are generated from the conjugation of VPA metabolites with hepatic GSH, have been identified in the urine of patients receiving therapeutic doses of VPA (Kassahun *et al.*, 1991; Gopaul *et al.*,

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2000a; Gopaul *et al.*, 2000b; Gopaul *et al.*, 2003). Although unproven, the formation of these GSH conjugates could theoretically lead to a reduction of GSH levels. The depletion of glutathione (GSH), an important scavenger of reactive oxygen species or electrophilic xenobiotics (Meister and Anderson 1983; Meister, 1994), might be responsible for the VPA-induced oxidative stress, mitochondrial dysfunction, or hepatotoxicity. In support of a role of abnormal GSH homeostasis in VPA-induced toxicity, elevated levels of the VPA-NAC metabolites were reported in the urine of individuals who have developed VPA-associated hepatotoxicity (Kassahun *et al.,* 1991) and in children who possessed the risk factors of VPA-induced liver injury (Gopaul *et al.,* 2003). However, the relationships between depletion of GSH and induction of mitochondrial dysfunction, oxidative stress, or hepatotoxicity from the administration of VPA have not been systematically studied.

The mechanism(s) by which VPA reduces the levels of hepatic GSH are not known, but evidence suggests that VPA biotransformation in the generation of reactive VPA metabolites might be involved. In support of a role of VPA metabolites in abnormal GSH homeostasis, the ester forms of (*E*)-2,4-diene-VPA were shown to readily conjugate with GSH in a reaction catalyzed by the glutathione-S-transferase (GST) enzymes (Tang and Abbott 1996a; Tang *et al.*, 1996c). Based on this reaction, the GSH-conjugates of (*E*)-2,4-diene-VPA have been identified in rats or rat hepatocytes administered relatively high doses of 4-ene-VPA or (*E*)-2,4-diene-VPA (Kassahun *et al.,* 1991; Kassahun and Abbott, 1993; Kassahun *et al.*, 1994; Tang *et al.*, 1995; Tang and Abbott 1996b; Tang *et al.,* 1996c; Jurima-Romet *et al.,*1996). Consistent with the notion that the formation of GSH conjugates could lead to a reduction of GSH levels, the administration of 4-ene-VPA, which generated GSH-conjugated metabolites (Kassahun and Abbott, 1993; Kassahun *et al.,*1994; Tang *et al.,*1995; Jurima-Romet *et al.,* 1996), also depleted hepatic GSH in rats (Tang *et al.,* 1995; Kassahun *et al.,* 1994) and cultured rat hepatocytes

(Jurima-Romet *et al.,* 1996). However, it is unknown whether the other metabolites of VPA also have an effect on the levels of hepatic GSH.

The role of VPA biotransformation in the reduction of GSH levels was demonstrated by the ß-oxidation of 4-ene-VPA in the production of (*E*)-2,4-diene-VPA (Tang *et al.,* 1995). This is based on the observation in rats where α-F-4-ene-VPA, which could not form the (*E*)-2,4-diene- metabolite, did not deplete hepatic GSH to the same extent as 4-ene-VPA (Tang *et al.,* 1995). However, subsequent experiments also indicated that the α-fluorinated analogues of VPA were also resistant to the formation of the glucuronide- (Tang *et al.*, 1997) or CoA- esters (Grillo *et al.,* 2001), suggesting a potential role for these biotransformation pathways in the depletion of GSH. With this in context, it should be noted that the role of VPA biotransformation in the depletion of GSH has not been systematically investigated. It is also unknown whether the *in situ* generated VPA metabolites from the administration of VPA are responsible for the depletion of GSH.

The present study examined the relationships between depletion of total cellular GSH and induction of mitochondrial dysfunction (WST-1 marker), oxidative stress (DCF marker), or cytotoxicity (LDH marker) in sandwich-cultured rat hepatocytes (SCRH) exposed to VPA. Initial experiments characterized the concentration- and time-dependent effects of VPA on the depletion of total cellular GSH in comparison to the effects of VPA on the WST-1, LDH, and DCF markers. The effects of GSH supplementation on the attenuation of VPA-associated mitochondrial dysfunction, oxidative stress, and cytotoxicity were also examined in SCRH. Furthermore, the abilities of the synthesized VPA metabolites (4-ene-VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 4-keto-VPA, and VPA-glucuronide) to deplete total cellular GSH were compared to that of VPA. Based on these data, quantitative correlations on the extent of oxidative stress, mitochondrial toxicity, and cytotoxicity were determined in relation to the extent of total cellular GSH depletion in SCRH. To further

investigate the role of VPA biotransformation in depletion of total cellular GSH, chemical inhibition experiments were designed to attenuate the CYP-mediated oxidative metabolism or the UGT-mediated glucuronidation of VPA. These VPA biotransformation pathways, or their associated VPA metabolites, are potentially involved in VPA-induced hepatotoxicity (Kingsley *et al.*, 1983; Kesterson *et al.*, 1984; Tang *et al.,* 1995; Tong *et al.*, 2005c). Several novel and intriguing findings from these experiments are discussed.

3.2 Materials and Methods

3.2.1 Chemicals

L-glutathione (reduced, > 98 %, cell culture tested), *meta*-phosphoric acid, and triethanolamine (reagent grade, 98 %) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The total cellular glutathione assay kit was obtained from the Cayman Chemical Company (Ann Arbor, MI, USA). The sources of all other materials used in these experiments were described in Chapter 2 of the present thesis.

3.2.2 Animal Care, Hepatocyte Isolation, Cell Culture, and Drug Treatment

All animal experiments were conducted according to the guidelines published by the University of British Columbia (UBC) Animal Care Committee. Adult male Sprague-Dawley rats (250 – 350 g), housed at 22 ºC with alternate 12 hour dark and light cycles, were provided water and the rat diet *ad libitum* (Labdiet 5001 rodent diet, PMI Feeds, Inc., Richmond IN). Sandwich cultured rat hepatocytes (SCRH) were prepared as described in Chapter 2 of the present thesis. Drug treatments were initiated after the formation of the sandwich culture, 24 hours after the initial plating of the hepatocytes. The concentrations of the test chemicals, the vehicles used, and the exposure periods are indicated in each figure legend. The chemical

inhibition experiments with 1-aminobenzotriazole (1-ABT) or ketoconazole (KTZ) were also described in Chapter 2 of the present thesis.

3.2.3 WST-1, 2´7´-Dichlorofluorescein (DCF), and Lactate Dehydrogenase (LDH) Assays

The WST-1, DCF, and the LDH markers were used for the measurement of mitochondrial function (Berridge *et al.*, 2005; Kikkawa *et al.*, 2005), oxidative stress (Tarpey *et al.*, 2004; Halliwell and Whiteman 2004; Bartosz, 2006), and cytotoxicity (Takeuchi *et al.*, 1988; Buchi *et al.*, 1984; Jurima-Romet *et al.,* 1996; Kingsley *et al.,* 1983) in SCRH, respectively. The specific assay conditions are described in Chapter 2 of the present thesis.

3.2.4 Determination of Total Cellular Glutathione (GSH)

Levels of total cellular glutathione were determined by a commercial kit (Cayman Chemical Company, Ann Arbor, MI) following the principles of the enzyme-recycling method (Tietze, 1969; Eyer and Podhradsky 1986). After drug exposure, hepatocytes were washed twice with warm phosphate buffered saline (pH 7.4) and immediately placed in the -80 ºC freezer for storage prior to the assay. On the day of the assay, cells were harvested in ice chilled phosphate buffered saline (pH 7.4) containing 1 mM of EDTA. Hepatocytes were pelleted by centrifugation (4 ºC, 10,000 x g, 5 minutes) and subsequently sonicated on ice (Sonic Dismembrator 60, Fisher Scientific, Pittsburgh, PA, USA). The resulting suspension was centrifuged at 10,000 x g (4 ºC, 15 minutes) and the supernatant was incubated with an equal volume of *meta*-phosphoric acid (10 % w/v in distilled water) at room temperature for 5 minutes. Subsequently, the mixture was further centrifuged (2,000 x g, 25 ºC, 5 minutes) for protein precipitation, and to the supernatant was added triethanolamine (5% v/v) immediately prior to the determination of GSH levels. To start the enzymatic reaction, a 50 µL aliquot of the processed sample was added to 150 µL of the reaction cocktail (provided by the Cayman

Chemical Company, Ann Arbor, MI, USA). The levels of the reaction product, 5-thio-2-nitrobenzoic acid (TNB) were monitored at regular intervals at an absorption wavelength of 450 nm on a Labsystems Multiskan Ascent® Multiwell plate reader (Thermo Electron Corp., Burlington, ON, Canada). The blank consisted of equal amounts of phosphate buffered saline (pH 7.4), EDTA, *meta*-phosphoric acid, and triethanolamine, in the absence of cells. Rates of TNB formation were calculated (with the blank readings subtracted) and compared to the calibration standards (0.5 – 16 μM, prepared from stock GSH standards provided by the manufacturer) to determine the levels of GSH present in each sample. The standard curves To determine the responsiveness of the assay in SCRH, positive control experiments were conducted with *tert*-butylhydroperoxide, an agent known to deplete glutathione in rat hepatocytes (Buc-Calderon *et al.*, 1991). Negative control experiments were conducted with octanoic acid, a straight chain, 8-carbon carboxylic acid.

3.2.5 Determination of VPA Metabolite Levels

Levels of (*E,E*)-2,3′-diene-VPA, (*E,Z*)-2,3′-diene-VPA, 4-ene-VPA, 3-ene-VPA, (*E*)-2-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA in the culture supernatant were determined by a gas-chromatography-mass-spectrometry (GC/MS) assay as described by Kiang *et al.* (2006). Levels of VPA-glucuronide in the culture supernatant were determined by a newly developed liquid-chromatography-tandem-mass spectrometry (LC/MS/MS) assay described in Chapter 2 of the present thesis.

3.2.6 Statistical Analysis

Data were analyzed by one way analysis of variance (ANOVA) (SigmaStat for Windows, Version 3.5, Systat Software, Inc.). In cases where the assumptions for a parametric test were violated, the Kruskal-Wallis test was used in place for one way ANOVA. The appropriate *post*

hoc analyses are indicated in each figure legend. The EC_{50} value was generated from sigmoidal curve fitting using the Hill-4 parameters equation; $y = yo + [ax^b / (c^b + x^b)]$, (SigmaPlot 2001 for Windows, version 7.0, SPSS, Inc.).

3.3 Results

3.3.1 Concentration-Dependent Effects of VPA on Depletion of Total Cellular Glutathione in Sandwich-Cultured Rat Hepatocytes.

To determine the concentration response of VPA on depletion of total cellular glutathione, hepatocytes were exposed to VPA at various concentrations for a period of 24 hours. VPA decreased total cellular GSH levels in SCRH in a concentration-dependent manner (Figure 3-1) with an EC_{50} value of 12.9 ± 1.3 mM (mean ± SEM, n = 4). The effects of VPA became significant at concentrations ≥ 12 mM when compared to the vehicle control. Treating the hepatocytes with 5 mM *tert*-butylhydroperoxide (positive control) for 24 hours decreased total cellular GSH levels by 98.8 ± 0.4 % (mean ± SEM, n = 4, $p < 0.05$) compared to the vehicle control. On the other hand, 12 mM of octanoic acid (negative control) did not decrease total cellular GSH levels after 24 hours of exposure in SCRH (Appendix, Figure A-9).

3.3.2 Effects of VPA Metabolites on Depletion of GSH in Comparison to VPA.

The effects of the individual synthesized metabolites (4-ene-VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 4-keto-VPA, and VPA-glucuronide) on the reduction of total cellular GSH levels were compared to that of the parent compound in SCRH. The concentration of exposure (12 mM) was based on the EC_{50} value of VPA for the GSH assay after 24 hours of treatment (Figure 3-1). As evident in Figure 3-2, of the metabolites tested, only (*E*)-2,4-diene-VPA was more effective than VPA in decreasing the levels of total cellular GSH in SCRH. All other metabolites tested in this experiment appeared less effective than VPA on the depletion of total cellular GSH, but these results were not statistically

significant based on only three individual experiments.

3.3.3 Temporal Relationships of the Effects of VPA on Mitochondrial Dysfunction (WST-1), Cytotoxicity (LDH), Oxidative Stress (DCF), and GSH Depletion.

To investigate the temporal relationships between depletion of total cellular GSH and induction of mitochondrial dysfunction (WST-1), oxidative stress (DCF), or cytotoxicity (LDH), the effects of VPA on these respective toxicity markers were determined at the following exposure times: 1, 6, 12, 20, and 24 hours. A VPA concentration of 12 mM was used for this experiment because it approximated the EC_{50} values of VPA for the GSH (Figure 3-1), LDH, and DCF markers (Figure 2-2). As evident in Figure 3-3, the effects of VPA on depletion of total cellular GSH were initially evident after 6 hours of exposure, whereas significant LDH release was not observed until 12 hours of VPA treatment. On the other hand, significant changes in DCF fluorescence or WST-1 product formation were evident after 1 hr of VPA exposure, and the levels of DCF remained constant between 1 – 24 hours.

3.3.4 Effects of Glutathione Supplementation on Attenuation of VPA-Associated Mitochondrial Dysfunction (WST-1), Cytotoxicity (LDH), and Oxidative Stress (DCF).

To further investigate the role of GSH homeostasis in VPA-induced toxicity, the effects of glutathione supplementation on VPA-associated mitochondrial dysfunction (WST-1), cytotoxicity (LDH), and oxidative stress (DCF) were studied in SCRH. The concentrations of exposure of VPA were based on the EC_{50} values for the individual assays (i.e. 12 mM for the LDH and DCF assays; 1 mM for the WST-1 assay; Figure 2-2). Preliminary experiments with equal molar concentrations *N*-acetylcysteine, L-cysteine, glutathione-ethyl ester, or L-glutathione, which are precursors to GSH (Griffith, 1999), indicated that supplementation with L-glutathione for 24 hours was the most effective regimen for the elevation of levels of total

cellular GSH in SCRH (Appendix, Figure A-10). As evident in Figure 3-4, positive control experiment with 12 mM of VPA treatment depleted the levels of total cellular GSH by approximately 50%, whereas L-glutathione elevated the levels of total cellular GSH in a concentration-dependent manner by an average of 0.7 fold (0.5 mM L-glutathione), 1.1 fold (1 mM), 2.8 fold (2.5 mM), or 5.4 fold (5 mM) when compared to the vehicle control. Similar fold increases of total cellular GSH were also obtained when L-glutathione was administered to the 12 mM VPA-treated hepatocytes (Figure 3-4).

As evident in Figure 3-5, L-glutathione, when administered up to 5 mM for 24 hours, did not attenuate the effects of VPA on the WST-1 or the LDH markers in SCRH. On the other hand, L-glutathione attenuated the VPA-associated DCF fluorescence in a dose-dependent manner, with the maximum concentration of L-glutathione (5 mM) leading to a reduction of DCF fluorescence to levels comparable to that of the vehicle control (Figure 3-5). However, L-glutathione alone also attenuated the basal DCF fluorescence, but the effects were small and apparently independent of concentration. At the 1 mM concentration, L-glutathione did not affect the basal DCF fluorescence, yet attenuated the effects of VPA on DCF fluorescence. Thus our data would suggest that VPA-induced oxidative stress, as measured by the DCF marker, is apparently attenuated by the exogenous supplementation of GSH in SCRH.

3.3.5 Rank Correlations between the Extent of Mitochondrial Dysfunction (WST-1), Cytotoxicity (LDH), Oxidative Stress (DCF), and GSH Depletion.

To investigate the association between total cellular GSH depletion and the induction of hepatotoxicity, correlations between the extent of mitochondrial dysfunction (WST-1), oxidative stress (DCF), or cytotoxicity (LDH) were determined in relation to the extent of total cellular GSH depletion in SCRH exposed to VPA or the individual synthesized metabolites of VPA (n = 9). The concentrations of exposure were 1 mM for the WST-1 assay, and 12 mM for the DCF,

LDH, and GSH assays. As evident in Figure 3-6, the correlation between the extent of WST-1 product formation and depletion of total cellular GSH was weak and not statistically significant ($r^2 = 0.23$, $p = 0.196$). On the other hand, significant correlations were observed between DCF formation and total cellular GSH depletion ($r^2 = 0.50$, $p = 0.033$) and between LDH release and total cellular GSH depletion ($r^2 = 0.89$, $p < 0.001$).

3.3.6 Effects of 1-Aminobenzotriazole and Ketoconazole on VPA-Associated GSH Depletion.

To determine whether CYP-mediated metabolism of VPA was associated with depletion of total cellular GSH, inhibition experiments were conducted with 1-aminobenzotriazole (1-ABT), a broad-spectrum, mechanism-based inactivator of CYP enzymes (Ortiz de Montellano and Mathews 1981). SCRH were pre-exposed to 1-ABT (0.5 mM) or vehicle (culture medium) for 30 minutes prior to exposure with the vehicle, 1-ABT (0.5 mM), VPA (12 mM), or the combination of VPA and 1-ABT for 24 hours. This treatment regimen of 1-ABT completely attenuated the production of the oxidative metabolites (i.e. 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA) without drastically affecting the levels of the other metabolites from the ß-oxidation or the glucuronidation of VPA (Figure 2-6). This specific 1-ABT treatment regimen also did not affect the basal levels of total cellular GSH in SCRH.

To examine whether the glucuronidation of VPA was associated with the depletion of total cellular GSH, chemical inhibition experiments were conducted with ketoconazole (KTZ), which attenuates both the CYP-mediated oxidative metabolism and the UGT-mediated glucuronidation of VPA (Figure 2-8). The hepatocytes were pre-exposed to KTZ (25 µM) or the vehicle (culture medium containing 0.5 % MeOH) for 60 minutes prior to exposure with the vehicle, KTZ (25 µM), VPA (12 mM), or the combination of VPA and KTZ for 24 hours. This KTZ treatment regimen completely attenuated the CYP-mediated oxidative metabolism and

partially reduced the UGT-mediated glucuronidation of VPA, without drastically affecting the levels of the ß-oxidation metabolites (Figure 2-8). KTZ alone reduced the levels of total cellular GSH at concentrations exceeding 25 μM in SCRH (Appendix, Figure A-11), thus limiting our use of higher concentrations of KTZ to maximally inhibit the formation of the VPA-glucuronide. Unfortunately, the effects of another glucuronidation inhibitor, (-)-borneol, were not determined, since this inhibitor reduced the levels of total cellular GSH at all concentrations required for the inhibition of VPA-glucuronide formation in SCRH (Appendix, Figure A-12). As evident in Figure 3-7, neither 1-ABT nor KTZ treatments attenuated the effects of VPA on the depletion of total cellular GSH in SCRH. On the other hand, KTZ enhanced the effects of VPA on the depletion of total cellular GSH (Figure 3-7).

3.3.7 Concentration-Dependent Effects of (*E*)-2,4-diene-VPA on Depletion of GSH.

Based on Figure 3-2, (*E*)-2,4-diene-VPA was the only metabolite tested that was more effective than VPA on the depletion of total cellular GSH in SCRH. In order to determine the potency of (*E*)-2,4-diene-VPA on the depletion of total cellular GSH, hepatocytes were exposed to various concentrations of the metabolite (0 – 12 mM) for 24 hours. As evident in Figure 3-8, (*E*)-2,4-diene-VPA decreased the levels of total cellular GSH in a concentration-dependent manner, and the effects of (*E*)-2,4-diene-VPA became significant at concentrations ≥ 0.1 mM when compared to the vehicle control. This corresponds to a 120-fold increase in potency when compared to the effects of VPA on the same marker (Figure 3-1).

3.3.8 Effects of α-F-VPA on Depletion of GSH.

To further investigate the role of biotransformation in VPA-associated total cellular GSH depletion, the effects of α-F-VPA were compared to that of VPA in SCRH. α-F-VPA can not form the (*E*)-2,4-diene-metabolite (Tang *et al.,*1995) and is resistant to the formation of the

glucuronide- (Tang *et al.,*1997) or CoA-esters (Grillo *et al.,* 2001). SCRH were exposed to the vehicle (culture medium), VPA (12 mM), or α-F-VPA (12 mM) for 24 hours. The concentration was based on the EC_{50} value of VPA for the GSH assay in SCRH (Figure 3-1). As evident in Figure 3-9, α-F-VPA treatment slightly elevated the levels of total cellular GSH, whereas VPA decreased the total cellular GSH levels by 58 ± 7% (n = 4, mean ± SEM), when compared to the vehicle control.

3.4 Discussion

GSH is an important cellular defence against reactive oxygen species or electrophilic xenobiotic intermediates (Meister, 1983; Meister, 1994). VPA is known to reduce the levels of hepatic GSH in various experimental models (Cotariu *et al.,* 1990; Seckin *et al.,* 1999; Jurima-Romet *et al.,* 1996; Klee *et al.,* 2000), but the consequences of GSH depletion in the development of VPA-associated hepatotoxicity have not been thoroughly investigated. Mitochondrial dysfunction or oxidative stress are associated with the administration of VPA (Sobaniec-Lotowska, 1997; Trost and Lemasters, 1996; Coude *et al.,* 1983; Tong *et al,* 2003; Tong *et al.,* 2005b; Turnbull *et al.,* 1986; Zimmerman and Ishak, 1982; Michoulas *et al.,* 2006; Schulpis *et al.,* 2006; Verrotti et al., 2008), and they have been suggested as potential mechanism(s) of VPA-induced hepatotoxicity (Fromenty and Pessayre 1995; Chang and Abbott 2006). The current research attempted to determine the relationships between the depletion of GSH and the induction of mitochondrial dysfunction, oxidative stress, or cytotoxicity from VPA treatment in a novel model of sandwich cultured rat hepatocytes (SCRH). We are also the first to systematically study the roles of VPA metabolites or VPA biotransformation in the depletion of total cellular GSH in SCRH. Several novel findings are discussed.

VPA decreased the levels of total cellular GSH in SCRH in a concentration-dependent manner with an EC_{50} value close to 12 mM (Figure 3-1). Dose-dependent effects of VPA on the

depletion of liver GSH levels have been demonstrated by Cotariu *et al.* (1990) in rats, and the EC_{50} value of total cellular GSH depletion from VPA treatment in SCRH is consistent with the observation from Jurima-Romet et al. (1996) in rat hepatocytes using comparable treatment conditions. The EC_{50} value of VPA on the depletion of total cellular GSH is similar to the effects observed of VPA on the release of lactate dehydrogenase (LDH) and the increase of 2′7′-dichlorofluorescein (DCF) in SCRH (Figure 2-2). The similar EC_{50} values of VPA on these markers would suggest that the depletion of total cellular GSH could be associated with the induction of oxidative stress or cytotoxicity in SCRH. On the other hand, the observed effects of VPA on mitochondrial function, as assessed by the WST-1 marker (Figure 2-2), were much more potent than its effects on the depletion of total cellular GSH. The implications of these findings are further discussed below.

We are the first to comprehensively characterize the effects of several synthesized VPA metabolites on the depletion of total cellular GSH in a single experimental model. Of the metabolites investigated in this study, only (*E*)-2,4-diene-VPA was more effective than VPA in depletion of total cellular GSH when tested on an equal molar basis (Figure 3-2). Consistent with this observation, (*E*)-2,4-diene-VPA was relatively more toxic than VPA with respect to markers of mitochondrial dysfunction (WST-1), oxidative stress (DCF), and cytotoxicity (LDH) in SCRH (Figure 2-3). Based on these data, the effects of (*E*)-2,4-diene-VPA on depletion of total cellular GSH may be associated with its effect on these other markers of toxicity, although further studies are needed to establish the cause-effect relationship. Another rational explanation for the effect of (*E*)-2,4-diene-VPA on total cellular GSH depletion is perhaps the tendency for the activated (*E*)-2,4-diene-VPA to conjugate with glutathione in a reaction catalyzed by GST enzymes (Tang *et al.,* 1996c). Based on this reaction, GSH conjugates of (*E*)-2,4-diene-VPA have been identified in the bile and urine of rats dosed with relatively high doses of 4-ene-VPA or (*E*)-2,4-diene-VPA (Kassahun and Abbott, 1993; Kassahun *et al.,*1994; Tang *et al.,* 1995),

and *N*-acetylcysteine conjugates have been found in the urine of human subjects receiving therapeutic doses of VPA (Kassahun *et al.,* 1991; Gopaul *et al.,* 2000b; Gopaul *et al.,* 2003). In support of the notion that the extent of GSH conjugate formation might be directly correlated with the extent of total cellular GSH depletion, levels of 5-GS-3-ene-VPA, a GSH conjugate of (*E*)-2,4-diene-VPA (Kassahun and Abbott, 1993), were found to be several-fold higher in SCRH exposed to (*E*)-2,4-diene-VPA than in hepatocytes exposed to its immediate precursors, 4-ene-VPA or (*E*)-2-ene-VPA (Appendix, Figure A-10). Obviously, further experiments incorporating the measurements of all known GSH conjugates of VPA (Kassahun *et al.,* 1994; Tang and Abbott F.S., 1996a) are needed to further explore the relationships between GSH conjugate formation and GSH depletion.

Jurima-Romet *et al.* (1996) also characterized the effects of both 4-ene-VPA and VPA on depletion of total cellular GSH in cultured rat hepatocytes. Although a direct comparison was not intended, VPA appeared slightly more effective than 4-ene-VPA on the depletion of total cellular GSH in their *in vitro* model (Jurima-Romet *et al.,* 1996). On the contrary, 4-ene-VPA was more effective than VPA in depleting GSH in rat liver homogenate (Kassahun *et al.,* 1994), suggesting a discrepancy in the observed effects of 4-ene-VPA between the *in vitro* and *in vivo* rat models. Consistent with this discrepancy, 4-ene-VPA is also more toxic than VPA, on the induction of hepatic steatosis, *in vivo* in rats (Kesterson *et al.,* 1984) and less toxic than VPA, on LDH release, *in vitro* in cultured-rat hepatocytes (Jurima-Romet *et al.,* 1996; Kingsley *et al.,* 1983). Likewise, 4-ene-VPA was equally or less toxic than VPA with respect to the WST-1, LDH, and DCF markers in our *in vitro* model of SCRH (Figure 2-3). The rationale for the observed discrepancies on the toxicities of 4-ene-VPA between the *in vitro* and *in vivo* models is not readily apparent. One might suggest that the physical limitations of cultured hepatocytes, where large amounts of 4-ene-VPA can not be cleared from the supernatant, may limit the

expression of 4-ene-VPA-associated toxicity, although more experiments are needed to investigate this proposed mechanism.

In the same study by Kassahun *et al.* (1994), (*E*)-2-ene-VPA was no more effective than VPA in depletion of GSH in the rat liver homogenate, which is also consistent with our results in SCRH (Figure 3-2). To the best of our knowledge, no one else has reported on the abilities of the oxygenated metabolites or VPA-glucuronide on the depletion of hepatic GSH. The minimal effects of these metabolites on total cellular GSH depletion (Figure 3-2) are consistent with their relative lack of toxicities with respect to the WST-1, DCF, and LDH markers in SCRH (Figure 2-3). Unlike (*E*)-2,4-diene-VPA, GSH conjugates of the oxygenated metabolites or VPA-glucuronide have not been reported, supporting the notion that GSH conjugate formation is a likely factor in GSH depletion. To test this hypothesis, studies are needed to determine whether treatments of oxygenated metabolites or VPA-glucuronide are associated with the formation of GSH conjugates in SCRH.

To further explore the relationships between the depletion of GSH levels and VPA-induced hepatotoxicity, the temporal relationships between the effects of VPA on markers of mitochondrial dysfunction, cytotoxicity, or oxidative stress were characterized in relation to total cellular GSH depletion in SCRH. Based on Figure 3-3, the effects of VPA on the DCF and WST-1 markers were evident after 1 hour of treatment, which preceded any evidence of total cellular GSH depletion (> 6 hours of VPA exposure), whereas a significant increase in LDH release was not detected until 12 hours of VPA treatment. The relatively early effects of VPA on DCF increase in SCRH is consistent with the findings of Tong *et al.* (2005b) where VPA elevated the levels of DCF and 15-F_{2t}-isoprostane, a marker of lipid peroxidation, after only 30 minutes of exposure. The levels of DCF from VPA treatment appeared to stay elevated after the initial rise in SCRH (Figure 3-3), which is in agreement with the similar temporal profile of the VPA-induced 15-F_{2t}-isoprostane elevation in the rat liver (Tong *et al.*, 2005a).

Our data on the early occurrence of mitochondrial dysfunction, as determined by the WST-1 marker, in SCRH exposed to VPA is consistent with the *in vivo* observation where early changes in the ultrastructure of the mitochondria were evident in the hepatocytes of rats chronically administered VPA (Sobaniec-Lotowska *et al.*, 1997). The relative late occurrence of LDH increase, a marker of hepatocyte necrosis, in SCRH exposed to VPA is also consistent with the *in vivo* observation where severe hepatic necrosis in rats was only evident after prolonged periods (i.e. 14 days) of VPA treatment (Tong *et al.,* 2005a). To the best of our knowledge, we are the first to explore the temporal relationships of VPA-associated mitochondrial dysfunction, cytotoxicity, or oxidative stress with respect to depletion of total cellular GSH in a single experimental model. The temporal sequence of the effects of VPA on these markers (Figure 3-3) would suggest that oxidative stress (DCF) and mitochondrial dysfunction (WST-1) are potential contributors, and cytotoxicity (LDH) a potential consequence, of VPA-associated total cellular GSH depletion in SCRH. To further study the role of GSH homeostasis in VPA-induced toxicity, attempts were also made to determine whether supplementation of exogenous GSH would attenuate the toxicity associated with VPA in SCRH.

Initial experiments demonstrated that L-glutathione was able to elevate, in a concentration-dependent manner, the total cellular GSH levels in SCRH (Figure 3-4). As demonstrated in Figure 3-5, L-glutathione, at all concentrations tested, did not attenuate the effects of VPA on the WST-1 marker in SCRH. This is supported by the observation in the literature where the effects of VPA on the WST-1 marker were mostly unaffected in GSH-depleted rat hepatocytes compared to the healthy controls (Tong *et al.,* 2005b). As evident in Figure 3-6, no correlation was obtained between the extent of WST-1 product formation and total cellular GSH depletion in SCRH exposed to VPA and its metabolites. As well, the concentration-dependent effects of VPA on mitochondrial function (Figure 2-2) were also several orders of magnitude less than the effects of VPA on total cellular GSH depletion (Figure

3-1). These data, in addition to the time-dependent effects of VPA on markers of mitochondrial toxicity and total cellular GSH depletion (Figure 3-3), would seem to suggest that VPA-associated total cellular GSH depletion in SCRH is unlikely a factor responsible for the induction of mitochondrial dysfunction.

In contrast, the EC_{50} value of VPA on total cellular GSH depletion (Figure 3-1) correlated with the EC_{50} value of VPA on LDH increase in SCRH (Figure 2-2). A strong correlation ($n = 9$, $r^2 = 0.89$) was also obtained between the extent of LDH release and total cellular GSH depletion in SCRH exposed to VPA or its metabolites (Figure 3-6). These observations, taken together with the temporal data where depletion of total cellular GSH preceded LDH elevation (Figure 3-3), would suggest that VPA-associated total cellular GSH depletion could be a potential factor for the induction of cytotoxicity in SCRH. On the other hand, despite data suggesting association between total cellular GSH depletion and induction of cytotoxicity, it was rather unexpected that L-glutathione supplementation did not attenuate the effects of VPA on LDH release in SCRH (Figure 3-5). These observations are consistent with the findings by Jurima-Romet *et al.* (1996) where rat hepatocytes depleted of GSH were not more susceptible to the cytotoxic effects of VPA than normal, healthy hepatocytes. Although these findings would suggest that glutathione depletion, *per se*, is not responsible for the VPA-induced cytotoxicity in cultured rat hepatocytes, depletion of total cellular GSH, in combination with other factors known to induce liver injury, may still contribute to the hepatotoxicity of VPA.

To support a multi-factoral hypothesis where depletion of total cellular GSH plays a partial role in VPA-induced cytotoxicity, depletion of total cellular GSH, in conjunction with pre-treatment of phenobarbital (PB), were shown to enhance VPA-induced LDH release in cultured rat hepatocytes (Jurima-Romet *et al.,* 1996). Conversely, neither depletion of total cellular GSH or pre-treatment of PB alone enhanced VPA-induced LDH release in the same model (Jurima-Romet *et al.,* 1996). PB pre-treatment is known to increase the levels of 4-ene-

VPA, a putative toxic metabolite (e.g. Rettie *et al.*, 1988; Loscher *et al.*, 1993; Tong *et al.,* 2003). One can hypothesize that the depletion of GSH, in conjunction with another factor (e.g. enhanced production of a toxic VPA metabolite from PB pre-treatment), together would contribute to the VPA-induced cytotoxicity. Further experiments are needed to test the hypothesis that VPA-induced GSH depletion plays a significant role in the VPA-induced cytotoxicity.

As evident in Figure 3-5, L-glutathione attenuated VPA-induced DCF fluorescence in a concentration-dependent manner in SCRH. Even though L-glutathione treatment alone reduced the basal DCF activity, the effect was minimal and apparently independent of concentration. The ability of L-glutathione to attenuate the VPA-induced DCF fluorescence, suggesting an association between abnormal glutathione homeostasis and the induction of oxidative stress, is supported by the strong correlation between the extent of total cellular GSH depletion and DCF formation obtained from the treatments of VPA and VPA metabolites (Figure 3-6). Our data are also consistent with the findings of Tong *et al.* (2005b) where rat hepatocytes deficient of GSH generated higher levels of DCF or 15-F_{2t}-isoprostane, a marker of lipid peroxidation, from VPA exposure compared to the normal controls. However, it is unlikely that depletion of total cellular GSH was responsible for the induction of oxidative stress, because the time course experiment indicated that the VPA-induced DCF fluorescence preceded any evidence of total cellular GSH depletion in SCRH (Figure 3-3). Rather, the temporal relationship between these two markers would suggest that GSH plays a defensive role against VPA-induced oxidative stress in SCRH. The attenuating effects of exogenously administered GSH on VPA-induced oxidative stress could simply be attributed to the antioxidant properties of GSH (Meister et al., 1994) or the ability of GSH to scavenge the electrophilic intermediates produced from the metabolism of VPA. Further mechanistic studies are warranted to test these possibilities.

A limitation of the current study is that GSH-depleted hepatocytes were not used. Preliminary experiments using buthionine-sulfoximine (BSO) and diethyl maleate (DEM) were carried out to deplete total cellular GSH in SCRH. BSO inhibits γ-glutamylcysteine synthetase (Griffith, 1981), a rate limiting enzyme for GSH synthesis, whereas DEM directly conjugates with GSH in a reaction mediated by the GST enzymes (Maellaro *et al.*, 1990; Plummer *et al.*, 1981). Because BSO or DEM resulted in toxicity in SCRH, further work is needed to establish conditions that would allow one to effectively reduce cellular GSH levels in the absence of toxicity. As well, one could use 1-bromoheptane, which has been suggested as an effective agent suitable for the depletion of GSH (Khan *et al.*, 1991). On the other hand, culture mediums (e.g. Eagle's Essential Medium or Williams E Medium) in the absence of added amino acids (cysteine, methionine) required for GSH synthesis can be used to complement the chemical modulator approach. Overall, these mechanistic approaches would allow one to determine whether cellular glutathione plays a role in VPA-associated toxicity in cultured hepatocytes.

To further understand the mechanism(s) associated with the effects of VPA on depletion of total cellular GSH, the role of VPA biotransformation was investigated for the first time. VPA is extensively metabolized (Abbott *et al.*, 1999) and CYP-generated metabolites, such as 4-ene-VPA or (*E*)-2,4-diene-VPA, might be associated with total cellular GSH depletion because their subsequent metabolites are capable of conjugating with hepatic GSH (Kassahun and Abbott, 1993; Kassahun *et al.,* 1994; Tang *et al.,* 1995; Tang *et al.,* 1996c). As well, UGT-mediated glucuronidation of VPA is of toxicological interest because the formation of VPA-glucuronide was apparently associated with induction of lipid peroxidation in rats administered VPA (Tong *et al.,* 2005c). The rationale behind the use of 1-ABT or KTZ, which inhibited the CYP- and UGT- mediated biotransformation of VPA, has already been discussed in Chapter 2. To recap, both 1-ABT and KTZ completely attenuated the CYP-mediated oxidative metabolism of VPA, and KTZ itself partially attenuated (~ 50%) the formation of the VPA-glucuronide (Figure 2-6;

Figure 2-8). Similar to their effects on the WST-1, DCF, and LDH markers, 1-ABT or KTZ did not attenuate the effects of VPA on total cellular GSH depletion in SCRH (Figure 3-7). On the other hand, KTZ actually enhanced the effects of VPA on total cellular GSH depletion (Figure 3-7), which is consistent, and perhaps associated, with the potentiating effects of KTZ on VPA-induced DCF formation (Figure 2-7). Overall, these data seem to suggest that CYP-mediated biotransformation or UGT-mediated glucuronidation of VPA was not associated with depletion of total cellular GSH in SCRH exposed to VPA. The same can be said of the *in situ* generated 4-ene-VPA or VPA glucuronide, because the co-administration of chemical inhibitors, which attenuated their production from VPA, did not reduce the effects of VPA on total cellular GSH levels in SCRH.

Based on Figure 3-2, (*E*)-2,4-diene-VPA was the only synthesized metabolite tested in the present study that was more effective than VPA in depleting total cellular GSH. For the following reasons, however, it is unlikely that the *in situ* generated (*E*)-2,4-diene-VPA from the administration of VPA would have played a role in the depletion of total cellular GSH in SCRH. The minimum concentration of (*E*)-2,4-diene-VPA required to deplete total cellular GSH in the SCRH model was 0.1 mM (Figure 3-8), which is many fold higher than the trace amounts of (*E*)-2,4-diene-VPA generated from the administration of VPA to cultured rat hepatocytes. As discussed in Chapter 2, the use of 1-ABT, a broad spectrum CYP inhibitor, would have further inhibited the formation of (*E*)-2,4-diene-VPA, because this metabolite was produced directly or indirectly by the actions of the CYP enzymes (Abbott and Anari, 1999). Treatment of 1-ABT did not attenuate the effects of VPA on the depletion of total cellular GSH, and this would lead one to conclude that (*E*)-2,4-diene-VPA did not play a role in the VPA-associated total cellular GSH depletion in SCRH. The lack of detection of (*E*)-2,4-diene-VPA in SCRH could be attributed to the reactivity of the metabolite toward glutathione or cellular proteins (Tang *et al.,* 1996c) and the possibility that the (*E*)-2,4-diene-VPA underwent covalent binding as fast as it

was generated *in situ* in SCRH. To test this hypothesis, one could quantify all known GSH conjugates of (*E*)-2,4-diene-VPA (Kassahun *et al.,* 1991; Kassahun *et al.,* 1994; Tang and Abbott 1996a) as surrogates for the metabolite, or determine the extent of cellular protein binding using radio-labelled derivatives of VPA.

In addition to the use of chemical inhibitors, we also studied the effects of α-F-VPA, which is resistant to the normal bioactivation reactions of VPA, in SCRH. The use of α-F-VPA was based on the rationale that this analogue could not form the (*E*)-2,4-diene- metabolite (Tang *et al.,* 1995) and is resistant to the formation of the glucuronide or Co-enzyme A (CoA) esters (Tang *et al.,*1997; Grillo *et al.*, 2001). Based on Figure 3-9, the α-F-VPA was significantly less effective in the depletion of total cellular GSH compared to VPA, which is consistent with the findings obtained with its effects on the WST-1, DCF, and LDH markers (Figure 2-12). It is not clear why α-F-VPA treatment would elevate the levels of total cellular GSH in SCRH over the vehicle control (Figure 3-9), although this apparent effect on the elevation of hepatic GSH was also observed *in vivo* in the α-F-4-ene-VPA-treated rats (Tang *et al.,* 1995). Based on the results of our chemical inhibition experiments, it can be suggested that the *in situ* generation of (*E*)-2,4-diene-VPA or VPA-glucuronide was not associated with the VPA-induced total cellular GSH depletion in SCRH. These findings, in conjunction with known characteristics of the α-F-VPA, would seem to suggest that the CoA ester of VPA, or the formation thereof, is potentially responsible for the depletion of total cellular GSH in SCRH. As well, it may be the acyl CoA esters of VPA metabolites that are responsible for the VPA-associated total cellular GSH depletion. In support of this concept, a CoA type ester of (*E*)-2,4-diene-VPA has been shown to be reactive toward hepatic GSH in a reaction catalyzed by the GST enzymes (Tang *et al.,* 1996c). The reactivity of the acyl CoA esters of the other VPA metabolites, however, remains to be established.

Overall, our chemical inhibition experiments suggest that CYP-mediated oxidative metabolism is not likely associated with the VPA-induced total cellular GSH depletion in SCRH. Mechanism(s) other than biotransformation may also contribute to the effects of VPA on the depletion of total cellular GSH. For example, reduced activities of glutathione reductase (Cotariu *et al.,* 1990; Cotariu *et al.*, 1992), increased activities of glutathione peroxidase (Kurekci *et al.*, 1995; Cengiz *et al.*, 2000; Graf *et al.*, 1998), or altered levels of trace elements serving as the co-factors for these enzymes (Graf *et al.,* 1998), associated with VPA treatment could all lead to a reduction of GSH levels. The effects of VPA on these GSH regulatory systems of GSH synthesis, however, are inconclusive due to the inconsistent data reported by different investigators (Verrotti *et al.*, 2002; Kurekci *et al.,* 1995; Graf *et al.,* 1998; Cengiz *et al.,* 2000; Cotariu *et al.,* 1992; Seckin *et al.,* 1999). Further studies are needed to address these possibilities.

In conclusion, we report the novel finding that exogenously added (*E*)-2,4-diene-VPA has a potent effect on total cellular GSH depletion in SCRH. Total cellular GSH depletion associated with VPA treatment does not appear to be a factor in the induction of mitochondrial dysfunction, but may play a partial role in VPA-induced cytotoxicity in SCRH. On the other hand, total cellular GSH may play a protective role against VPA-induced oxidative stress. Finally, our results appear to demonstrate that the *in situ* CYP-mediated oxidative metabolism of VPA to 4-ene-VPA or (*E*)-2,4-diene-VPA and the UGT-mediated glucuronidation of VPA to VPA glucuronide are not associated with depletion of total cellular GSH in VPA-treated SCRH.

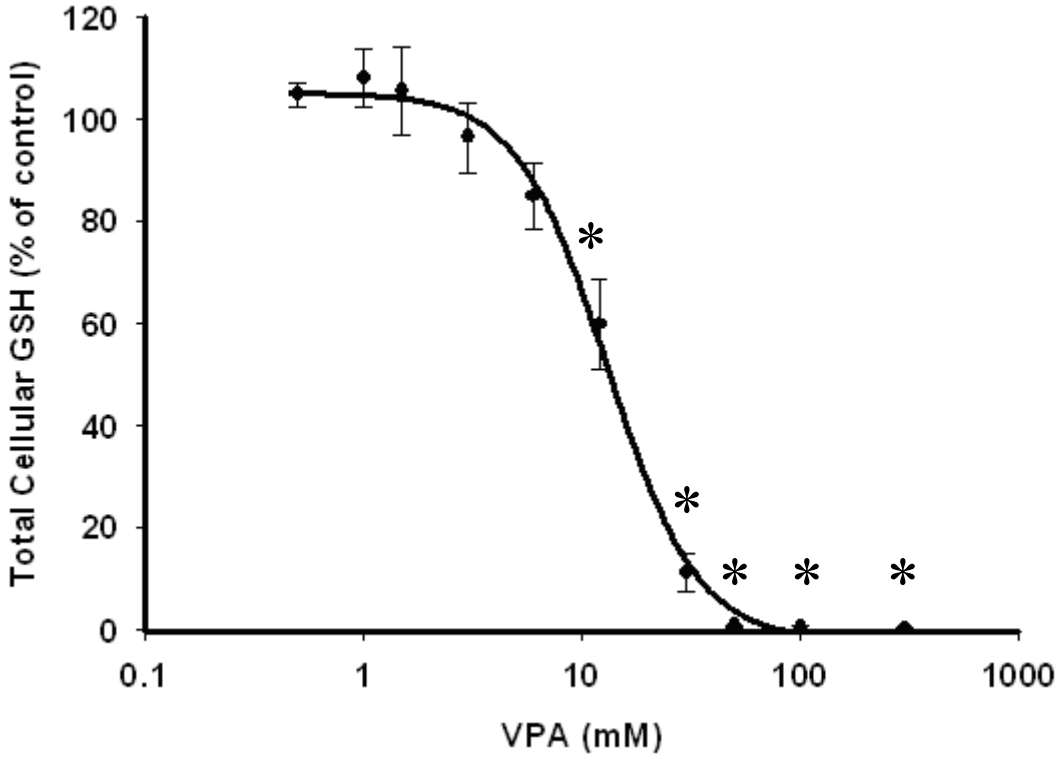

Figure 3-1. Concentration-dependent effects of VPA on the depletion of total cellular GSH in SCRH. Hepatocytes were exposed to the vehicle (culture medium), or various concentrations of VPA (0 – 300 mM) for 24 hours. The GSH assay was conducted as described in the Experimental Section. The EC_{50} value of the effect of VPA on total cellular GSH depletion was 12.9 ± 1.3 mM (mean ± SEM, n = 4), and the effects of VPA became significant at concentrations ≥ 12 mM. *$p < 0.05$ vs. the vehicle control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total cellular GSH levels in the vehicle-treated cells were 8.88 ± 0.89 µM (mean ± SEM).

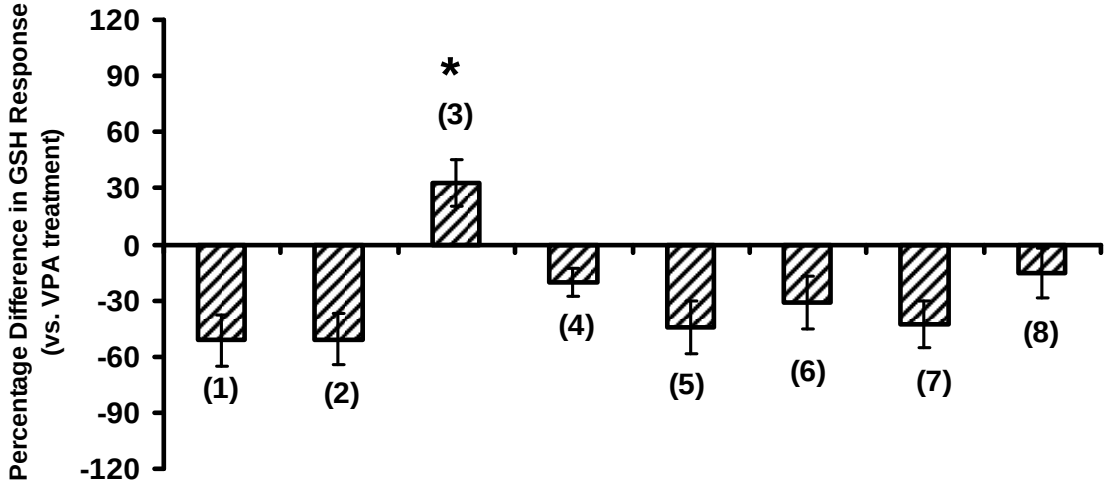

Figure 3-2. Effects of (1) 4-ene-VPA, (2) (*E*)-2-ene-VPA, (3) (*E*)-2,4-diene-VPA, (4) 5-OH-VPA, (5) 4-OH-VPA, (6) 4-keto-VPA, (7) 3-OH-VPA, and (8) VPA-glucuronide on the depletion of total cellular GSH relative to the parent compound in SCRH. Rat hepatocytes were exposed to the vehicle (culture medium), VPA metabolites, or VPA for 24 hours. The concentration of exposure was 12 mM, based on the EC_{50} value of VPA for the GSH assay (Figure 3-1). The GSH assay was conducted as described in the Experimental Section and the data are expressed as the difference (in percentage points) of the effects of each metabolite compared to that of VPA (mean ± SEM, n = 3). Positive values indicate enhanced toxicity (vs. VPA). Negative values indicated reduced toxicity relative to VPA. *p < 0.05 vs. the baseline control (i.e. VPA treatment) based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total cellular GSH levels in the vehicle-treated cells were 7.50 ± 0.90 µM (mean ± SEM) and in the VPA-treated cells were 3.50 ± 0.60 µM. . See Appendix Figure A-13 for values in reference to the vehicle control.

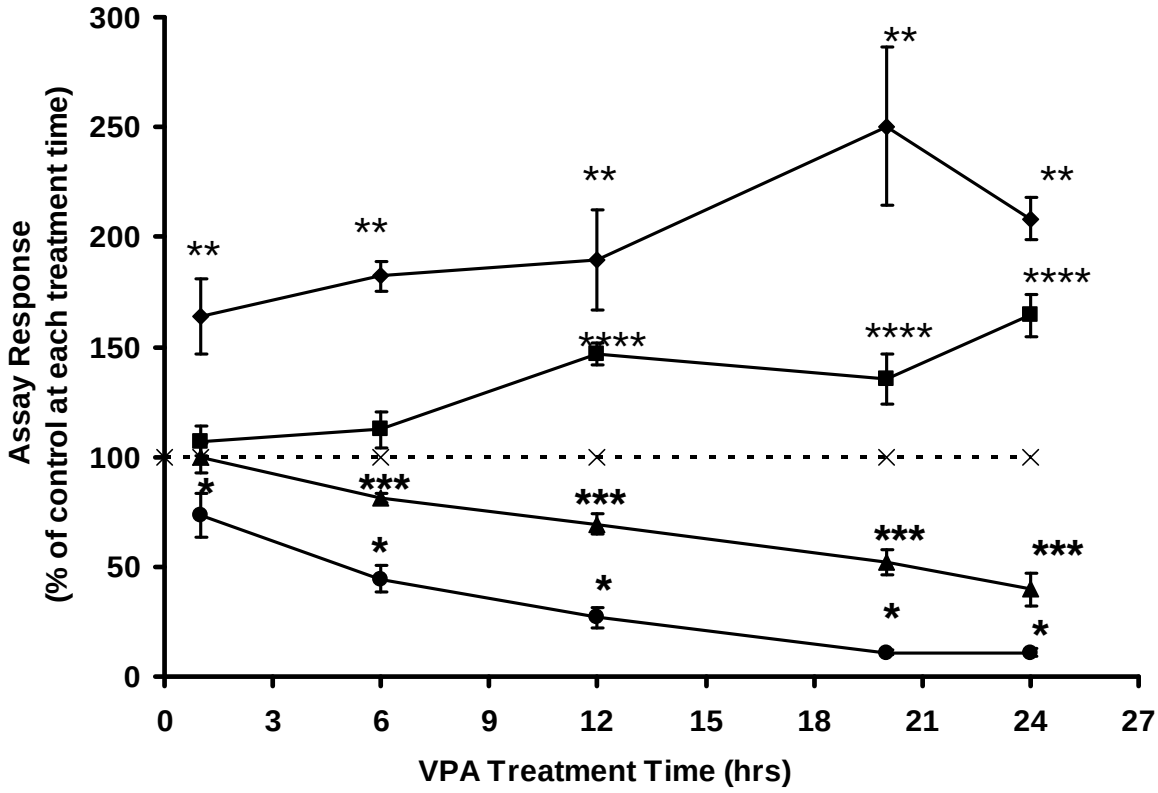

Figure 3-3. Temporal relationships of the effects of VPA on mitochondrial dysfunction (WST-1 ●, N = 3**), cytotoxicity (LDH ■,** N = 4**), oxidative stress (DCF ♦,** N = 3**), and GSH (▲,** N = 4**) depletion in SCRH. Hepatocytes were exposed to the vehicle (culture medium) or 12 mM VPA for 1, 6, 12, 20, and 24 hours. The individual assays were conducted as described in the Experimental Section. The effects of VPA became significant at 1 hr, 1 hr, 6 hr, and 12 hrs for the WST-1, DCF, GSH, and LDH markers, respectively. Data are expressed as the percentage of the vehicle control at each treatment time for the individual markers (mean ± SEM). *p < 0.05 vs. the vehicle control for the WST-1 marker; **p < 0.05 vs. the control for the DCF marker; ***p < 0.05 vs. the control for the GSH marker; and ****p < 0.05 vs. the control for the LDH marker, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. For the WST-1 marker, the vehicle control values were 6.7 x 10^{-3} ± 0.1 x 10^{-3} (1 hr treatment, mean ± SEM) absorption unit / min, 6.0 x 10^{-3} ± 0.1 x 10^{-3} (6 hr treatment), 5.4 x 10^{-3} ± 0.1 x 10^{-3} (12 hr treatment), 4.9 x 10^{-3} ± 0.0 x 10^{-3} (20 hr treatment), and 3.6 x 10^{-3} ± 0.1 x 10^{-3} (24 hr treatment). For the DCF marker, the vehicle control values were 1.6 x 10^{-3} ± 0.4 x 10^{-3} (1 hr treatment, mean ± SEM) µmole DCF / min, 1.6 x 10^{-3} ± 0.2 x 10^{-3} (6 hr treatment), 2.8 x 10^{-3} ± 0.1 x 10^{-3} (12 hr treatment), 1.6 x 10^{-3} ± 0.4 x 10^{-3} (20 hr treatment), and 2.1 x 10^{-3} ± 0.5 x 10^{-3} (24 hr treatment). For the GSH marker, the vehicle control values were 10.0 ± 1.4 (1 hr treatment, mean ± SEM) µM, 9.7 ± 1.3 (6 hr treatment), 9.8 ± 1.3 (12 hr treatment), 9.9 ± 1.5 (20 hr treatment), and 10.0 ± 1.5 (24 hr treatment). For the LDH marker, the vehicle control values were 7.4 ± 1.1 % of total LDH activity (1 hr treatment, mean ± SEM), 19.6 ± 4.4 % (6 hr treatment), 22.0 ± 3.7 % (12 hr treatment), 34.2 ± 5.7 % (20 hr treatment), and 33.1 ± 4.0 % (24 hr treatment). Graphical representations of the data are illustrated in Appendix A, Figure A-14.**

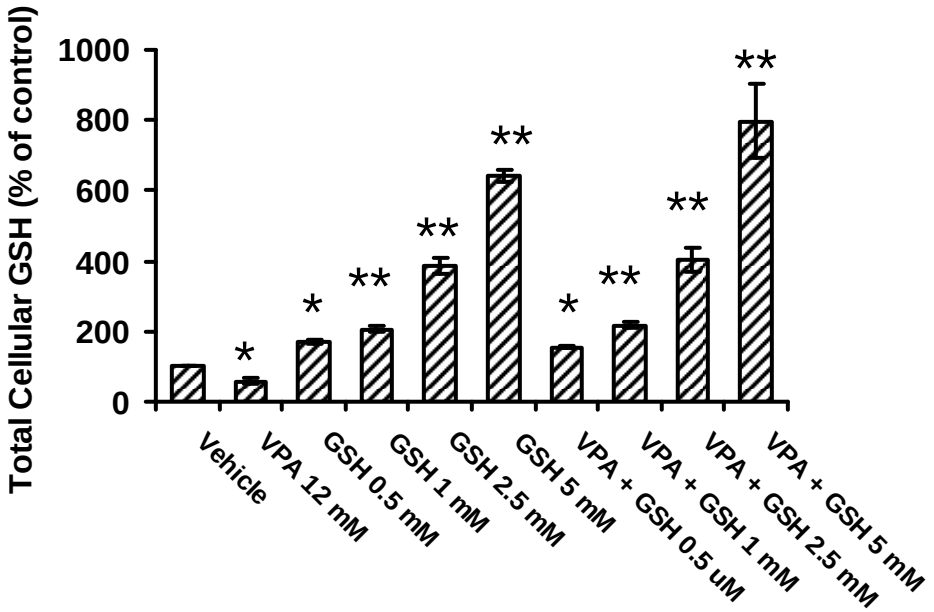

Figure 3-4. Concentration-dependent effects of L-glutathione (GSH) on the elevation of total cellular GSH levels in SCRH. Hepatocytes were exposed to the vehicle (culture medium), 12 mM VPA (as a positive control), various concentrations of GSH (0.5, 1, 2.5, or 5 mM) or the combination of VPA and GSH for 24 hours. The GSH assay was conducted as described in the Experimental Section and the data are expressed as the percentage of the vehicle control (mean ± SEM, n = 6). *$p < 0.05$ vs. the vehicle control; **$p < 0.05$ vs. the vehicle control and equivalent treatments groups (i.e. VPA or vehicle) with lower concentrations of L-glutathione, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total cellular GSH levels in the vehicle-treated cells were 10.9 ± 1.2 µM (mean ± SEM).

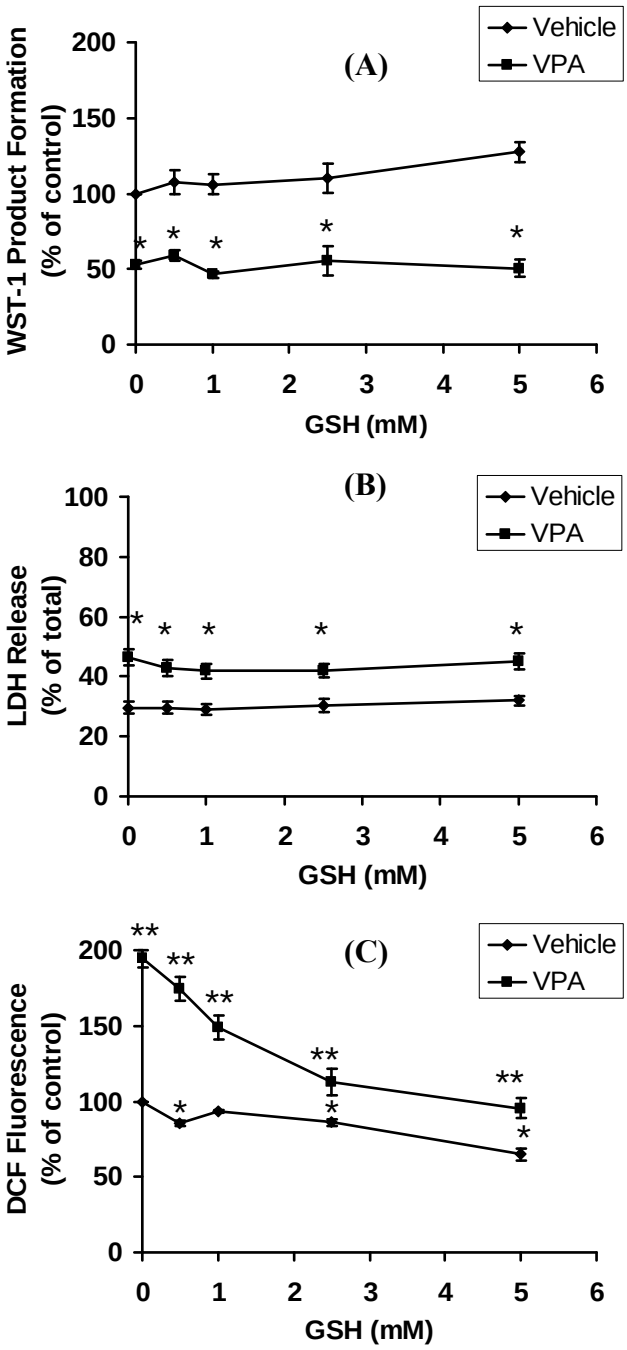

Figure 3-5. Concentration-dependent effects of L-glutathione (GSH) on the (A) WST-1 (N = 6), (B) LDH (N = 7), and (C) DCF (N = 5) markers in SCRH treated with the vehicle or VPA. Hepatocytes were exposed to the vehicle (culture medium), 1 mM VPA (for the WST-1 assay), 12 mM VPA (for all other assays), GSH (0.5, 1, 2.5, and 5 mM), or the combination of VPA and GSH for 24 hours. The WST-1, LDH, and DCF assays were conducted as described in the Experimental Section, and the data are expressed as the percentage of the vehicle control (mean ± SEM). *p < 0.05 vs. the vehicle control; **P < 0.05 vs. all other treatment groups, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $3.8 \times 10^{-3} \pm 0.6 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; 0.035 ± 0.004 (supernatant activity) and 0.115 ± 0.007 (total activity) absorption unit / min for the LDH marker; and $1.3 \times 10^{-3} \pm 0.2 \times 10^{-3}$ µmole DCF / min for the DCF marker.

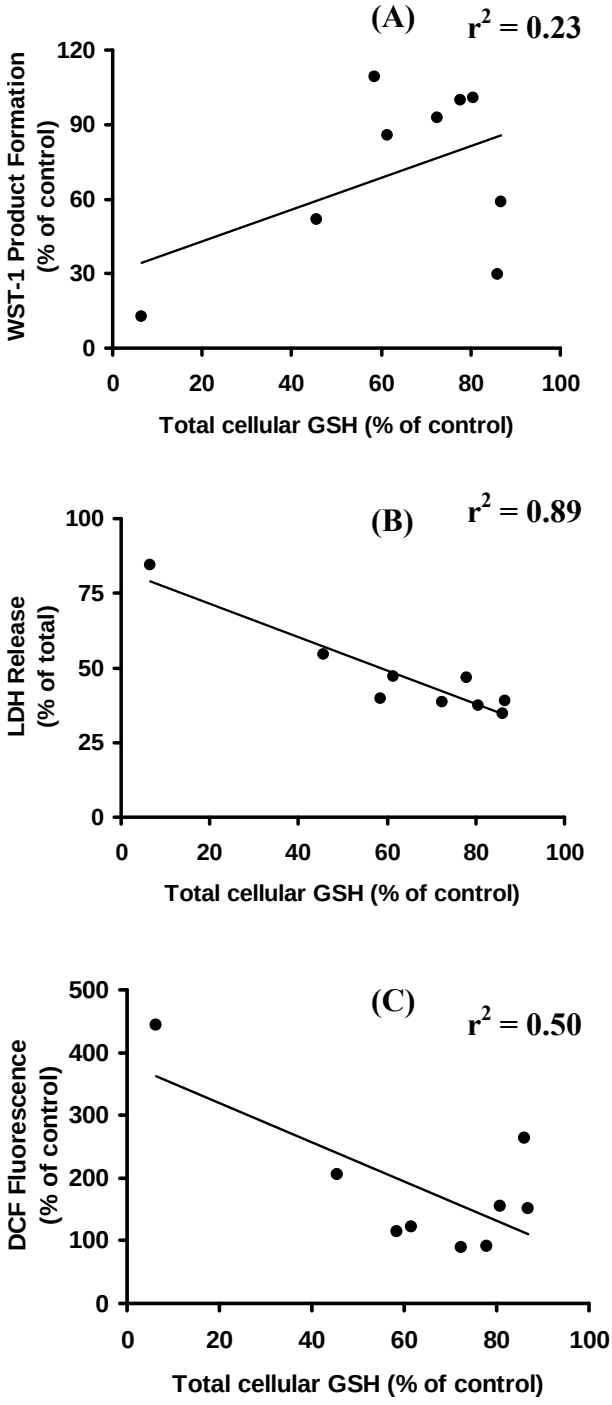

Figure 3-6. Rank correlations between the extent of (A) WST-1 product formation, (B) LDH release, and (C) DCF fluorescence with respect to the depletion of total cellular GSH in SCRH treated with VPA and eight VPA metabolites. Hepatocytes were exposed to the vehicle (culture medium), VPA, or the individual VPA metabolites for 24 hours. The concentrations of exposure were 1 mM (WST-1 assay) and 12 mM (DCF, LDH, and GSH assays). The individual assays were conducted as described in the Experimental Section. (A) Lack of correlation between WST-1 product formation and total cellular GSH depletion ($n = 9$, $r^2 = 0.23$, $p > 0.05$). (B) Significant correlation between LDH release and total cellular GSH depletion ($n = 9$, $r^2 = 0.89$, $p < 0.05$). (C) Significant correlation between DCF fluorescence and total cellular GSH depletion ($n = 9$, $r^2 = 0.50$, $p < 0.05$).

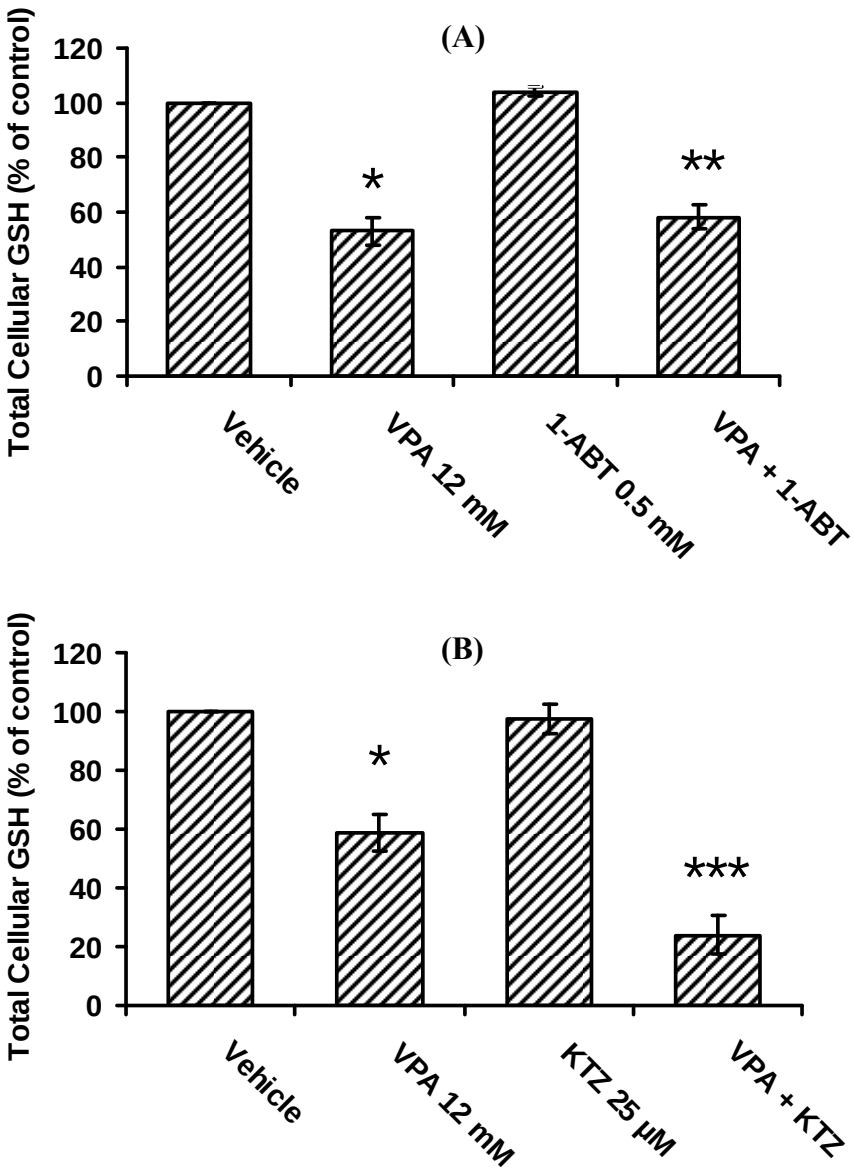

Figure 3-7. Effect of 1-aminobenzotriazole (1-ABT) or ketoconazole (KTZ) on VPA-associated depletion of total cellular GSH in SCRH. Hepatocytes were pre-treated with the vehicle, 1-ABT (0.5 mM) for 30 minutes, or KTZ (25 µM) for 60 minutes prior to treatments with vehicle, VPA, 1-ABT, KTZ, VPA + 1-ABT, or VPA + KTZ for 24 hours. The concentration of VPA was 12 mM, which was based on the EC_{50} value of VPA for the depletion of total cellular GSH in SCRH (Figure 3-1). The 1-ABT was dissolved in the culture medium, whereas KTZ was dissolved in the culture medium containing 0.5 % of MeOH. The GSH assay was conducted as described in the Experimental Section and the data are presented as the percentage of the vehicle control (mean ± SEM) from 6 (Figure A) to 8 (Figure B) individual experiments. *$p < 0.05$ vs. the vehicle control, **$p < 0.05$ vs. the vehicle and 1-ABT control, ***$p < 0.05$ vs. the vehicle, VPA, and KTZ control; based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total cellular GSH levels in the vehicle-treated cells were 8.7 ± 1.0 µM (mean ± SEM) for Figure 3-7A and 8.1 ± 1.0 µM for Figure 3-7B.

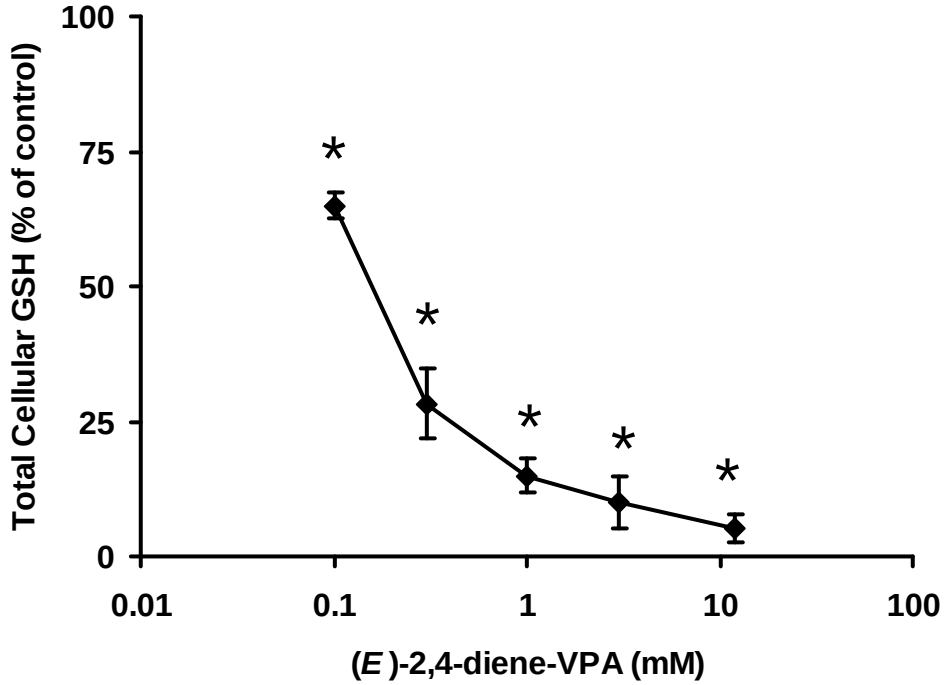

Figure 3-8. Concentration-dependent effect of (*E*)-2,4-diene-VPA on the depletion of total cellular GSH in SCRH. Hepatocytes were exposed to the vehicle (culture medium) or various concentrations of (*E*)-2,4-diene-VPA (0 – 12 mM) for 24 hours. The GSH assay was conducted as described in the Experimental Section, and the data are expressed as the mean ± SEM from 3 individual experiments. The minimum concentration of (*E*)-2,4-diene-VPA tested that had an effect on total cellular GSH depletion was 0.1 mM. *$p < 0.05$ vs. the vehicle control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total cellular GSH levels in the vehicle-treated cells were 6.4 ± 1.8 µM (mean ± SEM).

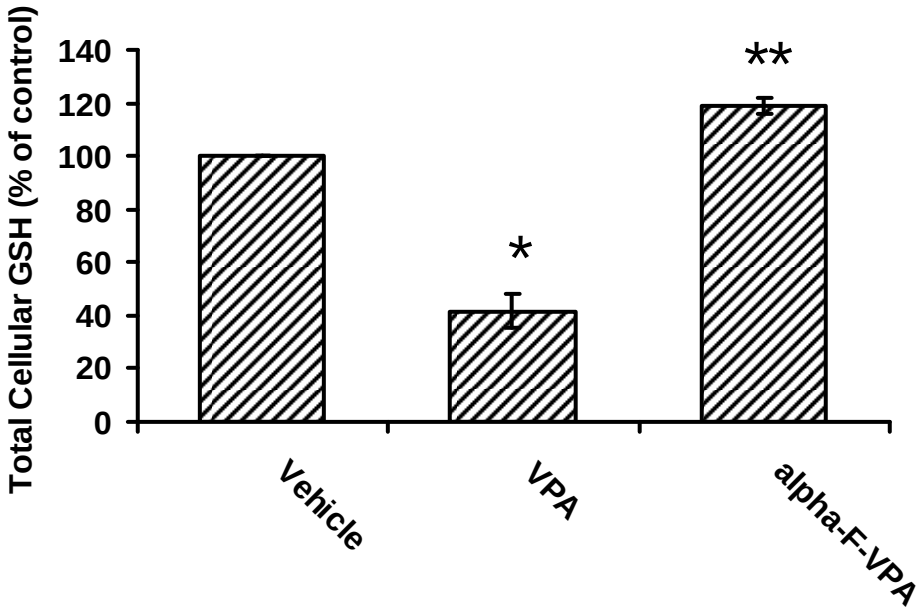

Figure 3-9. Effect of α-F-VPA on depletion of total cellular GSH in SCRH. Hepatocytes were exposed to the vehicle (culture medium), α-F-VPA, or VPA for 24 hours. The concentration of exposure was 12 mM, which was based on the EC_{50} value of VPA for the depletion of total cellular GSH (Figure 3-1). The GSH assay was conducted as described in the Experimental Section, and the data are expressed as the mean ± SEM from 4 individual experiments. *p < 0.05 vs. the vehicle control, **P < 0.05 vs. the vehicle and VPA controls; based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total cellular GSH levels in the vehicle-treated cells were 8.1 ± 2.2 μM (mean ± SEM).

3.5 References

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4 Correlational Analysis of Urinary Levels of Valproic Acid Metabolites and 15-F_{2t}-isoprostane in Children on Valproic Acid[3]

4.1 Introduction

Valproic acid (VPA) is a broad spectrum anticonvulsant that is also indicated in the management of migraine headache, bipolar disorder, and nerve pain (Johannessen and Johannessen 2003). Despite its broad usefulness, the administration of VPA is associated with liver abnormalities. The extent of liver injury may vary from mild, reversible elevations of liver transaminases (Genton and Gelisse 2002) to full blown idiosyncratic hepatotoxicity (Zimmerman and Ishak 1982; Kuhara *et al.*, 1990; Konig *et al.*, 1994; Bryant and Dreifuss 1996). Based on a series of retrospective studies conducted in the 1980's – 1990's, the incidence of VPA-induced idiosyncratic hepatotoxicity is higher in the pediatric population (< 2 years old) and in individuals receiving concurrent enzyme-inducing antiepileptic drugs (Dreifuss *et al.*, 1987; Dreifuss *et al.*, 1989; Bryant and Dreifuss, 1996). However, Koenig *et al.* (2006) reported that the majority of patients who had developed VPA-induced liver toxicity in a German population were greater than 5 years of age; thus the risk groups of VPA-associated liver toxicity may be more complex than previously thought. The mechanism(s) behind VPA-induced hepatotoxicity, however, remain unknown.

The biotransformation of VPA (Figure 4-1) in the production of toxic metabolites is a potential mechanism of VPA-induced liver toxicity. The terminal olefin, 4-ene-VPA, and its β-oxidation metabolite, (*E*)-2,4-diene-VPA, have been suggested as putative toxic species since

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they are potent inducers of hepatic steatosis in the rat (Kesterson *et al.*, 1984). The 4-ene-VPA metabolite is toxic to cultured rat hepatocytes (Kingsley *et al.*, 1983), and its effects are markedly exacerbated when total cellular glutathione is depleted (Jurima-Romet *et al.*, 1996). The reactivity of (*E*)-2,4-diene-VPA is verified when its glutathione and *N*-acetylcysteine (NAC) adducts are identified in rats exposed to 4-ene-VPA or (*E*)-2,4-diene-VPA (Gopaul *et al.*, 2000b;Gopaul *et al.*, 2000a; Tang and Abbott 1996a; Kassahun *et al.*, 1991; Kassahun and Abbott 1993; Kassahun *et al.*, 1994) and in human subjects taking VPA (Gopaul *et al.,* 2000b;Gopaul *et al.,* 2000a; Kassahun *et al.,* 1991; Gopaul *et al.*, 2003). Elevated levels of these NAC metabolites are also found in a few subjects who have developed VPA-associated hepatotoxicity, and in patients possessing the risk factors (i.e. young age and polytherapy) of VPA-induced hepatotoxicity (Gopaul *et al.,* 2003).

The administration of VPA is associated with the induction of oxidative stress. At relatively high doses, VPA increases the hepatic and plasma levels of 15-F_{2t}-isoprostane (Tong *et al.*, 2003), a sensitive marker of lipid peroxidation, in the rat (Fam and Morrow 2003). Similar effects are evident in cultured rat hepatocytes where VPA elevates the levels of 15-F_{2t}-isoprostane and 2′,7′-dichlorofluorescein (DCF), the latter a non-specific intracellular marker of oxidative stress (Halliwell and Whiteman 2004). Reduced levels of glutathione (GSH) and decreased activities of glutathione peroxidase have also been associated with VPA exposure in rat hepatic tissues (Cotariu *et al.*, 1990; Klee *et al.*, 2000). Similar to its effects in animal models, the administration of VPA for therapeutic purposes is also associated with the induction of oxidative stress in human subjects. Serum levels of 8-hydroxy-2-deoxyguanosine, a product of DNA oxidative damage, have been found to correlate with VPA levels in epileptic children on steady state VPA therapy (Schulpis *et al.*, 2006). Chronic VPA therapy also decreases the levels of vitamin E, an antioxidant, and increases the levels of malondialdehyde, a marker of lipid peroxidation, in the serum of obese children compared to healthy, age-matched controls (Verrotti

et al., 2008). Recently, our laboratory discovered that urinary levels of 15-F_{2t}-isoprostane are elevated in children receiving steady state VPA therapy compared to children receiving carbamazepine, clobazam, or the healthy controls (Michoulas *et al.*, 2006). This result is consistent with the finding in the rat model where VPA increases the hepatic and plasma levels of 15-F_{2t}-isoprostane in a dose-dependent manner (Tong *et al.,* 2003).

The biotransformation of VPA to produce reactive metabolites might logically be associated with the induction of oxidative stress, as evident in the rat model where inhibitors of glucuronidation apparently attenuated the VPA-induced elevation of 15-F_{2t}-isoprostane (Tong *et al.,* 2005b). Although abnormal patterns of VPA metabolism have been reported in human subjects who have developed VPA-associated hepatotoxicity (Eadie *et al.*, 1990; Siemes *et al.*, 1993; Kuhara *et al.,* 1990; Fisher *et al.*, 1992; Kondo *et al.*, 1992; McLaughlin *et al.*, 2000), the roles of biotransformation and / or specific metabolites in VPA-induced oxidative stress in humans remain unknown. To extend our observation in children where VPA therapy is associated with elevations of urinary 15-F_{2t}-isoprostane (Michoulas *et al.,* 2006), we characterized the correlations between the levels of VPA metabolites and 15-F_{2t}-isoprostane in the urine of children (< 18 years old) on VPA therapy. The correlations between key metabolite ratios, which represent VPA metabolism through the individual biotransformation pathways, and the levels of 15-F_{2t}-isoprostane were also determined. Based on these analyses, we report the novel finding that the glucuronide and NAC metabolites of VPA are extremely weak, but statistically significant, predictors of 15-F_{2t}-isoprostane levels in the urine of pediatric patients. The implications of this intriguing finding are discussed.

4.2 Materials & Methods

4.2.1 Study Design and Patient Recruitment

Pediatric patients (< 18 years old) receiving VPA therapy for seizure disorders or other conditions requiring the use of VPA were recruited from Vancouver (BC, Canada), Kansas City (MO, USA), Salt Lake City (UT, USA), and Louisville (KA, USA) under the supervision of Dr. Kevin Farrell (Neurologist, B.C. Children's Hospital, Vancouver, B.C., Canada) and Dr. James Steven Leeder (Professor, Pediatrics and Pharmacology, University of Missouri, Kansas City, MO, USA). Patients unable to void urine or non compliant with their VPA regimen were excluded from the study. Informed consent from legal guardians were obtained prior to sample collection. Sample collections were compliant with the ethical guidelines of the University of British Columbia Human Ethics Committee and the University of Missouri-Kansas City Health Sciences Pediatric Institutional Review Board. Overnight or morning urine samples were collected and shipped on dry ice to the University of British Columbia, where the analytical assays were conducted.

4.2.2 Chemicals

Sodium valproate (2-propyl-pentanoic acid), picric acid, and anhydrous creatinine were obtained from Sigma-Aldrich (Oakville, ON, Canada). Acetonitrile, ethyl acetate, phosphoric acid (85 %), ammonium acetate, and anhydrous ethyl ether of the highest analytical grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Dimethylformamide, pentafluorobenzyl bromide, and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide were purchased from Pierce Chemicals (Rockford, IL). Authentic VPA metabolites and internal standards were synthesized in our laboratory (Zheng, 1993;Gopaul *et al.,* 2000b). The 15-F_{2t}-isoprostane EIA kit was obtained from Cayman Chemical Company (Ann Arbor, MI, USA).

4.2.3 Urine Creatinine Assay

Levels of creatinine in the urine samples were assessed using a colourmetric assay in a 96 well microplate, based on the Jaffe method (Owen *et al.*, 1954). The urine samples were first vortex mixed and centrifuged (1000 x g for 10 minutes) to pellet the impurities. A 10 μL volume of each urine sample was added to 90 μL of the assay medium containing 2 mM NaCl, 0.0009 M picric acid, and 0.19 M NaOH. The mixture was incubated at 25 ºC for 15 minutes, and the absorbance of the reaction product, creatinine picrate, was quantified at 490 nm (Labsystems Multiskan Ascent® Multi well plate reader, Thermo Electron Corp., Burlington, O.N., Canada). The levels of VPA, VPA metabolites, and 15-F_{2t}-isoprostane were normalized to creatinine.

4.2.4 Urine 15-F_{2t}-isoprostane Assay

15-F_{2t}-isoprostane is a product of free radical-catalyzed lipid peroxidation of arachidonic acid, a common phospholipid in the plasma membrane of mammalian cells (Montuschi *et al.*, 2004). Levels of 15-F_{2t}-isoprostane in the urine were quantified by a competitive enzyme immunoassay (15-F_{2t}-isoprostane EIA Kit, Cayman Chemical Company). The same assay kit was employed for the assessment of urinary 15-F_{2t}-isoprostane levels in our lab in a previous study for children on VPA (Michoulas *et al.,* 2006). All other assay conditions have been described by Tong *et al.* (2003).

4.2.5 Urine VPA and VPA Metabolite Assays

Levels of (*E*)-2,4-diene-VPA, (*E,E*)-2,3´-diene-VPA, 4-ene-VPA, 3-ene-VPA, (*E*)-2-ene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 3-keto-VPA, and 4-keto-VPA were determined by a gas chromatography-mass spectrometry assay (GC/MS) as described previously by Kiang *et al.* (2006). Due to the large differences in the levels of VPA metabolites within or between individuals, each sample was assayed multiple times with different volumes. This ensures that

levels of metabolites were within the linear regions of the calibration curves. All of the other assay and instrument conditions were identical to that reported by Kiang *et al.* (2006).

Levels of VPA and VPA-glucuronide were determined by liquid chromatography-mass spectrometry (LC/MS) assay similar to that described by Tong *et al.* (2005b) and Michoulas *et al.* (2006). An assay mixture (1 mL) containing the urine sample, water, and saccharolactone (5 mM) was acidified with 20 μL of phosphoric acid and spiked with 50 μL of the internal standard containing 0.1 mg / mL of [$^2H_6$]-VPA and [$^2H_6$]-VPA-glucuronide. The mixture was then subjected to liquid-liquid extraction with ethyl acetate (4 mL) and anhydrous ethyl ether (4 mL). The organic layer was subsequently transferred to a glass test tube prefilled with 600 μL of distilled water containing 10 mM ammonium acetate. The organic layer was evaporated under nitrogen (0.5 psi at 25 ˚C, Zymark Turbo Vap LV Evaporator, Zymark, Hopkinton, MA) and the remaining aqueous layer was mixed with 200 μL of acetonitrile (ACN). A 10 μL volume of the mixture was then injected into the Waters Acquity UPLC fitted with a Phenomenex Gemini C_{18} column (150 x 2 mm, I.D., 5 μm). The mobile phase, delivered at a flow rate of 0.2 mL / min, consisted of a mixture of ACN and water, containing 10 mM ammonium acetate. The percentage of ACN was increased linearly from 25 % to 90 % over 9 minutes and sharply reduced and held at 25 % for 4 minutes prior to the next injection. Detection was achieved with a Waters Micromass Quattro Premier™ Tandem Mass Spectrometer (Micromass Ltd., Montreal, Canada) under the negative electrospray mode. The following ions were monitored: m/z 143 for VPA, m/z 149 for [$^2H_6$]-VPA, m/z 319 for VPA-glucuronide, and m/z 325 for [$^2H_6$]-VPA-glucuronide. The instrument had been optimally tuned for this assay at the following conditions: source temperature,150 ˚C; capillary voltage, 3 kV; cone voltage, 20 V; and the multiplier voltage, 650V.

Levels of 2-propyl-5-(N-acetylcystein-S-yl) pent-3-enoic acid (5-NAC-3-ene-VPA), 2-propyl-5-(N-acetylcystein-S-yl) pent-2-enoic acid (5-NAC-2-ene-VPA), and their isomers were

determined by liquid chromatography-tandem mass spectrometry (LC/MS/MS) assay modified from that of Gopaul *et al.* (2000a). A 1 mL volume of each urine sample was acidified with 20 µL of phosphoric acid and 10 µL of the internal standard (5 µg / mL of 2-fluoro-2-propylpentanoylglutamine) was added. The mixture was subjected to liquid-liquid extraction with ethyl acetate (4 mL) and anhydrous ethyl ether (4 mL). The organic layer was then transferred to a test tube containing 250 µL of distilled water and evaporated under a stream of nitrogen (0.5 psi at 25 ºC). The remaining aqueous layer was mixed with 25 µL of ACN, and 10 µL of the mixture was injected into the Waters Acquity UPLC. Chromatographic separation was achieved with an Acquity UPLC BEH C18 column (100 x 2.1 mm, I.D. 1.7 µm) fitted to a guard column (2 x 2.1 mm, I.D. 1.7 µm) using a flow rate of 0.25 mL / min at 25 ºC. The mobile phase consisted of a mixture of ACN and water, containing 0.2 % formic acid. The gradient consisted of 18.5 % ACN for the initial 14.5 minutes, followed by an increase to 30 % over 4 minutes, a sharp increase to 90 % over 2.5 minutes, then a return to 18.5 % for a total run time of 26 minutes. Detection was achieved with the Waters Micromass Quattro Premier™ Tandem Mass Spectrometer using the positive electrospray mode. Ions of interest were detected under multiple reaction monitoring of the transitions from m/z 304 → 123 for the NAC conjugates and m/z 291 → 130 for the internal standard. The sum of the levels of 5-NAC-2-ene-VPA, 5-NAC-3-ene-VPA, and their isomers is subsequently referred to as NACtotal in this report. The instrument had been optimally tuned at the following conditions: source temperature, 150 ˚C; capillary voltage, 3.5 kV; cone voltage, 15 V; and the multiplier voltage, 650V.

4.2.6 Statistical Analysis

Statistical analyses were conducted on $\log_{10}$ transformed data (SPSS Statistical Software, version 15.0 for Windows). The correlation statistics were determined by forward stepwise multiple linear regression analyses. The (arbitrary) order by which the variables were added was

as follows: VPA levels, polytherapy status, levels of (*E*)-2,4-diene-VPA, (*E,E*)-2,3′-diene-VPA, 4-ene-VPA, 3-ene-VPA, (*E*)-2-ene-VPA, 4-OH-VPA, 5-OH-VPA, 3-keto-VPA, 4-keto-VPA, VPA-G, and NACtotal. The levels of significance are indicated in each figure legend.

4.3 Results

4.3.1 Patient Demographics

Urine samples from 123 children were analyzed in the current study. The average age of the group was 9.5 ± 4.0 (mean ± SD) years. The distribution of race, which was categorized according to the guidelines of the US Census Bureau, in the current study was as follows: Caucasians (n = 92), African Americans (n = 12), Asians (n = 5), Hispanic Whites (n = 4), Brazilian (n = 1), mixed (n = 4), and uncharacterized (n = 5). There were 69 males, 53 females, and 1 of unknown gender. Of the 123 patients, 78 children were on VPA monotherapy , 38 children were on VPA with non-inducing anticonvulsants, and 7 were on VPA with enzyme inducing anticonvulsants such as phenobarbital, phenytoin, and carbamazepine. The average age in years in each group was similar: VPA monotherapy (9.5 ± 3.8, mean ± SD); VPA with non-inducing anticonvulsants (8.9 ± 4.4), and VPA with enzyme inducing anticonvulsants (11.4 ± 4.0). However, the average dose of VPA (mg/kg/day) in those individuals who were co-administered enzyme inducing anticonvulsants was significantly higher (50 ± 41, mean ± SD) compared to that VPA monotherapy (20 ± 8) and VPA with the non-inducers (25 ± 12). No differences were observed in the distribution of gender or race between the groups. Table 4-1 summarizes the relevant clinical information on the patients who were tested.

4.3.2 Correlational Analyses of VPA Metabolites

The mean urinary levels of VPA and the individual metabolites in 123 patients are presented in Table 4-2. The most abundant metabolite recovered in the urine was VPA-

glucuronide, and the least recoverable was the (*E,Z*)-isomer of 2,3′-diene-VPA that was below the limit of quantification. To verify the internal consistency of the metabolite data, correlational analyses were conducted on the metabolites: strong correlations were obtained between the metabolite precursors and their immediate products (Figure 4-2); whereas weak correlations were obtained between products that are two or more steps removed in the metabolic pathway, which are consistent with the known metabolism patterns of VPA (Figure 4-1). The coefficient of determinations are summarized in Table 4-3.

4.3.3 Multiple Linear Regression Analyses of Factors Associated with 15-F_{2t}-isoprostane

The mean urinary 15-F_{2t}-isoprostane levels in this pediatric patient study was 761 ± 589 pmole / mmole creatinine (mean ± SD), which is consistent with 15-F_{2t}-isoprostane levels in the urine of human subjects as reported in the literature (e.g. Wu *et al.*, 2008; Rossner *et al.*, 2008; Rossner *et al.*, 2006; Il'vasova *et al.*, 2005; Helmersson and Basu 1999). Initial stepwise multiple linear regression analysis incorporating "gender", "race", "VPA dose", and "age" indicated that none of these patient factors predicted the levels of 15-F_{2t}-isoprostane in the current study population; thus these variables were omitted from the subsequent regression models. To determine which metabolites were associated with elevations of 15-F_{2t}-isoprostane, levels of all VPA metabolites characterized in this study ((*E*)-2,4-diene-VPA, (*E,E*)-2,3′-diene-VPA, 4-ene-VPA, 3-ene-VPA, (*E*)-2-ene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 3-keto-VPA, 4-keto-VPA, VPA-glucuronide, and NAC_{total}) were incorporated into a multiple linear regression model. As it was not known whether VPA levels or polytherapy status would affect the correlations, these variables were also included in the regression model. Individuals were classified as either on VPA monotherapy, VPA polytherapy with non-inducing drugs, or VPA polytherapy with enzyme-inducing drugs, as presented in Table 4-1. Of the metabolites

analyzed, only VPA-glucuronide ($r^2 = 0.037$, $p = 0.008$) and NACtotal ($r^2 = 0.073$, $p = 0.042$) predicted the levels of 15-F_{2t}-isoprostane. A positive correlation was obtained between the levels of VPA-glucuronide and 15-F_{2t}-isoprostane, whereas NAC_{total} correlated with the marker inversely. On the other hand, VPA levels or polytherapy status were not predictors of the levels of 15-F_{2t}-isoprostane in these regression models.

The flux of VPA through the individual biotransformation pathways can be characterized by the specific metabolite ratios. To characterize the metabolism through the glucuronidation pathway, the ratios of VPA glucuronide to VPA or the other primary metabolites (i.e. 2-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 4-ene-VPA) were used. For the ß-oxidation pathway, the ratios of 2-ene-VPA, a primary β-oxidation metabolite, to VPA or the other primary metabolites of VPA were examined. Of particular interest is the ratio between 2-ene-VPA and 3-keto-VPA, which has been used as an indicator of mitochondrial ß-oxidation function by various investigators (Siemes *et al.,* 1993; Eadie *et al.,*1990). To characterize the cytochrome P450 (CYP)-mediated VPA metabolism, the ratios of 4-OH-VPA (as a surrogate for the other CYP metabolites) to VPA, 2-ene-VPA, and VPA-glucuronide were employed. We also included the ratio between 4-ene-VPA and 4-OH-VPA in the "CYP group", because these two metabolites are produced from a common mechanism (Rettie *et al.*, 1988), but exhibit markedly different toxicity characteristics (Kingsley *et al.,*1983). To determine whether the formation of the NAC metabolites were related to the elevation of 15-F_{2t}-isoprostane, we characterized the ratios of NACtotal to their precursor metabolites (i.e. (*E*)-2,4-diene-VPA, 4-ene-VPA, and (*E*)-2-ene-VPA). For these analyses, VPA levels and polytherapy status were also included in the regression model.

Based on forward stepwise multiple linear regression analysis, none of the metabolite ratios representing the glucuronidation, ß-oxidation, and the CYP-oxidation of VPA predicted the levels of 15-F_{2t}-isoprostane in the present study population. On the other hand, the ratio of

NAC_{total} to 4-ene-VPA inversely correlated with the levels of 15-F_{2t}-isoprostane ($r^2 = 0.040$, $p = 0.034$).

4.4 Discussion

Recently, our research group reported that urinary levels of 15-F_{2t}-isoprostane, a sensitive marker of lipid peroxidation, were elevated in children receiving VPA monotherapy compared to age-matched healthy controls (Michoulas *et al.,* 2006). This finding was consistent with the dose-dependent effects of VPA on the elevation of 15-F_{2t}-isoprostane in rats (Tong *et al.,* 2003). The present study extended the observations from Michoulas *et al.* (2006) by examining the correlations between VPA metabolite levels or specific metabolite ratios and the elevation of 15-F_{2t}-isoprostane in the urine of children (< 18 years old) receiving VPA therapy. The sample size of the present study (N = 123) is larger than that of Michoulas *et al.* (2006) (N = 25 for VPA treatment), and this would ensure enough power to have confidence in the multiple variable regression models. The hypothesis being tested is that the levels of certain VPA metabolites in the urine or abnormal VPA metabolism patterns can affect the urinary levels of 15-F_{2t}-isoprostane observed in pediatric patients receiving VPA therapy.

In order to test this hypothesis, urinary levels of various VPA metabolites representing the major biotransformation pathways of VPA (Figure 4-1) were quantified using GC/MS, LC/MS, and LC/MS/MS assays developed in our laboratory. The use of GC/MS assays for the sensitive and simultaneous quantification of multiple oxidative metabolites is well-established in our lab (Anari *et al.*, 2000; Kiang *et al.,* 2006; Yu *et al.*, 1995; Kassahun *et al.*, 1990; Kassahun *et al.*, 1989; Singh *et al.*, 1987). The LC/MS or LC/MS/MS assays are also reliable methods for the detection of relatively polar phase II conjugated VPA metabolites (Tong *et al.,* 2005b; Gopaul *et al.,* 2000a; Tang and Abbott, 1996a; Tang *et al.*, 1996b; Kassahun *et al.,* 1991). A strength of our approach is that we are one of the first to measure VPA-glucuronide levels in

human tissue using a direct approach. The traditional approach, where levels of VPA-glucuronide are estimated from the amount of VPA present after enzyme- or pH-catalyzed hydrolysis, can underestimate the true glucuronide levels. Moreover, using the higher resolving power of UPLC, we were able to detect novel isomers of the VPA-NAC metabolites in the present study, in addition to the two major isomers previously reported by Gopaul *et al.* (2000b). The relative abundance of urinary VPA metabolites, the actual metabolite levels, or key metabolite ratios in the present study are considered comparable to the reported literature values in human subjects (Gopaul *et al.,* 2003; Dickinson *et al.*, 1989; Eadie *et al.,* 1990; Anderson *et al.*, 1992; Sugimoto *et al.*, 1996; Fisher *et al.,* 1992). As well, further verification of the internal consistency of the metabolite data was determined by the correlations between the individual VPA metabolites. Stronger correlations were obtained between metabolite precursors and their immediate biotransformation products, whereas weaker correlations were obtained between metabolites that are two or more steps removed in the biotransformation pathway (Figure 4-1, Figure 4-2, Table 4-3). These findings support the established biotransformation patterns of VPA in humans (Abbott *et al.*, 1999) and suggest that the metabolite data obtained in this study are reliable.

The 15-F_{2t}-isoprostane is a free radical catalyzed breakdown product of the common phospholipid, arachidonic acid, and its formation is believed to be independent of the cyclooxygenase (COX) enzymes (Fam and Morrow, 2003; Basu, 2004; Basu and Helmersson 2005). There is no diurnal variability in the production of 15-F_{2t}-isoprostane, which simplifies the urine collection regimen in human subjects (Helmersson and Basu, 1999). Because 15-F_{2t}-isoprostane is a sensitive and reliable marker for the VPA-induced oxidative stress in rats (Tong *et al.,* 2003; Tong *et al.*, 2005a; Tong *et al.,* 2005b) and was reported to be elevated in the urine of children receiving VPA (Michoulas *et al.,* 2006), it was reasonable to use the same marker for

the measurement of oxidative stress in the present study. The present study was in a sense an extension of the earlier observations from Michoulas *et al.* (2006).

Multiple regression analysis of the urinary 15-F_{2t}-isoprostane data indicated that patient "age", "gender", "race", "VPA dose", "VPA levels", and "polytherapy status" did not predict the levels of 15-F_{2t}-isoprostane in the present study. This is consistent with the results of Michoulas *et al.* (2006) with respect to the patient factors "age", "VPA dose", and "VPA levels". On the other hand, conflicting reports on the effects of "gender" or "race" on the urinary excretion of 15-F_{2t}-isoprostane in humans have been published (Block *et al.*, 2002; Keaney *et al.*, 2003; Kauffman *et al.*, 2003; Ke *et al.*, 2003; Meghdadi *et al.*, 2003; Lopes *et al.*, 2003). The unequal distribution of "race" and "polytherapy status" in the present study also limited the power of the correlational analysis. As such, further studies are needed to clarify the effects of the individual patient factors on the urinary excretion of 15-F_{2t}-isoprostane in humans.

Based on the multiple regression analysis of all metabolites characterized in the present study, only VPA-glucuronide and NACtotal predicted the levels of 15-F_{2t}-isoprostane. From these findings, a positive correlation was obtained with VPA-glucuronide ($r^2 = 0.037$), whereas NACtotal ($r^2 = 0.073$) inversely predicted the levels of the lipid peroxidation marker. Because the correlations with 15-F_{2t}-isoprostane by these two metabolites were extremely weak, these results may be chance observations and point out that other factors related to VPA in the present study were responsible for the elevation of the lipid peroxidation marker.

Having said that, the significant, but weak, correlation found between the levels of VPA-glucuronide and 15-F_{2t}-isoprostane in this human study is intriguing given that inhibitors of glucuronidation apparently attenuated the VPA-induced elevation of 15-F_{2t}-isoprostane in the rat model (Tong *et al.,* 2005b). Consistent with these findings, the testing of α-F-VPA, which undergoes minimal glucuronidation, also did not elevate the levels of 15-F_{2t}-isoprostane in the rat (Tong *et al.,* 2005b). Nevertheless, a mechanism leading to an association between VPA-

glucuronide formation and the elevation of 15-F_{2t}-isoprostane remains unknown. Although acyl-glucuronides can be reactive and electrophilic species, the VPA glucuronide is relatively inert (Williams *et al.*, 1992; Bailey and Dickinson 2003; Bailey and Dickinson 1996). As well, in yet to be published studies from our laboratory, exogenously added VPA-glucuronide, even at relatively high concentrations, was not toxic to cultured rat hepatocytes with respect to markers for mitochondrial dysfunction, oxidative stress, cytotoxicity, and total cellular glutathione (Chapters 2 & 3 of the present thesis). Thus, these observations would suggest that factors other than the VPA glucuronide metabolite itself may be responsible for the elevation of 15-F_{2t}-isoprostane.

One possible mechanism for the association between VPA-glucuronide and lipid peroxidation is the intramolecular rearrangements of VPA-glucuronide in the formation of potentially reactive, ß-glucuronidase resistant, species (Williams *et al.,* 1992). Supporting this argument, the levels of these rearranged glucuronides were found elevated in a patient with VPA-induced hepatotoxicity (Dickinson *et al.*, 1985), although the cause-effect relationship between metabolite formation and toxicity was not established. Another potential mechanism is the enterohepatic recycling of VPA-glucuronide and the subsequent release of glucuronic acid (Tong *et al.,* 2005b), the latter is also reported to be an inducer of oxidative stress (Kim *et al.*, 2004). If this were the case, patients co-administered VPA and a carbapenem antibiotic, which reduces the enterohepatic recycling of VPA-glucuronide (Mori *et al.*, 2007), might result in lower levels of urinary 15-F_{2t}-isoprostane in comparison to similar patients receiving VPA monotherapy. To probe this hypothesis, mechanistic experiments using carbapenem antibiotics are being conducted in our laboratory in the rat model. Furthermore, the glucuronide conjugates of various VPA metabolites, which were not characterized in this study, may also contribute to the elevation of 15-F_{2t}-isoprostane. Although it has already been established that the (*E*)-2,4-diene-VPA-glucuronide in rats is reactive toward glutathione or cellular proteins (Tang *et al.,*

1996b), the reactivity of other VPA metabolite glucuronides has yet to be determined. As such, any role of VPA metabolite glucuronides other than (*E*)-2,4-diene-VPA glucuronide in depletion of cellular glutathione and induction of oxidative stress remains to be studied.

The GSH conjugates of (*E*)-2,4-diene-VPA-CoA, which are formed in the mitochondria, undergo sequential metabolism through the mercapturic acid pathway to produce the urinary NAC metabolites of VPA that were analyzed in the present study (Kassahun *et al.,* 1991; Gopaul *et al.,* 2000b). As the urine levels of VPA-NAC metabolites have been reported to be higher in patients who have developed VPA-associated hepatotoxicity (Kassahun *et al.,* 1991) and in children possessing the risk factors of VPA-induced hepatotoxicity (Gopaul *et al.,* 2003), the inverse association found between the levels of NAC metabolites (NAC_{total}) and 15-F_{2t}-isoprostane in the present study was rather unexpected. A potential explanation for this apparent contradiction could be that the amount of thio-conjugated metabolites generated from VPA is a direct reflection of the mitochondrial GSH capacity in an individual. This concept has been demonstrated in an *in vitro* model where levels of thio-conjugaged (*E*)-2,4-diene-VPA produced from the exposure of 4-ene-VPA are several-fold higher in normal rat hepatocytes compared to GSH-depleted hepatocytes (Jurima-Romet *et al.,* 1996). Based on this logic, individuals who generate more NAC metabolites would actually have a higher mitochondrial GSH capacity, and therefore be less likely to develop oxidative stress from VPA exposure. To the best of our knowledge, no reports are available in the literature describing the association between levels of thio-conjugated xenobiotics and GSH capacity in humans.

In addition to the VPA metabolite levels, we also examined the correlations between key VPA metabolite ratios, which represent the biotransformation pathways of VPA, and the elevation 15-F_{2t}-isoprostane. Based on multiple regression analysis, none of the metabolite ratios representing the glucuronidation, β-oxidation, and CYP-mediated oxidation of VPA predicted the levels of 15-F_{2t}-isoprostane in this study population. Although various ratios

representing the formation of VPA-NAC metabolite (i.e. NAC_{total} to 4-ene-VPA, (*E*)-2-ene-VPA, and (*E*)-2,4-diene-VPA) were analyzed, only the NAC_{total} to 4-ene-VPA ratio correlated , albeit inversely, with the levels of 15-F_{2t}-isoprostane. The lack of prediction of 15-F_{2t}-isoprostane levels by the NAC_{total} to 2-ene-VPA ratio may be explained by the fact that these NAC metabolites arise predominately from 4-ene-VPA through ß-oxidation, and not through (*E*)-2-ene-VPA by CYP oxidation, as demonstrated in rats (Kassahun *et al.,* 1994). The ratio of the NAC metabolites to the CoA ester of (*E*)-2,4-diene-VPA might have been a better marker, but only the free acid of the diene was quantified in the present study. As well, the multiple steps in the mercapturic acid pathway required for the formation of NAC metabolites from 4-ene-VPA may also explain the relatively weak associations obtained of this NAC metabolite ratio and the elevation of 15-F_{2t}-isoprostane. Consistent with the findings of the NAC_{total}, the observed inverse correlation between the NAC_{total} to 4-ene-VPA ratio and the levels of 15-F_{2t}-isoprostane might suggest that the extent of NAC metabolite formation is likely correlated with the GSH capacity of an individual. Thus, it can be postulated that patients who are deficient of GSH, a critical antioxidant, would not be able to generate as much of the NAC metabolites, and are likely more susceptible to the oxidative stress associated with VPA. Further studies are needed to investigate this hypothesis.

Of particular interest was the lack of correlation between 4-ene-VPA, a putative toxic metabolite (Kesterson *et al.,* 1984; Tang *et al.*, 1995), and levels of 15-F_{2t}-isoprostane in the present study. This is consistent with the results from Tong *et al.* (2003) in rats where the formation of 4-ene-VPA was determined not to be a factor in the VPA-induced elevation of 15-F_{2t}-isoprostane. As well, the majority of the human cases with documented VPA-associated toxicity also have not reported abnormal 4-ene-VPA levels (McLaughlin et al*.,* 2000; Kondo et al*.,* 1992; Tennison *et al.*, 1988; Paganini *et al.*, 1987; Kuhara et al*.,* 1990; Siemes *et al.,* 1993; Fisher et al*.,* 1992; Eadie *et al.,* 1990), although these studies did not specifically characterize the

induction of oxidative stress from VPA treatment. Taken together, these observations would suggest that the *in situ* generated 4-ene-VPA was unlikely involved in the VPA-induced oxidative stress, and that the 4-ene-VPA component in the NAC_{total} / 4-ene-VPA metabolite ratio was unlikely responsible for the correlation obtained with the 15-F_{2t}-isoprostane marker.

Several limitations could have influenced the results obtained in this human study. The urinary excretion of 15-F_{2t}-isoprostane is more or less the same throughout the day (Helmersson and Basu, 1999), but the urinary clearance of some VPA metabolites is subject to diurnal variations in humans (Reith *et al.*, 2001). The diurnal variability, compounded with the inconsistent urine collection times (overnight or one time morning sample), may have contributed to the large inter-individual variability in the levels of VPA metabolites observed in this study, which maybe one reason for the weak correlations obtained with the metabolites and 15-F_{2t}-isoprostane. However, this problem would likely be difficult to remedy because clinical studies that have resorted to extended periods of sample collection also report large variability in the levels of VPA metabolites in human samples (Fisher *et al.,* 1992; Dickinson *et al.,* 1989). Nevertheless, future studies should take into consideration the diurnal variability of VPA metabolite levels and properly design sample collection procedures to minimize the large variability in the levels of VPA metabolite typically observed in human patients.

With respect to our marker of oxidative stress, diet, vitamin intake, or various pathophysiological conditions can all affect the levels of 15-F_{2t}-isoprostane in humans (Basu and Helmersson, 2005). In children on routine VPA therapy, exposure to second-hand smoke was also shown to elevate the urinary levels of 15-F_{2t}-isoprostane (Michoulas *et al.,* 2006). However, it is not known whether any of these factors influenced the results of the present study since the initial patient selection did not take these variables into consideration. Furthermore, a potential interaction between VPA and elimination of 15-F_{2t}-isoprostane could have also confounded the results obtained in the present investigation. VPA and some of its metabolites are potent

inhibitors of fatty acid β-oxidation, as demonstrated in rats (Silva *et al.*, 2001; Kesterson *et al.,* 1984; Ponchaut *et al.*, 1992), and the mechanism of inhibition is non-specific, as VPA sequesters the co-factors needed for the ß-oxidation of all fatty acids (Ponchaut *et al.,* 1992). Given that a significant amount (i.e. ~ 30 %) of 15-F_{2t}-isoprostane is rapidly oxidized *via* β-oxidation in humans (Chiabrando *et al.*, 1999; Roberts *et al.*, 1996), it is possible that VPA, independent of its effects on oxidative stress, could have potentially elevated the levels of 15-F_{2t}-isoprostane *via* inhibition of ß-oxidation of 15-F_{2t}-isoprostane. However, one might expect the levels of VPA to correlate somewhat with that of 15-F_{2t}-isoprostane if this interaction were statistically significant, which was not the case in the present study. Nevertheless, further studies are needed to adder ss this potential interaction between VPA and 15-F_{2t}-isoprostane metabolism, and future studies that examine VPA-induced oxidative stress in patients might better employ alternate markers of oxidative stress to support any findings obtained with the 15-F_{2t}-isoprostane marker.

In conclusion, VPA-glucuronide and NAC metabolites are extremely weak but statistically significant predictors of 15-F_{2t}-isoprostane levels in the urine of children on routine VPA therapy. This intriguing association is likely too weak to be clinically relevant, but our findings may provide further insights into potential mechanisms of VPA-induced oxidative stress in humans.

Table 4-1. The Demographic Information of Patients Enrolled in the Study
Individuals on VPA Monotherapy (N = 78)

Patient ID	Gender	Race	VPA dose (mg/kg/day)	Age (Yrs)	Concurrent AED
L-03-1	F	C	12.4	10.9	none
L-19-1	M	C	25.0	13.8	none
L-28-1	F	C	15.3	3.1	none
L-31-1	F	C	22.2	N/A	none
L-36-1	M	AA	1.5	11.6	none
M-01-1	F	C	28.4	8.2	none
M-04-1	F	C	18.9	4.2	none
M-08-6	M	C	16.3	13.0	none
M-13-1	M	C	21.7	12.9	none
M-18-1	F	C	8.7	10.3	none
M-26-1	F	AA	18.1	6.8	none
M-30-1	M	AA	13.0	15.3	none
M-32-1	M	C	26.2	10.4	none
M-41-1	M	C	14.5	12.0	none
M-44-1	F	AA	13.8	11.7	none
M-49-1	M	C	21.8	17.3	none
M-59-1	M	C	15.7	8.4	none
M-60-1	F	AA	16.5	12.0	none
M-62-1	M	Asian	16.3	11.5	none
M-66-1	M	C	18.6	9.0	none
M-68-1	M	Hispanic-W	16.6	14.4	none
M-70-1	M	C	24.1	5.5	none
M-71-1	M	AA	48.4	5.6	none
M-72-1	M	AA	24.6	1.8	none
M-76-1	M	AA	28.6	9.8	none
M-78-2	M	Hispanic-W	15.4	13.9	none
M-83-1	M	AA	15.6	6.8	none
M-88-1	M	AA	13.4	9.6	none
M-89-1	M	C	42.6	4.9	none
U-03-1	F	C	19.6	5.7	none
U-14-1	F	C	13.3	13.4	none
U-26-2	F	C	18.5	8.5	none
U-36-1	M	C	31.4	4.2	none
U-37-1	M	C	13.9	17.3	none
U-43-1	M	C	17.5	5.5	none
U-44-1	F	Brazilian	18.2	16.1	none

Patient ID	Gender	Race	VPA dose (mg/kg/day)	Age (Yrs)	Concurrent AED
U-52-1	M	C	37.5	7.2	none
U-53-2	M	C	12.4	13.6	none
U-60-1	M	C	22.0	16.2	none
U-62-1	F	C	18.0	11.5	none
U-67-1	M	C	9.8	13.3	none
U-65-1	M	C	12.7	8.6	none
U-70-3	M	C	29.1	6.4	none
U-81-2	M	C	20.6	5.0	none
U-87-1	F	Hispanic-W	20.6	6.5	none
U-88-2	F	C	22.0	10.8	none
U-90-2	M	C	22.3	5.6	none
U-94-1	F	C	19.2	11.2	none
U-95-1	M	C	22.0	14.6	none
U-96-1	M	C	17.2	10.4	none
U-100-1	M	C	36.2	3.8	none
U-106-1	M	C	23.4	7.5	none
U-108-1	F	AA/C	22.2	16.1	none
2	M	C	22.6	8.2	none
4	F	C	40.1	8	none
8	F	N/A	26	13.7	none
10	M	Asian	17.9	8.8	none
13	M	C	18.3	4	none
22	F	C (Italian/Mexican)	11.8	7.1	none
41	M	C	8.3	14.4	none
44	F	Asian	28.8	4	none
51	M	C	17	4.6	none
56	F	C	8	10.4	none
61	F	C	20.5	8.7	none
67	F	C	20.4	11.9	none
73	M	C	23.8	6.8	none
79	M	C	27.2	6.5	none
81	M	C	14.8	8.7	none
4C	M	C	15.8	12.1	none
5C	M	N/A	19.2	6.1	none
8C	F	C	55.0	8	none
82	F	C	31.9	6.6	none
84	M	C	16.6	14.3	none
87	F	C	16.7	15.1	none
91	F	C	24.4	11.2	none

Patient ID	Gender	Race	VPA dose (mg/kg/day)	Age (Yrs)	Concurrent AED
92	M	C	27.4	3.9	none
95	M	N/A	5.2	8.7	none
104	F	Asian (Fijian)	18.9	8.7	none
Individuals on VPA and non-inducing anticonvulsants (N = 38)					
Patient ID	**Gender**	**Race**	**VPA dose (mg/kg/day)**	**Age (Yrs)**	**Concurrent AED**
L-07-1	F	C	16.5	4.7	levetiracetam
L-13-1	F	C	38.4	7.1	levetiracetam, clonazepam
L-24-1	M	C	35.0	8.8	clonidine
L-29-1	M	C	19.2	7.9	topiramate, clonazepam
L-32-1	M	C	15.3	7.1	gabapentin
L-39-1	F	C	19.4	11.5	levetiracetam, ethosuximide, clonazepam
L-40-1	F	AA	16.7	13.4	lamotrigine, oxcarbazepine, clonazepam
M-14-1	F	C	35.0	8.1	clonazepam
M-15-1	F	C	18.9	7.2	oxcarbazepine
M-19-1	F	C	14.3	15.7	lamotrigine, zonisamide
M-27-1	F	Mixed	17.4	9.0	felbamate, topiramate
M-34-1	F	C	47.2	5.1	topiramate
M-42-1	M	C	20.7	7.1	atomoxetine, concerta
M-56-1	M	C	19.5	15.3	midazolam (prn)
M-73-1	M	C	36.9	14.5	felbamate
M-87-1	F	C	34.2	3.6	zonisamide
U-08-1	F	C	71.4	10.4	oxcarbazepine, escitalopram
U-16-1	M	C	21.8	4.4	zonisamide
U-17-2	M	AA	22.5	11.1	lamotrigine
U-18-1	M	C	30.8	9.6	lamotrigine, zonisamide, lorazepam
U-19-1	M	C	17.4	16.7	lamotrigine
U-24-2	F	Mixed	21.2	12.0	lamotrigine
U-47-1	F	C	11.1	14.9	oxcarbazepine
U-48-2	F	C	38.2	3.9	diazepam (rectal)
U-73-2	M	C	35.0	2.8	topiramate
U-77-1	F	C	34.4	4.8	lamotrigine
U-83-2	M	C	36.8	3.0	topiramate
U-86-2	F	C	21.4	11.9	oxcarbazepine, zonisamide
U-91-1	F	C	16.4	1.9	chlorazepate
U-99-1	M	C	34.2	4.1	levetiracetam
U-110-1	N/A	N/A	21.3	16.5	lamotrigine
34	F	Asian (Indian)	17.2	15.1	keppra
69	M	C	18.1	6.1	clobazam

Patient ID	Gender	Race	VPA dose (mg/kg/day)	Age (Yrs)	Concurrent AED
93	M	C	23.9	3.9	lamotrigine
97	M	N/A	7.1	12.7	lamotrigine
105	F	C	9.4	12.5	clonazepam
106	M	C	16.2	9.1	lamotrigine
L-07-1	F	C	16.5	4.7	levetiracetam

Individuals on VPA with enzyme inducing anticonvulsants (N = 7)

Patient ID	Gender	Race	VPA dose (mg/kg/day)	Age (Yrs)	Concurrent AED
M-63-1	M	C	138.9	10.2	phenytoin
M-86-1	M	C	35.0	14.7	phenytoin
U-04-2	F	C	24.8	16.5	carbamazepine, topiramate
U-06-1	M	C	61.3	4.2	phenobarbital
U-21-2	F	C	35.1	11.3	clonazepam, phenobarbital
U-30-1	M	Hispanic-W	38.9	10.1	carbamazepine, levetiracetam
37	F	C	16	12.5	carbamazepine

C (Caucasian), AA (African American), Hispanic-W (Hispanic White); N/A (data not available); AED (antiepileptic drug)

Table 4-2. Urinary Levels of VPA Metabolites and VPA

	(μmole / mmole creatinine)
4-ene-VPA	0.03 ± 0.06
3-ene-VPA	0.15 ± 0.19
(*E*)-2-ene-VPA	0.65 ± 2.39
(*E*)-2,4-diene-VPA	0.13 ± 4.65
(*E,E*)-2,3′-diene-VPA	2.09 ± 6.27
3-keto-VPA	61.28 ± 38.90
4-keto-VPA	15.29 ± 16.06
3-OH-VPA	13.40 ± 9.02
4-OH-VPA	48.11 ± 36.99
5-OH-VPA	24.98 ± 21.80
VPA-glucuronide	452.32 ± 493.49
NACtotal	0.69 ± 0.59
VPA	9.08 ± 22.46

Data are presented as the mean ± S.D. (n = 123). NAC total is the sum of all isomers of 5-NAC-3-ene-VPA and 5-NAC-2-ene-VPA. The individual metabolite assays are described in Materials & Methods.

Table 4-3. Coefficient of Determinations (r^2) of the Correlations between the Individual VPA Metabolites

(*E*)-2,4-diene-	(*E*,*E*)-2,3´-diene-	4-ene-	3-ene-	2-ene-	4-OH-	3-OH-	5-OH-	3-keto-	4-keto-	VPA	VPA-G	NACtotal	
1	0.75^{***}	0.60^{***}	0.63^{***}	0.85^{***}	0.42^{***}	0.24^{***}	0.43^{***}	0.22^{***}	0.41^{***}	0.58^{***}	0.17^{***}	0.18^{***}	**(*E*)-2,4-diene-**
	1	0.45^{***}	0.69^{***}	0.84^{***}	0.33^{***}	0.36^{***}	0.34^{***}	0.20^{***}	0.28^{***}	0.65^{***}	0.16^{***}	0.21^{***}	**(*E*,*E*)-2,3´-diene-**
		1	0.66^{***}	0.44^{***}	0.41^{***}	0.18^{***}	0.49^{***}	0.16^{***}	0.49^{***}	0.69^{***}	0.29^{***}	0.09^{**}	**4-ene-**
			1	0.61^{***}	0.40^{***}	0.33^{***}	0.45^{***}	0.27^{***}	0.40^{***}	0.62^{***}	0.37^{***}	0.21^{***}	**3-ene-**
				1	0.36^{***}	0.29^{***}	0.36^{***}	0.16^{***}	0.29^{***}	0.59^{***}	0.25^{***}	0.21^{***}	**2-ene-**
					1	0.55^{***}	0.86^{***}	0.49^{***}	0.80^{***}	0.38^{***}	0.37^{***}	0.30^{***}	**4-OH-**
						1	0.49^{***}	0.27^{***}	0.39^{***}	0.33^{***}	0.16^{***}	0.18^{***}	**3-OH-**
							1	0.39^{***}	0.72^{***}	0.39^{***}	0.37^{***}	0.24^{***}	**5-OH-**
								1	0.56^{***}	0.17^{***}	0.11^{***}	0.24^{***}	**3-keto-**
									1	0.39^{***}	0.23^{***}	0.20^{***}	**4-keto-**
										1	0.27^{***}	0.07^{**}	**VPA**
											1	0.16^{**}	**VPA-G**
												1	**NACtotal**

Based on the bivariate correlation analysis of the $\log_{10}$ transformed metabolite data collected from 123 individual patients. $p < 0.01^{**}$ or 0.001^{***}. VPA-G (VPA-glucuronide); NACtotal (sum of all isomers of 5-NAC-3-ene-VPA and 5-NAC-2-ene-VPA). The individual metabolite assays are described in Materials and Methods.

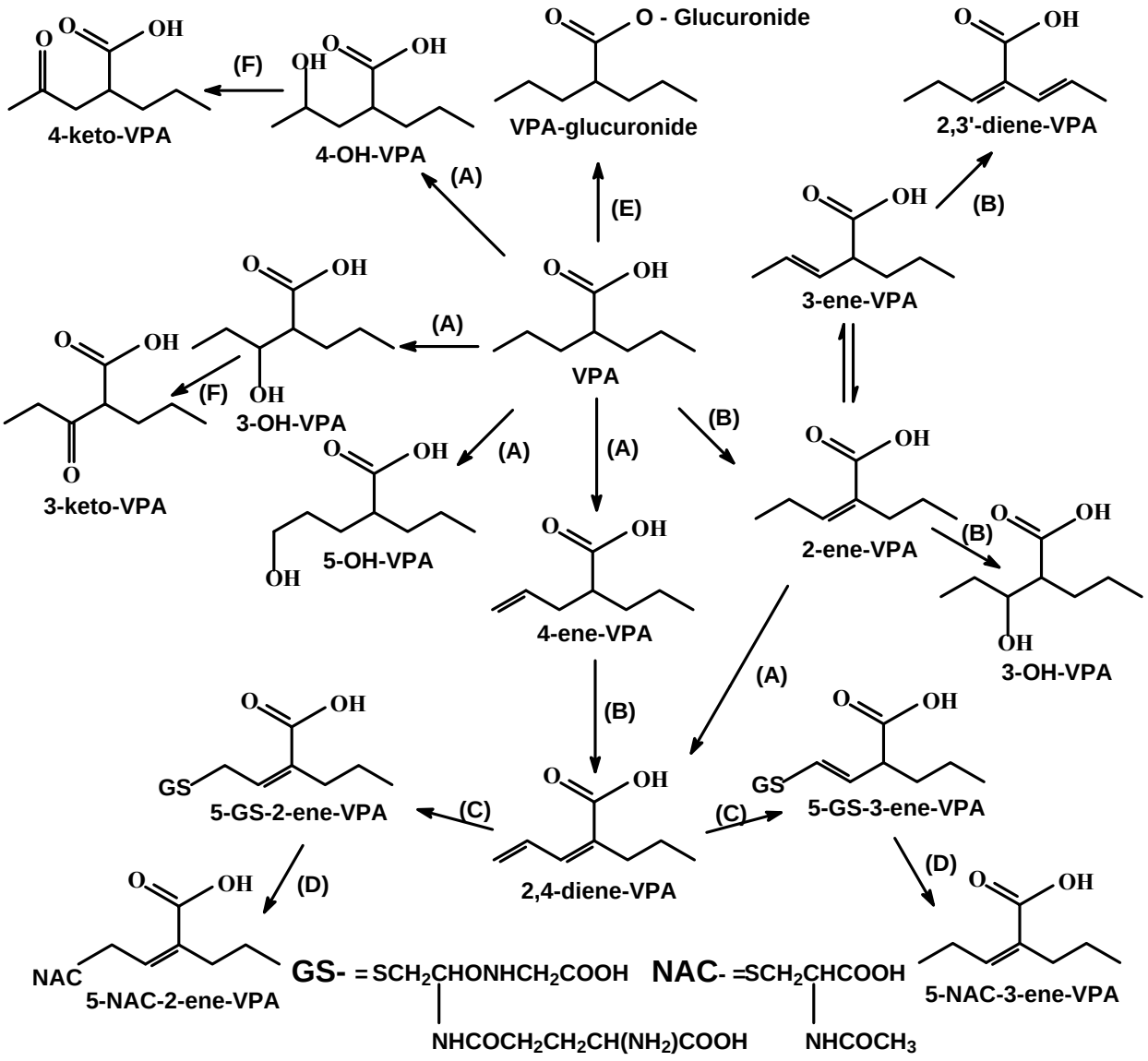

Figure 4-1. A summary of the metabolic pathways of VPA. (A) CYP oxidation, (B) ß-oxidation, (C) GSH conjugation, (D) mercapturic acid pathway, (E) glucuronidation, (F) alcohol dehydrogenation. (*E*)- and (*Z*)-isomers may exist for 2-ene-VPA, 3-ene-VPA, and 2,4-diene-VPA. (*E,E*)- and (*E,Z*)-isomers may exist for 2,3´-diene-VPA. Based on the review by Abbott and Anari (1999). GS (glutathione); NAC (*N*-acetylcysteine).

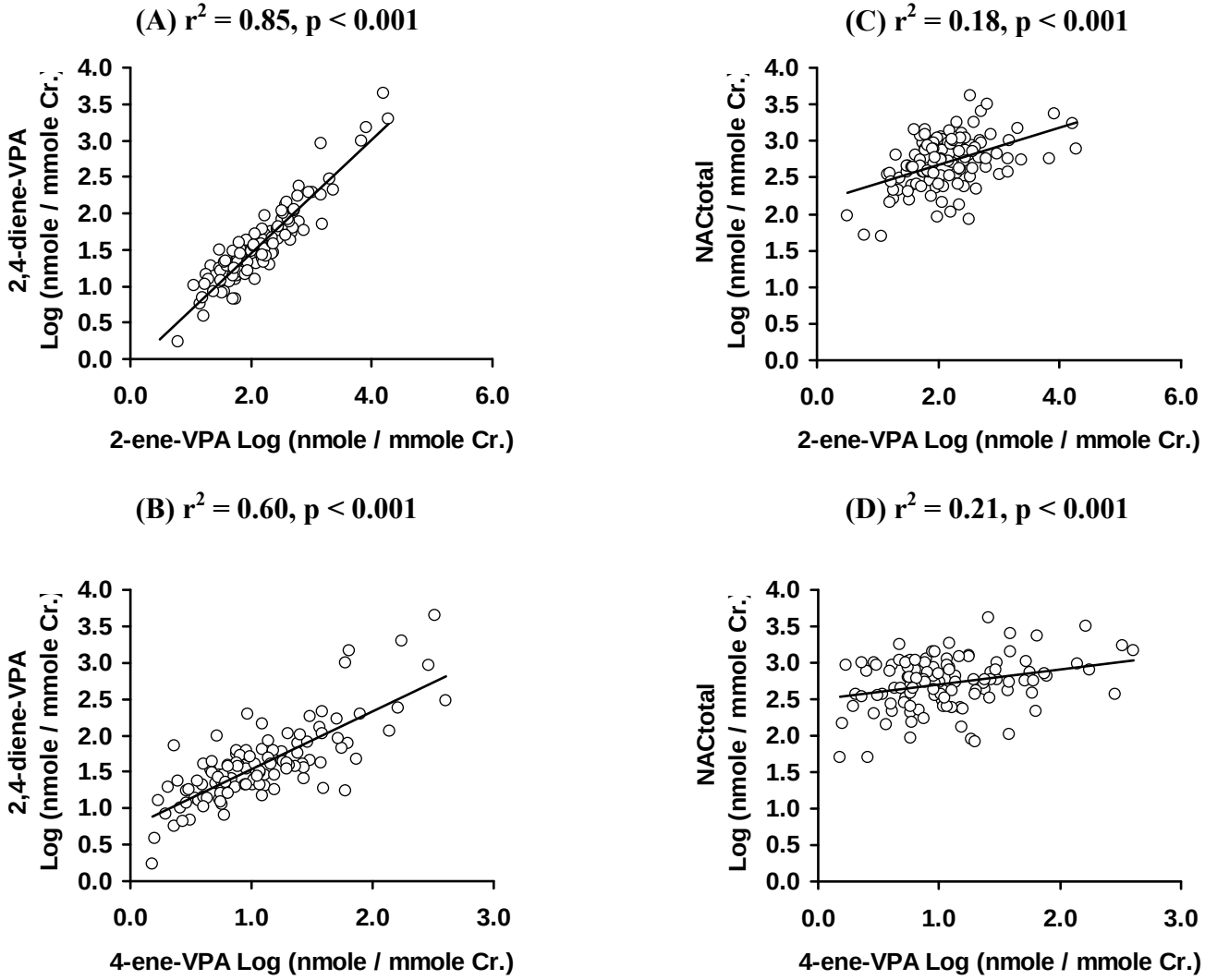

Figure 4-2. Correlations of metabolite levels between (A & B) metabolite precursors and their immediate products, or (C & D) products that are two or more steps removed in the biotransformation pathway of VPA. NACtotal is the sum of all isomers of 5-NAC-3-ene-VPA and 5-NAC-2-ene-VPA. The individual assays are described in Materials & Methods.

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5 Contribution of CYP2C9, CYP2A6, and CYP2B6 to Valproic Acid Metabolism in Hepatic Microsomes from Individuals with the CYP2C9*1/*1 Genotype[4]

5.1 Introduction

Valproic acid (2-propylpentanoic acid or dipropylacetic acid, VPA) is an antiepileptic drug, and its other indications include bipolar disorder, neuropathic pain, and migraine (Loscher, 1999). Current interest in VPA is based on its inhibition of histone deacetylation (Phiel *et al*., 2001) and its potential therapeutic application in cancer (Minucci and Pelicci, 2006) and human immunodeficiency virus infection (Lehrman *et al*., 2005). The clinical use of VPA is associated with a rare, but potentially fatal, hepatotoxicity (Loscher, 1999). Based on retrospective studies (Bryant III and Dreifuss, 1996), this idiosyncratic toxicity has been linked to several risk factors, including the concurrent administration of a cytochrome P450 (CYP)-inducing drug (e.g. phenobarbital), especially in patients younger than two years of age. The mechanism of VPA-associated hepatotoxicity is still not well-understood, but it may be associated with reactive metabolites of VPA, such as 4-ene-VPA (Baillie, 1988) and its β-oxidation product, 2,4-diene-VPA (Kassahun *et al.*, 1991; Tang *et al*., 1995).

VPA undergoes terminal desaturation, ω-2 hydroxylation, ω-1 hydroxylation, and ω-hydroxylation to form 4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA, respectively (Prickett and Baillie, 1984; Rettie et al., 1987). Studies conducted with individual cDNA-

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expressed human CYP enzymes indicated that CYP2A6, CYP2B6, and CYP2C9 catalyzed VPA terminal desaturation, whereas little or no metabolite was formed by several other CYP enzymes, including CYP2E1 and CYP3A4 (Sadeque *et al*., 1997; Anari *et al*., 2000). Very little information is available on the role of human hepatic microsomal CYP enzymes in VPA terminal desaturation. Hepatic microsomal CYP2A6 and CYP2C9 have been implicated in the formation of 4-ene-VPA, based on a study with microsome samples from two individual livers indicating that 4-ene-VPA formation was decreased by coumarin and sulfaphenazole (Sadeque *et al*., 1997), which were employed to inhibit the catalytic activity of CYP2A6 (Messina *et al*., 1997) and CYP2C9 (Newton *et al*., 1995), respectively. However, the contribution of these and other CYP enzymes to the formation of 4-ene-VPA in individual hepatic microsomes remains to be determined. Even less is known about the role of human CYP enzymes in VPA hydroxylation reactions. According to experiments with cDNA-expressed enzymes, CYP2C9 catalyzes VPA 4-hydroxylation and VPA 5-hydroxylation (Ho *et al*., 2003). However, it is not known whether other human CYP enzymes are capable of catalyzing the formation of 3-OH-VPA, 4-OH-VPA, or 5-OH-VPA or which specific CYP enzymes are responsible for these enzymatic reactions in human hepatic microsomes.

In the present study, we conducted a detailed, systematic investigation on the role of specific human CYP enzymes in the *in vitro* oxidative metabolism of VPA. A complementary approach was employed that involved: 1) individual cDNA-expressed human CYP enzymes; 2) chemical inhibitors of specific CYP enzymes; 3) CYP-specific inhibitory monoclonal antibodies; 4) individual human hepatic microsomes; and 5) correlational analysis. Our primary aim was to determine the contribution of specific CYP enzymes to VPA metabolism in human hepatic microsomes. Similar to the findings of other CYP2C9 substrates (Tracy *et al*., 2002), our previous study showed that the CYP2C9*2 and CYP2C9*3 allelic variants catalyzed VPA termination desaturation and hydroxylation reactions with substantially reduced capacity, when

compared to the wild-type CYP2C9*1 (Ho *et al*., 2003). Furthermore, chemical inhibition of CYP2C9 may be allele-dependent (Melet *et al*., 2003). Therefore, the present study was conducted with microsomes obtained only from donors with the CYP2C9*1/*1 genotype.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

VPA (the acid form, 99% pure) was supplied by Arcos Organics (Morris Plains, NJ), and the sodium salt of VPA was prepared (Olson et al., 1986). NADPH, sulfaphenazole, triethylenethiophosphoramide (thio-TEPA), coumarin, *N,N'*-diisopropylethylamine, and *tert*-butyldimethylsilyl chloride were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dimethylformamide, pentafluorobenzyl bromide, and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide were purchased from Pierce (Rockford, IL). Authentic VPA metabolites (4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA) and heptadeuterated internal standards (4-ene-[$^2H_7$]VPA, 3-OH-[$^2H_7$]VPA, 4-OH-[$^2H_7$]VPA, and 5-OH-[$^2H_7$]VPA) were synthesized in our laboratory (Zheng, 1993).

5.2.2 cDNA-expressed enzymes, hepatic microsomes, and monoclonal antibodies

Microsomes isolated from baculovirus-infected cells co-expressing NADPH-cytochrome P450 reductase and CYP1A1 (cat. no. 456211), CYP1A2 (cat. no. 456203), CYP1B1 (cat. no. 456220), CYP2A6 (cat. no. 456254), CYP2B6 (cat. no. 456255), CYP2C8 (cat. no. 456212), CYP2C9*1 (cat. no. 456218), CYP2C19 (cat. no. 456219), CYP2D6 (cat. no. 456217), CYP2E1 (cat. no. 456206), CYP3A4 (cat. no. 456202), CYP3A5 (cat. no. 456235), CYP4A11 (cat. no. 456221), CYP4F2 (cat. no. 456272), CYP4F3A (cat. no. 456273), or CYP4F3B (cat. no. 456274), control insect cell microsomes (cat. no. 456201), and individual human hepatic microsomes (HG24, HG30, HG88, HG95, HH13, HH18, HH47, HH64, HH74, HH91, HK37)

with the CYP2C9*1/*1 genotype were purchased from BD GENTEST Corp. (Woburn, MA). Among the eleven donors, eight were female and three were male. The mean ± S.D. age of the donors was 51 ± 17 years (the youngest was 28 years old and the oldest was 78 years old). Monoclonal antibody against CYP2A6 (MAb-2A6, clone 151-45-4) (Sai *et al*., 1999), monoclonal antibody against CYP2B6 (MAb-2B6, clone 49-10-20) (Yang *et al*., 1998), monoclonal antibody against CYP2C9 (MAb-2C9, clone 763-15-5) (Krausz *et al*., 2001), and control monoclonal antibody against lysozyme (Hy-Hel-9) (Krausz *et al*., 2001) were provided by Dr. H. V. Gelboin and his colleagues at the National Cancer Institute, National Institutes of Health (Bethesda, MD).

5.2.3 VPA metabolism assay

Each standard 200 μL incubation mixture contained 60 mM Tris buffer (pH 7.4), 1.8 mM $MgCl_2$, 1 mM sodium VPA, cDNA-expressed human CYP enzyme (40 pmol) or human hepatic microsomes (45 pmol total CYP), and 1 mM NADPH. The complete incubation mixture (but without NADPH) was pre-warmed to 37 ^{o}C for 5 min in a water bath with gentle shaking. The enzymatic reaction was initiated by the addition of NADPH and terminated 40 min later (unless indicated otherwise) by the addition of ice-cold 0.1 M phosphoric acid (75 μl). The substrate concentration (1 mM) was chosen to approximate steady-state plasma VPA concentrations achieved in humans administered maintenance doses of the drug (Loscher, 1999). Preliminary experiments were performed to delineate the incubation conditions whereby the VPA metabolism assay was linear with respect to incubation time and amount of enzyme. Standard curves were constructed with samples containing known amounts of authentic 4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA.

5.2.4 VPA metabolite analysis by gas chromatography – mass spectrometry

At the termination of the VPA metabolism assay, 50 μL of an internal standard mixture, which contained 2 μg/ml of 4-ene-[$^2H_7$]VPA, 3-OH-[$^2H_7$]VPA, 4-OH-[$^2H_7$]VPA, and 5-OH-[$^2H_7$]VPA, and 675 μl of distilled water, were added to each incubation mixture. To acidify the samples, 1 ml of 1 M KH_2PO_4 (pH 3.0) was added. Samples were extracted with ethyl acetate (8 ml) by gentle rotation for 30 min and then centrifuged at 3000 × *g* for 10 min at room temperature. The organic layer was transferred to a clean borosilicate glass screw-top test tube, dried with anhydrous sodium sulfate, rotated for 30 min, and centrifuged at 3000 × *g* for 10 min at room temperature. The organic layer was transferred to a new test tube and the ethyl acetate was evaporated to a volume of 100-200 μl under a gentle stream of nitrogen (0.5 p.s.i. at 25°C; Zymark Turbo Vap® LV evaporator, Zymark, Hopkinton, MA). To conduct the pentafluorobenzyl ester derivatization of the carboxylic acid groups, *N*,*N*′-diisopropylethylamine (30 μl) and pentafluorobenzyl bromide (10 μl) were added to each of the extracted samples followed by vortex-mixing and heating at 45 °C for 60 min. Subsequently, the mixture was cooled to room temperature. To perform the *tert*-butyldimethylsilylation of hydroxyl groups, 20 μl of dimethylformamide and 40 μl of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide containing 2% *tert*-butyldimethylsilyl chloride were added to the sample mixture and heated at 65 °C for 2 h. The samples were cooled to room temperature and then concentrated by applying a gentle stream of nitrogen (0.5 p.s.i. at 25°C for 30 min). The residue was mixed with 200 μl of *n*-hexane (gas chromatography grade) by vortexing for 20 s, and the mixture was centrifuged at 3000 × *g* for 10 min at room temperature. Subsequently, 1 μl of the hexane layer was analyzed for VPA metabolites. The analytical system consisted of an HP6890 gas chromatograph, an HP5973 mass selective detector, and an HP7683 autosampler (Hewlett-Packard, Avondale, PA). The HP Enhanced Chemstation Software G1701BA (V. B.01.00) was used. The operating

conditions and the specifications of the chromatographic columns were the same as those described previously (Ho *et al*., 2003).

5.2.5 Chemical inhibition experiments

Sulfaphenazole (20 μM), coumarin (50 μM), or methanol (0.5% v/v final concentration; vehicle control) was added to each incubation mixture (without preincubating the inhibitor with microsomes and NADPH), and the VPA metabolism assay was performed as described above. Thio-TEPA (50 μM) or distilled water (vehicle control) was preincubated with human hepatic microsomes (67.5 pmol total CYP) and NADPH (1 mM) for 15 min at 37 ^{o}C in a volume of 30 μl. Subsequently, a 20 μl aliquot was transferred to a tube containing 60 mM Tris (pH 7.4), 1.8 mM $MgCl_2$, 1 mM sodium VPA, and 1 mM NADPH. The reaction was stopped 40 min later by the addition of ice-cold 0.1 M phosphoric acid (75 μl). VPA metabolite analysis was performed as described above.

5.2.6 Immunoinhibition experiments

The general steps in the immunoinhibition experiments were performed according to a published protocol (Krausz et al., 2001). Briefly, individual human hepatic microsomes (45 pmol total CYP) were preincubated with MAb-2A6, MAb-2B6, MAb-2C9, or the corresponding level of control MAb (as indicated in each figure legend) in 60 mM Tris buffer (pH 7.4) containing 1.8 mM $MgCl_2$ for 5 min at 37 ^{o}C prior to the addition of sodium VPA (1 mM) and NADPH (1 mM). The reaction was terminated 40 min later (unless indicated otherwise) by the addition of ice-cold 0.1 M phosphoric acid (75 μl). VPA metabolite analysis was performed as described above.

5.2.7 Statistical analysis

The difference between the means of the groups was analyzed by the one-tailed, paired t-test. The level of significance was set *a priori* at $p < 0.05$.

5.3 Results

5.3.1 VPA Metabolism by Individual cDNA-expressed Human CYP Enzymes

To identify individual human CYP enzymes competent in catalyzing VPA terminal desaturation and hydroxylation reactions, the VPA metabolism assay was performed with individual cDNA-expressed CYP enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9*1, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A11, CYP4F2, CYP4F3A, and CYP4F3B). Among these enzymes, only CYP2C9*1, CYP2A6, and CYP2B6 catalyzed the formation of 4-ene-VPA (Figure 5-1). These three enzymes also catalyzed VPA 4-hydroxylation and VPA 5-hydroxylation. Minimal levels of 4-OH-VPA and 5-OH-VPA were formed by CYP1A1, CYP1A2, CYP1B1, CYP2C8, CYP2C19, CYP2D6, CYP2E1, CYP4A11, CYP4F2, CYP4F3A, and CYP4F3B (only 1-8% of the levels by CYP2C9*1). Only CYP1A1, CYP2A6, CYP2B6, CYP4F2, and CYP4F3B catalyzed VPA 3-hydroxylation (Figure 5-1).

5.3.2 VPA Metabolism by Human Hepatic Microsomes

The results from the experiment with cDNA-expressed enzymes indicated that CYP2A6, CYP2B6, and CYP2C9 were the major catalysts of VPA metabolism and suggested a role by the corresponding hepatic microsomal enzyme. Our initial experiment with hepatic microsomes was to determine VPA metabolism in samples characterized for their CYP2A6, CYP2B6, and CYP2C9 catalytic activities, as assessed by coumarin 7-hydroxylation, (*S*)-mephenytoin *N*-demethylation, and diclofenac 4′-hydroxylation, respectively (Table 5-1). Each individual hepatic microsome sample catalyzed VPA terminal desaturation, VPA 4-hydroxylation, VPA-5-

hydroxylation, and VPA-3-hydroxylation. The ratio was 1: 10: 10: 0.6 for the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA. As shown by correlational analyses (Table 5-2), a positive correlation was obtained between 4-ene-VPA formation and CYP2C9-mediated diclofenac 4′-hydroxylation ($r^2 = 0.55$, $p = 0.009$), and between 3-OH-VPA formation and CYP2A6-mediated coumarin 7-hydroxylation ($r^2 = 0.47$, $p = 0.02$).

5.3.3 Effect of Chemical Inhibitors of CYP2A6, CYP2B6, and CYP2C9 on VPA Metabolism by Human Hepatic Microsomes

To determine whether human hepatic microsomal CYP2A6, CYP2B6, and CYP2C9 played a role in catalyzing VPA terminal desaturation and hydroxylation reactions, the VPA metabolism assay was conducted with individual hepatic microsome samples in the presence of coumarin (to inhibit CYP2A6) (Messina *et al*., 1997), thio-TEPA (to inhibit CYP2B6) (Rae *et al*., 2002), sulfaphenazole (to inhibit CYP2C9) (Newton *et al*., 1995), or the respective vehicle (control). Sulfaphenazole decreased the group mean formation of 4-ene-VPA (by 54 ± 4%; mean ± S.E.M., Figure 5-2), 4-OH-VPA (by 62 ± 4%), and 5-OH-VPA (by 66 ± 6%), but not 3-OH-VPA. In contrast, coumarin did not reduce the group mean formation of 4-ene-VPA (Figure 5-2), 4-OH-VPA, or 5-OH-VPA, but it decreased 3-OH-VPA by 57 ± 9%. By comparison, thio-TEPA did not affect the group mean formation of any of these metabolites. However, in one microsomal sample (HH74), which had the greatest CYP2B6-mediated (*S*)-mephenytoin *N*-demethylation activity in our panel of microsome samples, thio-TEPA reduced the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by 26%, 15%, and 29%, respectively. Control experiments verified the inhibition of CYP2A6, CYP2B6, and CYP2C9 by coumarin, thio-TEPA, and sulfaphenazole, respectively, as evaluated by incubations containing VPA and the corresponding cDNA-expressed enzyme (data not shown).

5.3.4 Effect of MAb-2A6, MAb-2B6, and MAb-2C9 on VPA Metabolism by Human Hepatic Microsomes

To assess the extent by which CYP2A6, CYP2B6, and CYP2C9 contributed to VPA terminal desaturation and hydroxylation reactions in human hepatic microsomes, we used inhibitory monoclonal antibodies (i.e. MAb-2A6, MAb-2B6, and MAb-2C9) of known specificity toward CYP2A6 (Sai *et al*., 1999), CYP2B6 (Yang *et al*., 1998), and CYP2C9 (Krausz *et al*., 2001). Initial experiments verified the inhibitory effect (> 90%) of these antibodies and determined the level that yielded maximal inhibition of human hepatic microsomal VPA metabolism (data not shown). MAb-2C9 decreased the group mean formation of 4-ene-VPA by 77 ± 1% (Figure 5-3), 4-OH-VPA by 75 ± 2%, and 5-OH-VPA by 80 ± 3%, but it did not affect 3-OH-VPA. In contrast, MAb-2A6 did not affect the group mean formation of 4-ene-VPA (Figure 5-3), 4-OH-VPA, or 5-OH-VPA, whereas it reduced 3-OH-VPA by 55 ± 6%. By comparison, MAb-2B6 did not affect the group mean formation of any of these metabolites (Figure 5-3). However, in one sample (HH74) with high CYP2B6-associated enzyme activity, it reduced the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by 26%, 18%, and 42%, respectively.

5.3.5 Combinatorial Immunoinhibition of VPA Metabolism by Human Hepatic Microsomes

To further illustrate that CYP2A6 and CYP2B6 contributed to the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA in human hepatic microsomes, we employed a combinatorial approach with monoclonal antibodies (Gelboin and Krausz, 2006) in a human hepatic microsome sample (HG24) from our panel that had a large CYP2A6 and CYP2B6 catalytic capacity. The combination of MAb-2A6 and MAb-2B6 yielded greater inhibition of 4-ene-VPA (21%, Figure 5-4), 4-OH-VPA (24%), and 5-OH-VPA formation (24%) when compared to the

effect by each antibody when added alone. In contrast, the effect on 3-OH-VPA formation by the combination of MAb-2A6 and MAb-2B6 was similar to that by MAb-2A6 alone, in accord with the lack of inhibition by MAb-2B6 (Figure 5-4). The combination of MAb-2A6, MAb-2B6, and MAb-2C9 decreased the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA by 92% (Figure 5-4), 85%, and 88%, respectively.

5.3.6 Association Between the Extent of Immunoinhibition of VPA Metabolism by MAb-2B6 and MAb-2A6 and the Levels of CYP2B6- and CYP2A6-mediated Enzyme Activities

We determined whether the extent of contribution by hepatic microsomal CYP2B6 and CYP2A6 to VPA metabolism was associated with the inherent catalytic capacity of these enzymes in microsomes. Positive correlation was obtained between MAb-2B6 inhibition of 4-OH-VPA formation and CYP2B6-mediated (*S*)-mephenytoin *N*-demethylation activity ($r^2 = 0.79$, $p = 0.001$, Figure 5-5), MAb-2B6 inhibition of 5-OH-VPA formation and CYP2B6-mediated (*S*)-mephenytoin *N*-demethylation activity ($r^2 = 0.79$, $p = 0.002$), and MAb-2A6 inhibition of 3-OH-VPA formation and CYP2A6-mediated coumarin 7-hydroxylation activity ($r^2 = 0.58$, $p = 0.02$).

5.4 Discussion

Previous studies with cDNA-expressed enzymes showed that CYP2C9 catalyzed the formation of 4-ene-VPA (Sadeque *et al*., 1997; Anari *et al*., 2000; Ho *et al*., 2003). The present study confirms this finding and further demonstrates that cDNA-expressed CYP2C9 catalyzes VPA 4-hydroxylation and VPA 5-hydroxylation. In the only study with human hepatic microsomes reported to date, it was concluded that CYP2C9 played a role in the formation of 4-ene-VPA (Sadeque *et al*., 1997), based on experiments with sulfaphenazole, a CYP2C9-selective inhibitor (Newton *et al*., 1995). However, that study was performed with microsome samples

from two individuals and their CYP2C9 genotype was not known (Sadeque *et al*., 1997). The percentage decrease in 4-ene-VPA formation by sulfaphenazole was 43% in one microsome sample, but 15% in the other sample. The present study was conducted with hepatic microsomes obtained from donors with the CYP2C9*1/*1 genotype. A novel finding is that CYP2C9*1 is the principal human hepatic microsomal CYP catalyst in the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA. This conclusion was based on our immunoinhibition data showing that MAb-2C9 decreased the group mean formation of each of these VPA metabolites by 75-80%, suggesting that CYP2C9*1 was largely (i.e., 75-80%) responsible for hepatic microsomal formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA. Consistent with this conclusion are the findings that: 1) a CYP2C9-selective inhibitor, sulfaphenazole, reduced the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA (Figure 5-2); 2) CYP2C9-mediated diclofenac 4′-hydroxylation activity correlated with 4-ene-VPA formation (Table 5-2); and 3) VPA competitively inhibited CYP2C9-mediated tolbutamide hydroxylation activity in human hepatic microsomes (apparent K_i = 0.6 mM) (Wen *et al*., 2001).

The contribution of CYP2A6 and CYP2B6 to the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA was minimal in hepatic microsomes from individuals with the CYP2C9*1/*1 genotype, as judged by the results from the immunoinhibition experiments with MAb-2A6 and MAb-2B6 and the chemical inhibition experiments with coumarin (to inhibit CYP2A6) (Messina *et al*., 1997) and thio-TEPA (to inhibit CYP2B6) (Rae *et al*., 2002). According to our combinatorial immunoinhibition analysis, CYP2A6 and CYP2B6 together accounted for 20-25% of the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA in the hepatic microsome sample that was analyzed. However, inter-individual variability existed in the contribution of CYP2B6 to these catalytic reactions, as illustrated by the positive correlation between CYP2B6-mediated (*S*)-mephenytoin *N*-demethylation activity and the percentage inhibition of VPA 4-hydroxylation (or VPA 5-hydroxylation) by MAb-2B6 in hepatic microsomes. The basis for this variability is

the substantial inter-individual differences in hepatic CYP2B6 expression (Code *et al*., 1997), which is due, in part, to the inducibility of this enzyme by drugs and other chemicals (Chang et al., 1997). Pharmacogenetics is another factor that may influence the relative contribution of specific CYP enzymes to a drug metabolism reaction. As an example, the CYP2C9*2 and CYP2C9*3 alleles are associated with substantially decreased VPA oxidative metabolism (Ho et al., 2003). Therefore, in an individual with a CYP2C9 poor metabolizer phenotype, CYP2A6 and CYP2B6 may account for a greater proportion of the enzymatic activity for the terminal desaturation, 4-hydroxylation, and 5-hydroxylation of VPA.

Our experiment with individual cDNA-expressed enzymes also identified other human CYP catalysts of VPA 4-hydroxylation and VPA 5-hydroxylation. However, the extent of 4-OH-VPA and 5-OH-VPA formation by CYP1A1, CYP1A2, CYP1B1, CYP2C8, CYP2C19, CYP2D6, CYP2E1, CYP4A11, CYP4F2, CYP4F3A, and CYP4F3B was only 1-8% of the levels by CYP2C9*1. Among these enzymes, CYP1A2, CYP2C8, CYP2C19, CYP2D6, CYP2E1, CYP4A11, CYP4F2 and CYP4F3B are expressed in human liver (Christmas *et al*., 2001; Donato and Castell, 2003). However, the contribution of these enzymes to 4-OH-VPA and 5-OH-VPA formation in human hepatic microsomes was likely minimal or negligible because as shown by our combinatorial immunoinhibition analysis, CYP2C9, CYP2A6, and CYP2B6 were responsible for virtually all of the VPA 4-hydroxylation and VPA 5-hydroxylation activities in human hepatic microsomes. Similarly, CYP1A1, CYP1B1, and CYP4F3A should not have accounted for any of the hepatic microsomal VPA 4-hydroxylation and VPA 5-hydroxylation activities because these enzymes are not expressed in human liver (Christmas et al., 2001; Chang et al., 2003).

An earlier study implicated CYP enzymes in the metabolism of VPA to form 3-OH-VPA (Prickett and Baillie, 1984). Another novel finding in the present study is that cDNA-expressed human CYP1A1, CYP2A6, CYP2B6, CYP4F2, and CYP4F3B are active catalysts of VPA 3-

hydroxylation. Based on the immunoinhibition experiment with MAb-2A6, we concluded that CYP2A6 contributed approximately 50% to VPA 3-hydroxylation activity in human hepatic microsomes. The extent of contribution by CYP2A6 was associated with the inherent catalytic activity in each microsome sample, as illustrated by the positive correlation between the percentage inhibition of 3-OH-VPA formation by MAb-2A6 and CYP2A6-mediated coumarin 7-hydroxylation activity. A role for hepatic microsomal CYP2A6 in VPA 3-hydroxylation is supported by our findings that: 1) an inhibitor of CYP2A6 activity, coumarin (Messina *et al*., 1997), reduced the formation of 3-OH-VPA in human hepatic microsomes (Figure 5-2); and 2) CYP2A6-mediated coumarin 7-hydroxylation activity correlated with the formation of 3-OH-VPA in human hepatic microsomes ($r^2 = 0.47$, $p = 0.02$, Table 5-2). Consistent with these results is the finding that VPA is a mechanism-based inactivator of CYP2A6 ($K_{inact} = 0.9$ mM) (Wen *et al*., 2001). The remainder of the VPA 3-hydroxylation activity in human hepatic microsomes was not due to CYP2B6 because the formation of 3-OH-VPA was not decreased by MAb-2B6 or a CYP2B6-selective chemical inhibitor (thio-TEPA). A role for CYP1A1 can also be ruled out because this enzyme is not expressed in human liver (Chang *et al.,* 2003). Candidate enzymes include CYP4F2 and CYP4F3B, which catalyze the ω-hydroxylation of arachidonic acid (Powell *et al*., 1998) and leukotriene B_4 (Christmas *et al*., 2001), respectively, and are expressed in human liver. The contribution of these two enzymes to VPA 3-hydroxylation in human hepatic microsomes was not determined because inhibitory antibody specific for CYP4F2 or CYP4F3B was not available.

Limited information is available on the role of other mammalian CYP enzymes in VPA metabolism. Immunologically purified rat CYP2B1 catalyzes the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA with a ratio of 1: 37: 5 (Rettie *et al.*, 1995). This pronounced preference for VPA 4-hydroxylation by rat CYP2B1 was not apparent in our experiment with

cDNA-expressed human CYP2B6, which yielded a ratio of 1: 47: 51. In contrast to rat CYP2B1, purified rabbit CYP4B1 has a greater preference for VPA 5-hydroxylation (a ratio of 1: 2: 117 for the formation of 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA) (Rettie *et al*., 1995). However, among the human CYP enzymes investigated in the present study, none of them showed a preference for VPA 5-hydroxylation over VPA 4-hydroxylation. Rat CYP3A enzymes account for the majority of VPA 3-hydroxylation activity in hepatic microsomes from rats treated with pregnenolone 16α-carbonitrile (an inducer of CYP3A) (Fisher *et al*., 1998). However, as shown in the present study, human CYP3A4 and CYP3A5 did not metabolize VPA, in accord with previous findings (Sadeque *et al*., 1997; Anari *et al*., 2000). Collectively, these results suggest species-dependent effects of CYP enzymes on VPA metabolism.

The biotransformation of VPA involves glucuronidation, β-oxidation, and CYP-catalyzed terminal desaturation and hydroxylation (Abbott and Anari, 1999). Although the CYP-catalyzed metabolism of VPA is quantitatively minor relative to the other two pathways, it is of toxicological interest because of the formation of 4-ene-VPA. As shown in previous studies conducted *in vitro* (cultured rat hepatocytes) (Kingsley et al., 1983; Jurima-Romet et al., 1996) and in *vivo* (rats) (Kesterson et al., 1984; Loscher et al., 1993), signs and symptoms of hepatic injury could be demonstrated under specific experimental conditions by the direct administration of relatively high doses of 4-ene-VPA. However, it is not entirely clear from human studies whether the *in situ* concentrations of the enzymatically formed 4-ene-VPA are sufficient to account for the hepatotoxicity in patients administered VPA (Nau et al., 1991; Siemes et al., 1993). This may relate to the notion that the hepatotoxicity of 4-ene-VPA is due to reactive metabolites produced by the β-oxidation of 4-ene-VPA (Tang et al., 1995). Future studies are needed to investigate directly whether modulation of CYP-catalyzed formation of 4-ene-VPA and the subsequent alteration in the levels of β-oxidation-derived reactive metabolites would influence the extent of toxicity in cultured human hepatocytes treated with VPA.

In summary, based on the *in vitro* VPA metabolism assay conducted at 1 mM substrate concentration and with human hepatic microsomes from individuals with the CYP2C9*1/*1 genotype: 1) CYP2C9*1 was responsible for the majority (75-80%) of VPA terminal desaturation, VPA 4-hydroxylation, and VPA 5-hydroxylation activities, whereas CYP2A6 and CYP2B6 contributed to the remainder of these reactions (Figure 5-6); 2) CYP2A6 accounted for approximately 50% of VPA 3-hydroxylation activity (Figure 5-6); and 3) the extent by which CYP2A6 and CYP2B6 contributed to VPA oxidative metabolism was associated with the inherent catalytic capacity of these enzymes in each microsome sample.

Table 5-1. VPA oxidative metabolism and CYP2A6-, CYP2B6-, and CYP2C9-associated enzyme activities in our panel of hepatic microsomes from individuals with the CYP2C9*1/*1 genotype

	Mean	S.D.	CV
	(pmol/min/nmol total CYP)		(%)
VPA terminal desaturation (4-ene-VPA)	13	5	38
VPA 4-hydroxylation	130	30	23
VPA 5-hydroxylation	128	52	41
VPA 3-hydroxylation	8	2	25
Coumarin 7-hydroxylation (CYP2A6)	1964	1463	74
(*S*)-Mephenytoin *N*-demethylation (CYP2B6)	135	147	109
Diclofenac 4′-hydroxylation (CYP2C9)	8752	2662	30

The VPA metabolism assay (1 mM substrate) was performed in duplicate at 37^{o}C for 40 min with individual human hepatic microsomes (45 pmol total CYP) and the formation of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, and 3-OH-VPA was determined as described under Materials and Methods. The coumarin 7-hydroxylation, (*S*)-mephenytoin *N*-demethylation, and diclofenac 4′-hydroxylation data were provided by the supplier. Shown are the mean, standard deviation (S.D.), and coefficient of variation (CV) in eleven individual samples of hepatic microsomes.

Table 5-2. Correlation analysis of VPA oxidative metabolism and CYP enzyme-selective activities in hepatic microsomes from individuals with the CYP2C9*1/*1 genotype

Substrate Reaction	Associated CYP Enzyme	Coefficient of Determination (r^2)			
		4-ene-VPA	4-OH-VPA	5-OH-VPA	3-OH-VPA
Phenacetin *O*-deethylation	CYP1A2	0.02	0.01	0.02	0.50*
Coumarin 7-hydroxylation	CYP2A6	0.02	0.03	0.00	0.47*
(*S*)-Mephenytoin *N*-demethylation	CYP2B6	0.00	0.05	0.00	0.00
Paclitaxel 6α-hydroxylation	CYP2C8	0.13	0.19	0.10	0.01
Diclofenac 4′-hydroxylation	CYP2C9	0.55*	0.23	0.32	0.18
(*S*)-Mephenytoin 4′-hydroxylation	CYP2C19	0.00	0.00	0.01	0.19
Bufuralol 1′-hydroxylation	CYP2D6	0.00	0.02	0.01	0.12
Chlorzoxazone 6-hydroxylation	CYP2E1	0.03	0.01	0.00	0.03
Testosterone 6β-hydroxylation	CYP3A	0.06	0.05	0.03	0.30
Lauric acid 12-hydroxylation	CYP4A11	0.00	0.00	0.00	0.08

Correlational analyses were performed with enzyme activity data from the same samples as those in Table 5-1. *$p < 0.05$

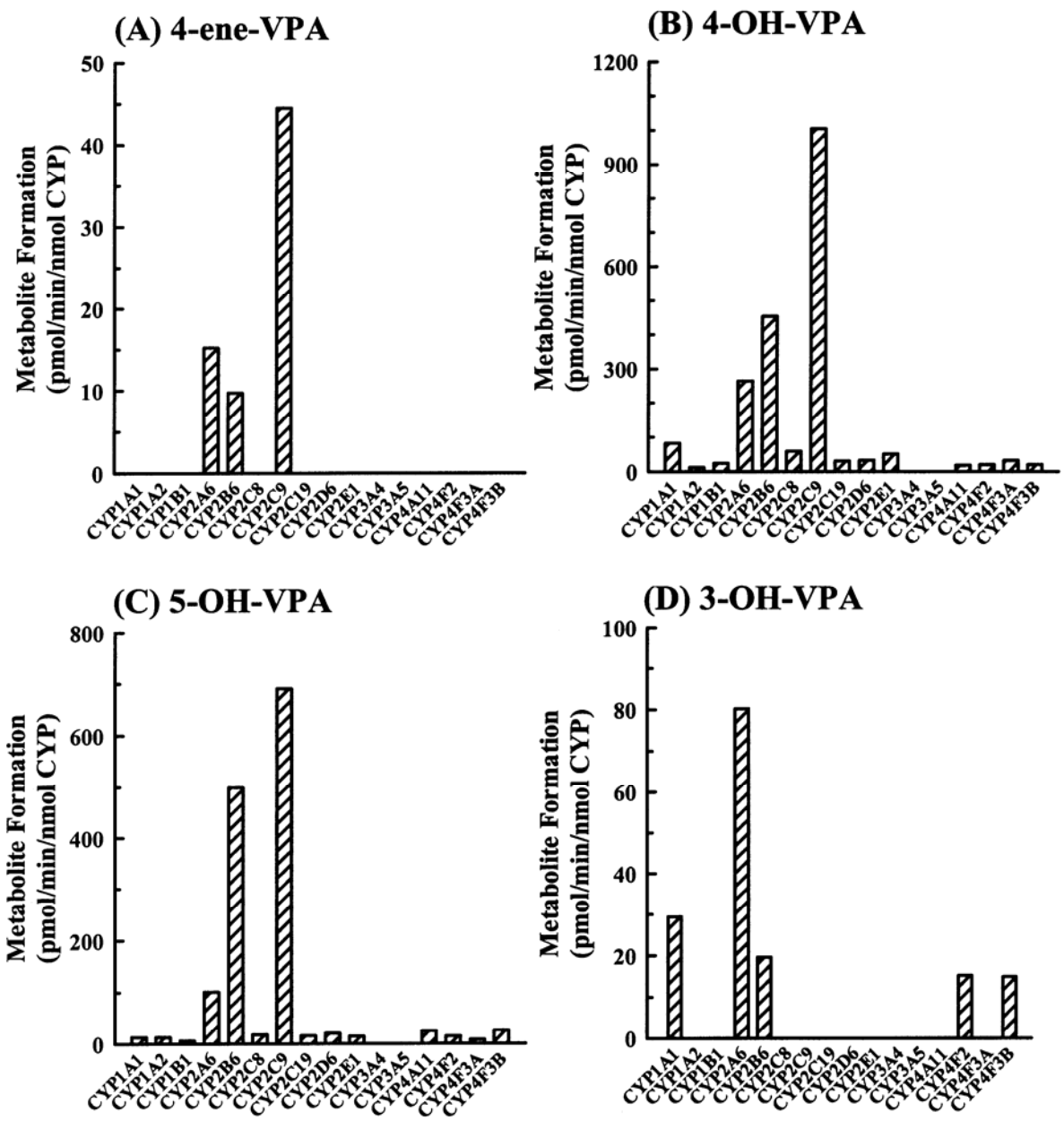

Figure 5-1. VPA terminal desaturation and hydroxylation reactions catalyzed by individual cDNA-expressed human CYP enzymes. The VPA metabolism assay (1 mM substrate) was performed at 37 ^{o}C for 30 min with individual cDNA-expressed human CYP enzymes (40 pmol). The levels of 4-ene-VPA (panel A), 4-OH-VPA (panel B), 5-OH-VPA (panel C), and 3-OH-VPA (panel D) were determined as described under Materials and Methods. Results are expressed as the mean of two incubations. The differences in metabolite formation between the duplicate incubations were <25%.

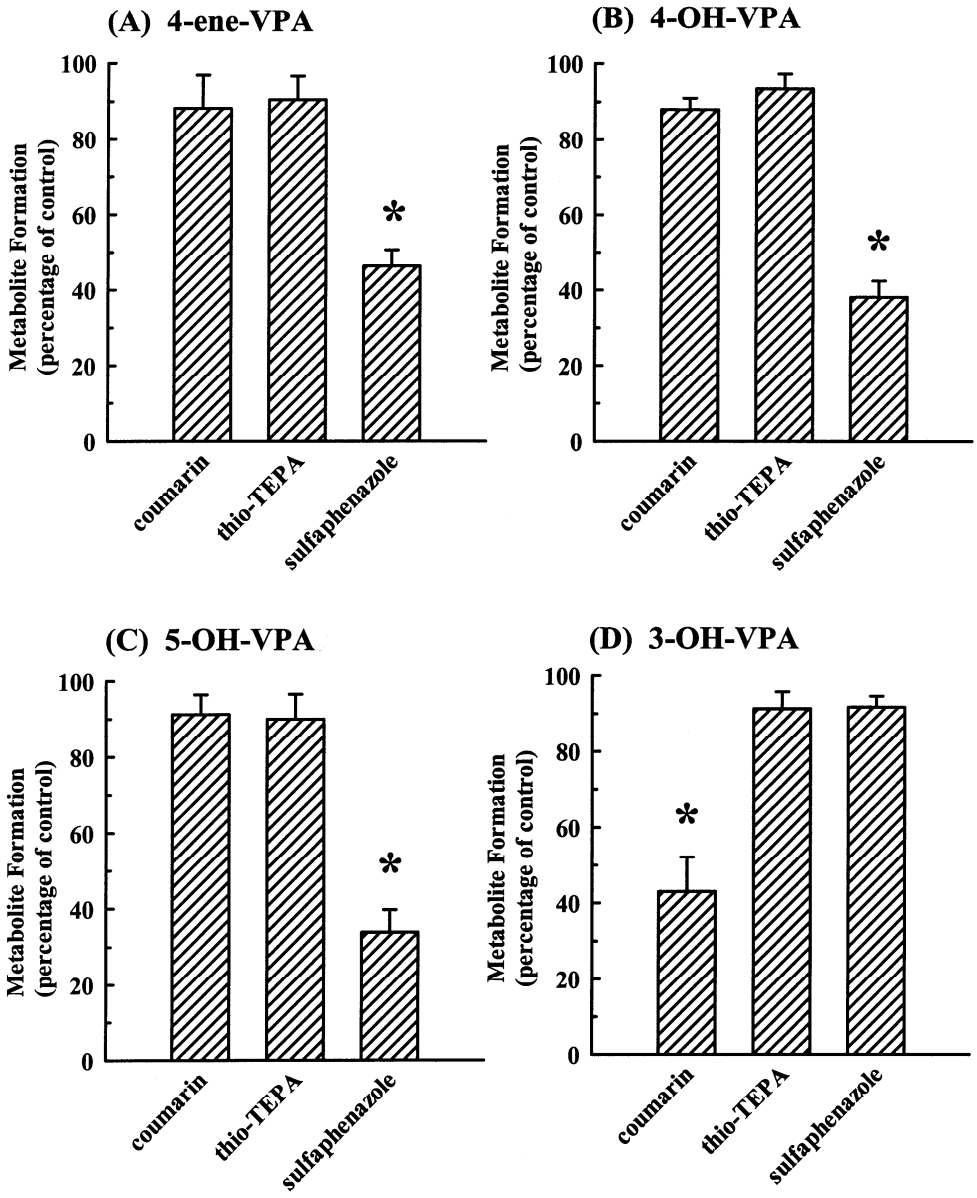

Figure 5-2. Effect of coumarin, thio-TEPA, and sulfaphenazole on VPA desaturation and hydroxylation reactions catalyzed by human hepatic microsomes. The VPA metabolism assay (1 mM substrate) was performed in duplicate at 37 ^{o}C with individual human hepatic microsomes (45 pmol total CYP) and coumarin (50 μM), thio-TEPA (50 μM), sulfaphenazole (20 μM), or vehicle (distilled water for thio-TEPA and 0.5% v/v methanol for the other two inhibitors). The incubation period was 40 min (but 70 min for the analysis of 3-OH-VPA in incubations containing coumarin). The levels of 4-ene-VPA (panel A), 4-OH-VPA (panel B), 5-OH-VPA (panel C), and 3-OH-VPA (panel D) were determined as described under Materials and Methods. Results are expressed as mean ± SEM metabolite formation (as a percentage of vehicle control) for four individual microsome samples. *$p < 0.05$ vs. the corresponding control group.

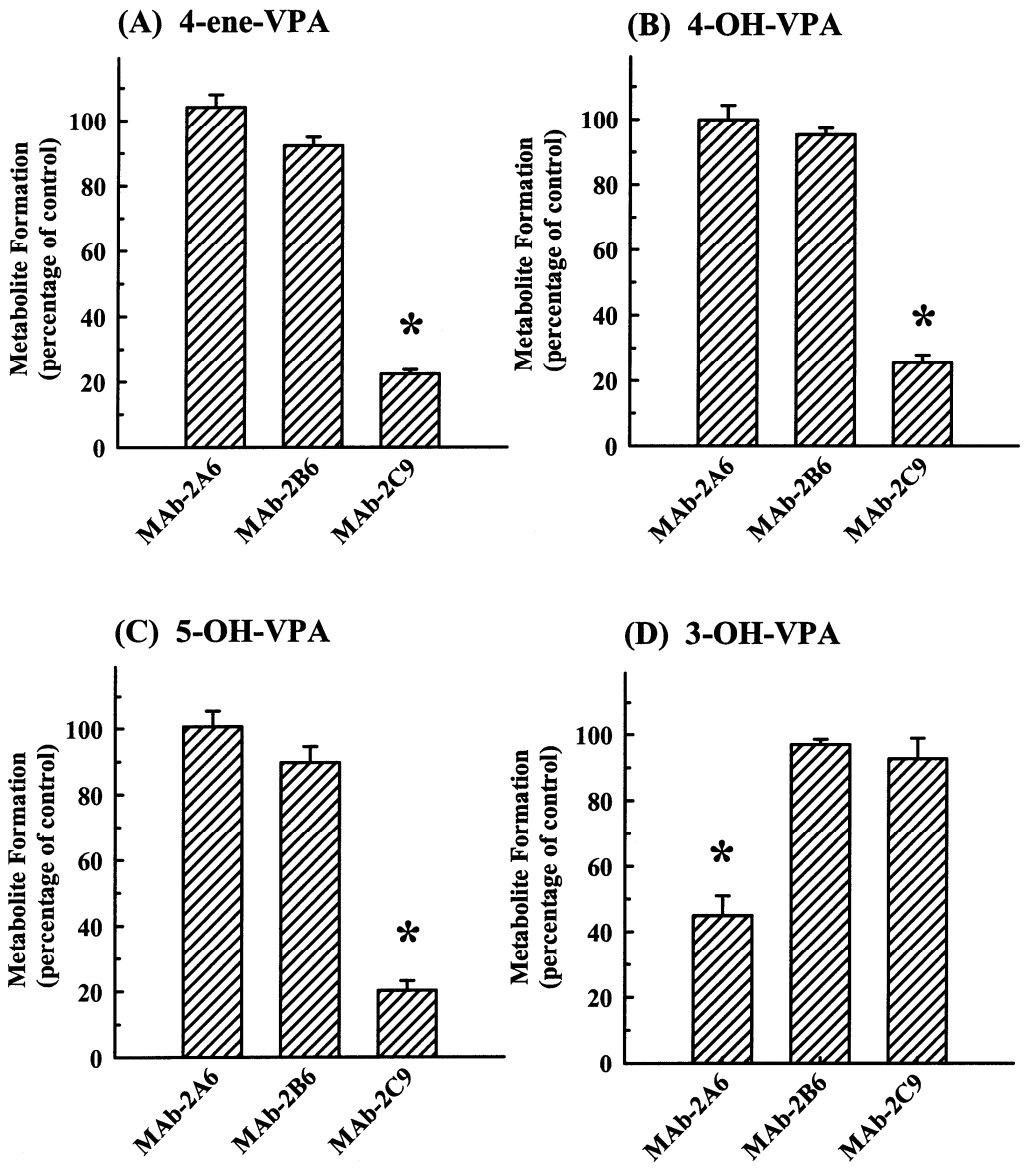

Figure 5-3. Immunoinhibition of VPA metabolism in a panel of individual human hepatic microsomes. The VPA metabolism assay (1 mM substrate) was performed in duplicate at 37 ^{o}C with individual human hepatic microsomes (45 pmol total CYP) and MAb-2A6 (3 µl), MAb-2B6 (5 µl), MAb-2C9 (3 µl), or an equivalent level of control MAb (Hy-Hel-9). The incubation period was 40 min (but 90 min for the analysis of 3-OH-VPA in incubations containing MAb-2A6 and for the analysis of the other three metabolites in incubations containing MAB-2C9). The levels of 4-ene-VPA (panel A), 4-OH-VPA (panel B), 5-OH-VPA (panel C), and 3-OH-VPA (panel D) were determined as described under Materials and Methods. Results are expressed as mean ± SEM metabolite formation (as percentage of control) for nine individual microsome samples. *$p < 0.05$ vs. the corresponding control group.

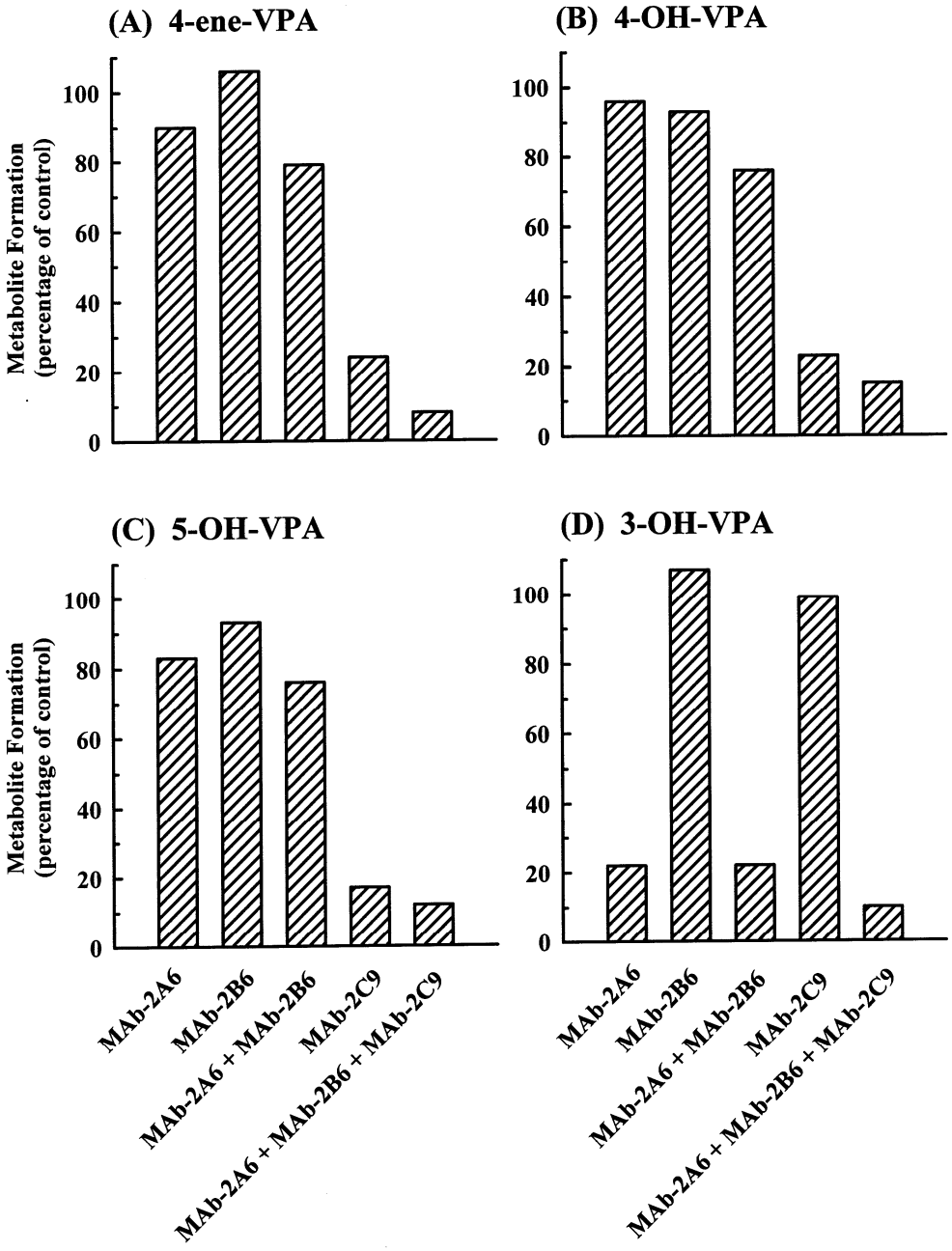

Figure 5-4. Combinatorial immunoinhibition of VPA metabolism catalyzed by human hepatic microsomes. VPA metabolism assay was performed as described in the legend to Figure 5-3, except that the additional groups were the combination of MAb-2A6 and MAb-2B6 and the combination of MAb-2A6, MAb-2B6, and MAb-2C9. The levels of 4-ene-VPA (panel A), 4-OH-VPA (panel B), 5-OH-VPA (panel C), and 3-OH-VPA (panel D) were determined in duplicate in an individual human hepatic microsome sample (HG24). Results are expressed as percentage of metabolite formation in control incubation containing an equivalent level of control MAb (Hy-Hel-9).

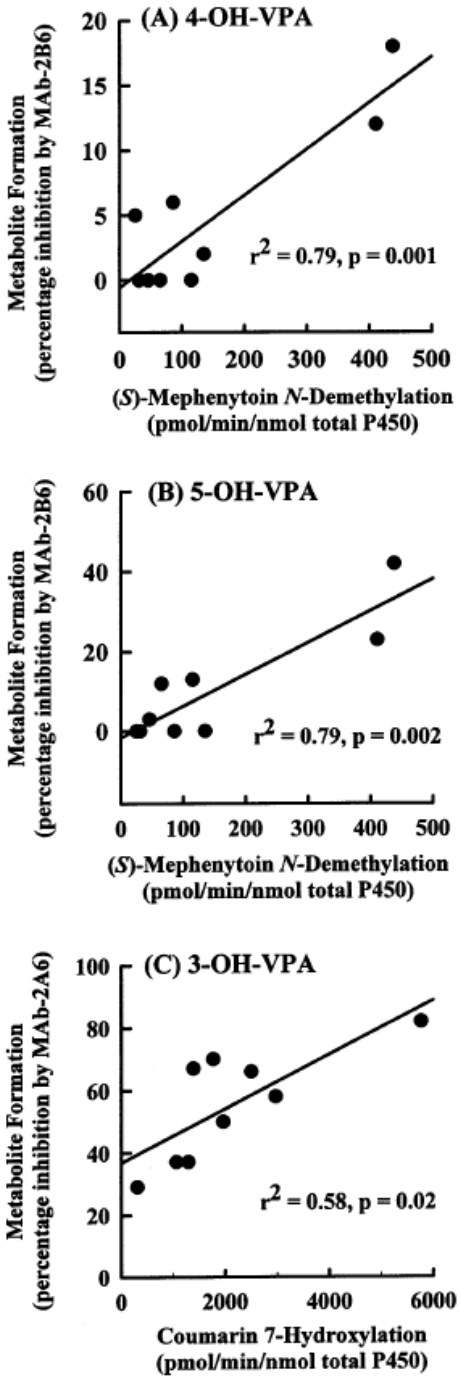

Figure 5-5. Association between immunoinhibition of VPA metabolism and CYP2B6- and CYP2A6-associated enzyme activities in human hepatic microsomes. Correlational analyses were performed between MAb-2B6 inhibition of 4-OH-VPA formation and CYP2B6-mediated (*S*)-mephenytoin *N*-demethylation activity (panel A), MAb-2B6 inhibition of 5-OH-VPA formation and CYP2B6-mediated (*S*)-mephenytoin *N*-demethylation activity (panel B), and MAb-2A6 inhibition of 3-OH-VPA formation and CYP2A6-mediated coumarin 7-hydroxylation activity (panel C) in a panel of nine individual human hepatic microsome samples (c.f., Figure 5-3 and Table 5-1).

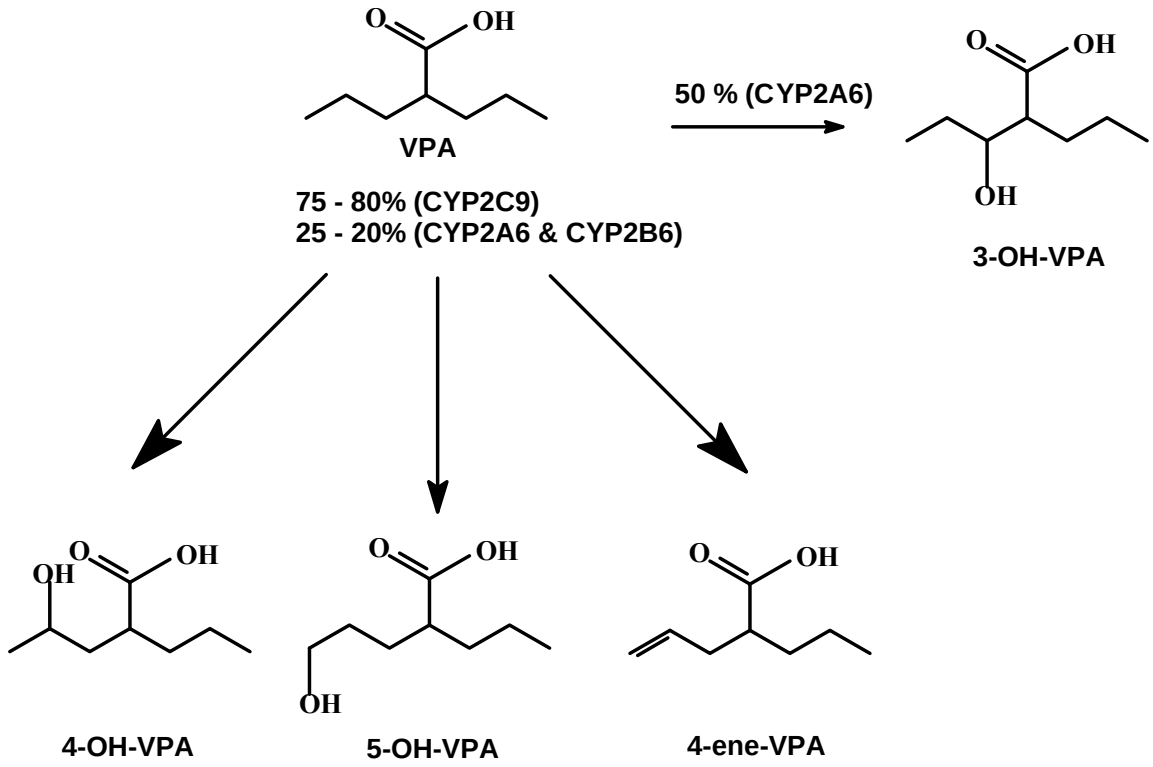

Figure 5-6. Relative contributions of CYP2A6, CYP2B6, and CYP2C9 in the oxidative metabolism of VPA in human liver microsomes (N = 9) obtained from individuals with the CYP2C9*1/*1 genotype.

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6 CONCLUSION

6.1 Overview and Objectives for the Present Thesis

Valproic acid (VPA) is a broad-spectrum anticonvulsant that is also indicated for the management of bi-polar disorder, migraine headache, and neurologic pain (Johannessen and Johannessen, 2003). It is relatively safe, but in rare instances is associated with severe, life threatening hepatotoxicity (Zimmerman and Ishar, 1982; Scheffner *et al.*, 1988; Kuhara *et al.*, 1990; Konig *et al.*, 1994; Bryant and Dreifuss 1996; Eadie *et al.*, 1988). The incidence of VPA-associated idiosyncratic hepatotoxicity is the highest in young children receiving concurrent enzyme-inducing anticonvulsants (Dreifuss *et al.*, 1987; Dreifuss *et al.*, 1989; Bryant and Dreifuss, 1996), but the exact mechanisms responsible for the hepatotoxicity remain unknown. Despite data suggesting the involvement of mitochondrial dysfunction (Fromenty *et al.*, 1995; Pessayre *et al.*, 1999), oxidative stress (as reviewed by Chang and Abbott, 2006), or abnormal glutathione homeostasis (Cotariu *et al.*, 1990) in the VPA-induced liver toxicity, the relationships between these pathological events have not been systematically examined. As well, VPA is extensively metabolized (Abbott and Anari, 1999) and toxic or reactive VPA metabolites may also contribute to the VPA-induced hepatotoxicity (Eadie *et al.,* 1988). Consequently, the role of VPA metabolites in VPA-induced hepatotoxicity requires further investigation.

The objectives of the present thesis were to i) examine the roles of VPA metabolites or VPA biotransformation pathways in the VPA-induced hepatotoxicity; ii) characterize the relationships between abnormal glutathione homeostasis and VPA-induced hepatotoxicity; and iii) investigate the contributions of individual human hepatic cytochrome P450 (CYP) enzymes in the oxidative metabolism of VPA. Sandwich cultured rat hepatocytes (SCRH), urine samples from pediatric patients receiving steady state VPA therapy, and human hepatic microsomes from

multiple donors were used to investigate these objectives. The toxicity markers employed were based on the known toxicology of VPA: mitochondrial function in SCRH was measured by the WST-1 marker (Berridge *et al.*, 2005), cytotoxicity of SCRH was determined by the release of lactate dehydrogenase (Jurima-Romet *et al.*, 1996; Kingsley *et al.*, 1983; Takeuchi *et al.*, 1988; Buchi *et al.*, 1984), and oxidative stress was determined by the 2′7′-dichlorofluorescein (DCF) or 15-F_{2t}-isoprostane markers (Halliwell and Whiteman, 2004), respectively, in SCRH or the human urine samples.

6.2 Toxicity of VPA Metabolites

The present thesis characterized the toxicities of a comprehensive panel of synthesized VPA metabolites in a single experimental model. Of the metabolites studied, only (*E*)-2,4-diene-VPA was consistently more toxic than VPA on the induction of mitochondrial dysfunction (WST-1), cytotoxicity (LDH), oxidative stress (DCF), and the depletion of total cellular glutathione in SCRH (Chapters 2 & 3). Subsequent concentration-response experiments also indicated (*E*)-2,4-diene-VPA to be at least 2.5, 6, 30, and 120 fold more potent than VPA with respect to the WST-1, LDH, DCF, and the GSH markers, respectively. Supporting the effects of (*E*)-2,4-diene-VPA on mitochondrial dysfunction in SCRH, (*E*)-2,4-diene-VPA is a potent inducer of hepatic steatosis or inhibitor of mitochondrial β-oxidation in various rat models (Kesterson *et al.*, 1984; Ponchaut *et al.*, 1992). However, to the best of our knowledge, no other investigators have reported on the effects of (*E*)-2,4-diene-VPA on any other markers of toxicity in the literature. In the human situation, indirect proof of a role of (*E*)-2,4-diene-VPA in the VPA-induced hepatotoxicity was obtained when the levels of the thio-conjugated (*E*)-2,4-diene-VPA were found to be elevated in individuals who have developed VPA-induced hepatotoxicity (Kassahun *et al.,* 1991) or in children possessing the risk factors of VPA-induced liver injury (Gopaul *et al.,* 2003).

Despite substantial evidence in the literature supporting the reactivity of (*E*)-2,4-diene-VPA toward cellular proteins or GSH (Kassahun *et al.,* 1991; Kassahun *et al.,* 1994; Tang *et al.,* 1995; Gopaul *et al.,* 2000a; Gopaul *et al.,* 2000b; Gopaul *et al.,* 2003), the mechanism(s) by which (*E*)-2,4-diene-VPA induces toxicity in SCRH remain unknown. The CoA- and glucuronide- activated (*E*)-2,4-diene-VPA are capable of conjugating with hepatic GSH (Kassahun *et al.*, 1991;Tang *et al.*, 1995; Tang and Abbott, 1996a), a reaction that could lead to the depletion of total cellular GSH in SCRH. As GSH is a critical defence against reactive oxygen species or reactive electrophilic intermediates (Meister, 1983; Meister, 1994), the depletion of GSH could be a mechanism for the pathogenesis of (*E*)-2,4-diene-VPA in SCRH. To test this hypothesis, attenuating the formation of CoA- (e.g. with trimethylacetic acid) or glucuronide- (e.g. with borneol) esters (Porubek *et al.*, 1989; Kretz-Rommel and Boelsterli, 1993; Li *et al.*, 2003) should decrease the toxicity associated with (*E*)-2,4-diene-VPA. As well, increasing (e.g. with L-glutathione) or decreasing (i.e. with diethylmaleate or buthionine sulfoximine) the GSH capacity of SCRH should attenuate or enhance, respectively, the toxic effects of (*E*)-2,4-diene-VPA. To further understand the role of GSH homeostasis in the pathogenesis of (*E*)-2,4-diene-VPA in SCRH, future studies should also characterize the time-dependent effects of (*E*)-2,4-diene-VPA on markers of oxidative stress, mitochondrial dysfunction, or cytotoxicity with respect to the depletion of GSH in SCRH.

Another metabolite of toxicological interest is the terminal olefin, 4-ene-VPA. An intriguing observation for 4-ene-VPA is that it is more toxic than VPA *in vivo* in rats (Kesterson *et al.,* 1984; Tang *et al.,* 1995; Kassahun *et al.*, 1994), but less cytotoxic than VPA in cultured-rat hepatocytes (Jurima-Romet *et al.,* 1996; Kingsley *et al.,*1983). In the present investigation, 4-ene-VPA was equally, or significantly less toxic than VPA with respect to mitochondrial dysfunction, cytotoxicity, oxidative stress, or total cellular GSH depletion in SCRH (Chapters 2 & 3). Unlike the *in vivo* situation, the majority of the administered 4-ene-

VPA would not be cleared from the culture supernatant, and this may explain why 4-ene-VPA is relatively less toxic when tested in cultured-rat hepatocytes. For 4-ene-VPA to induce toxicity, further bioactivation by the CYP or ß-oxidation enzymes to produce the 4,5-epoxide-VPA (Kassahun *et al.,*1994) or (*E*)-2,4-diene-VPA (Tang *et al.,*1995), respectively, maybe required. However, 4-ene-VPA itself also destroys the CYP and inhibits the mitochondrial ß-oxidation enzymes, as demonstrated in various rat preparations (Prickett and Baillie, 1986; Kesterson *et al.,*1984; Silva *et al.*, 2001; Ponchaut *et al.,* 1992). Thus the large amounts of 4-ene-VPA in an *in vitro* environment may inhibit its own bioactivation and reduce the formation of reactive metabolites that are presumed to mediate the toxicity of 4-ene-VPA. To test this hypothesis, one could compare the extent of enzyme inhibition or reactive metabolite formation from 4-ene-VPA treatment in various *in vitro* and *in vivo* experimental models.

Unlike (*E*)-2,4-diene-VPA and 4-ene-VPA, little toxicity data is available in the literature for (*E*)-2-ene-VPA, the hydroxylated metabolites of VPA, and VPA-glucuronide. The relative lack of effects of (*E*)-2-ene-VPA or the hydroxylated metabolites on the induction of mitochondrial dysfunction or cytotoxicity in SCRH is in agreement with the data in the literature (Loscher *et al.*, 1993; Kesterson *et al.,* 1984; Jurima-Romet *et al.,* 1996; Kassahun *et al.,* 1994; Kingsley *et al.,*1983). On the other hand, we are the first to report the effects of VPA-glucuronide on all markers of toxicity studied in SCRH. The observed lack of toxicity could not be attributed to the inadequate exposure of the cells to VPA-glucuronide, because control experiments proved that the metabolite was capable of gaining entry into the hepatocytes. As well, to the best of our knowledge, reactive forms of VPA-glucuronide or the hydroxylated metabolites of VPA have not been reported, which may explain the relative lack of toxicity of these metabolites. In order to examine the association between the formation of reactive metabolite and the induction of hepatotoxicity, studies are ongoing in SCRH to determine

whether the hydroxylated metabolites or the glucuronide of VPA are capable of conjugating with hepatic GSH, a reaction that would indicate the formation of a reactive metabolite.

The strength of our approach in SCRH is the use of multiple markers tailored to the known toxicology of VPA (i.e. mitochondrial dysfunction, oxidative stress, cytotoxicity, and glutathione depletion). Such an approach allowed us to characterize the relationships between these pathological events in a single experimental setting, which to the best of our knowledge, has never been done for VPA. As well, the toxicity data of the synthesized VPA metabolites obtained in SCRH were in most cases consistent with the *in vitro* or *in vivo* toxicology data for the same metabolites in the literature. Thus, the predictability of the SCRH, in addition to the simplicity of an *in vitro* set up, would suggest that our model is suitable for the high-throughput, hepatotoxicity screening of novel VPA analogues. As to be discussed further, such studies are already underway for a number of novel fluorinated VPA that were specifically designed in our laboratory to minimize the bioactivation-mediated hepatotoxicity. On the other hand, as it would appear that our *in vitro* culture conditions and toxicity assays are suitable for the examination of VPA-induced hepatotoxicity in rat hepatocytes, we are also attempting to extend the culture model to toxicity testing in the human hepatocytes. This would allow us to compare, head-to-head, any species-related differences with respect to the hepatotoxicity induced by VPA or the metabolites of VPA, predict the hepatotoxicity of novel VPA analogues, and conduct mechanistic experiments to further investigate the VPA-induced hepatotoxicity in the human model.

Based on the data obtained from the treatments of VPA and the individual VPA metabolites in SCRH, we were able to observe significant correlations between the extent of oxidative stress (DCF) and mitochondrial dysfunction (WST-1) or oxidative stress and cytotoxicity (LDH) (Chapters 2 & 3). This is the first instance where an association between mitochondrial dysfunction and oxidative stress in rat hepatocytes has been observed for VPA.

As it is well established that mitochondrial function is adversely affected by VPA (Ponchaut *et al.,*1992; Silva *et al.,*2001; Trost and Lemasters, 1996; Becker and Harris, 1983; Coude *et al.*, 1983; Turnbull *et al.*, 1986; Pessayre *et al.,*1999; Fromenty and Pessayre, 1995) and that the mitochondria are the major source of reactive oxygen species in hepatocytes (Pessayre *et al.,*1999), the relationship between VPA-induced mitochondrial dysfunction and oxidative stress warrants further investigation. Furthermore, the strong correlation obtained between the extent of oxidative stress and cytotoxicity in SCRH supports the hypothesis that oxidative stress is associated with the induction of cytotoxicity. Although Buchi *et al.* (1984) indicated that antioxidant treatments were able to attenuate the VPA-induced cytotoxicity in cultured-rat hepatocytes, the effects of VPA on the induction of oxidative stress or that of the antioxidants on the attenuation of VPA-induced oxidative stress were not determined in their investigation. Aside from the study by Buchi et al. (1984), no other mechanistic investigations that examine the association between the induction of oxidative stress and the manifestation of VPA-induced hepatotoxicity are available. As such, further studies are needed to support this observed relationship.

6.3 Role of Biotransformation in VPA-Induced Hepatotoxicity

The majority of toxicity studies on VPA metabolites have been done on exogenously added, synthesized metabolites at concentrations far exceeding those obtained after the therapeutic administration of VPA. The kinetic characteristics of exogenously administered metabolites in cell culture also likely differ significantly from the same metabolites generated *in situ* from the metabolism of exogenously administered VPA. With these limitations in mind, we set out to determine the roles of *in situ* generated VPA metabolites in the VPA-induced toxicity in SCRH. Specifically, the CYP-mediated oxidative metabolism and/or the UGT-mediated glucuronidation of VPA were studied because these biotransformation pathways, and their

associated metabolites, are reported to be involved in the VPA-induced hepatotoxicity (as reviewed in the introduction section). Furthermore, to extend our experiments to the human situation, we also characterized the associations between the levels of VPA metabolites and the elevation of 15-F_{2t}-isoprostane, a marker of lipid peroxidation (Basu, 2004), in the urine of children receiving chronic VPA therapy. Based on our laboratory's expertise on the quantitative analysis of VPA metabolites, we were able to determine the levels of a comprehensive panel of VPA metabolites representing the major biotransformation pathways of VPA. The large number of subjects (n = 123) in our clinical study also ensured adequate power to have enough confidence for the multiple variable regression models. The results of these investigations are further discussed.

Our data from the 1-aminobenzotriazole, ketoconazole, and (-)-borneol experiments provide reasonably clear evidence that the CYP-mediated oxidative metabolism of VPA to 4-ene-VPA or (*E*)-2,4-diene-VPA and the UGT-mediated-glucuronidation of VPA to VPA-glucuronide are not associated with the VPA-induced mitochondrial dysfunction, cytotoxicity, oxidative stress, or glutathione depletion in SCRH. The strength of our approach is that we were able to determine the selectivity, or lack thereof, of the individual chemical inhibitors by examining the levels of VPA metabolites generated from the individual biotransformation pathways of VPA. Based on these experiments, it was determined that 1-aminobenzotriazole was selectively inhibitory toward the CYP-mediated oxidative metabolism of VPA, whereas ketoconazole or (-)-borneol affected multiple biotransformation pathways. Aside from the lack of selectivity, ketoconazole and borneol were also inherently toxic to the SCRH when administered at concentrations required to completely inhibit VPA-glucuronide formation (Chapter 2 and 3). As such, we were only able to reduce the formation of VPA-glucuronide by approximately 50 % in our experiments. Further work is obviously needed to identify selective and potent modulators to inhibit the glucuronidation of VPA in SCRH.

The discrepancy between our results in SCRH and that of Tong *et al.* (2005b) in rats with respect to the role of VPA-glucuronide in the induction of oxidative stress could be attributed to differences between the two models. As it has been proposed that the entero-hepatic recirculation of VPA-glucuronide and the subsequent release of glucuronic acid in rats might be a mechanism by which VPA-glucuronide induces lipid peroxidation (Tong *et al.,*2005b), such a mechanism would be difficult to produce in cultured rat hepatocytes given the physical limitations of an *in vitro* model. On the other hand, it was subsequently revealed that the glucuronidation inhibitor (i.e. (-)-borneol) used by Tong *et al.* (2005b) was not selective toward the glucuronidation of VPA (unreported observation). Thus the non-specific effects of the chemical modulators used by Tong *et al.* (2005b) could be another reason for the discrepancy in results observed between the two experimental models. Further studies should be undertaken to determine whether the entero-hepatic recirculation of VPA-glucuronide is indeed the mechanism associated with the induction of oxidative stress in rats. As well, future *in vivo* studies should take into consideration the non-specific inhibitory effects of (-)-borneol toward biotransformation pathways other than glucuronidation.

In the case of the human study, a correlation was found between the levels of VPA-glucuronide and 15-F_{2t}-isoprostane, a marker of lipid peroxidation, in the urine of children receiving chronic VPA therapy (Chapter 4). The weak correlation ($r^2 = 0.037$) may very well be a chance effect, but the fact that it is significant would suggest a potential but minor role of VPA-glucuronide, or some aspect of the glucuronidation pathway, in the induction of oxidative stress in humans. However, a few limitations of our clinical study, which could have potentially confounded the results obtained, should be addressed. Factors known to affect the levels of 15-F_{2t}-isoprostane in humans, such as second hand smoke exposure, diet, vitamin intake, or certain medical conditions (Basu and Helmersson, 2005; Michoulas *et al.*, 2006), were not accounted for during the initial patient recruitment process. As well, VPA, being a non-specific inhibitor of

mitochondrial ß-oxidation (Silva *et al.*, 2008), could potentially have elevated the levels of 15-F_{2t}-isoprostane, which is extensively cleared via mitochondrial ß-oxidation (Chiabrando *et al.*, 1999; Roberts *et al.*, 1996), in a mechanism independent of oxidative stress induction. Although an interaction between VPA and 15-F_{2t}-isoprostane is still unproven, a better marker for the measurement of oxidative stress in the current study would have been the major ß-oxidation metabolite of 15-F_{2t}-isoprostane, 2,3-dinor-5,6-dihydro-8-iso-prostaglandin $F2_{\alpha}$ (Roberts *et al.,*1996). The measurement of this 15-F_{2t}-isoprostane metabolite, in addition to 15-F_{2t}-isoprostane, would help determine the validity of the proposed interaction and / or provide a better indicator for the VPA-induced oxidative stress. In any case, future studies that examine VPA-induced oxidative stress might better employ other biomarkers to support the findings obtained with the measurement of the 15-F_{2t}-isoprostane in tissue.

Based on the limited amount of data in the literature (Tong *et al.,* 2005b) and the conflicting results obtained in our work, the role of VPA-glucuronide formation in the VPA-induced hepatotoxicity in rats or humans remains inconclusive. To further investigate this matter, the contributions of individual hepatic UGT enzymes toward the glucuronidation of VPA should be characterized in both rat and human species. Only with this information in hand, would it then be possible to choose or design inhibitors that are selective and inhibitory toward the glucuronidation of VPA.

In contrast to the data obtained for VPA-glucuronide, our chemical inhibition experiments demonstrated clearly that the *in situ* generated 4-ene-VPA was not associated with VPA-induced mitochondrial dysfunction, cytotoxicity, oxidative stress, or glutathione depletion in SCRH. Our finding in the SCRH model is significant because we believe this is one of the first comprehensive, mechanistic studies that have provided evidence against a role of *in situ* generated 4-ene-VPA in the VPA-induced hepatotoxicity. The idea that 4-ene-VPA is unlikely involved in the VPA-induced hepatotoxicity was initially suggested by Loscher *et al.* (1993)

based on the observation that plasma levels of this metabolite did not correspond to the extent of hepatic steatosis in rats. As well, in the human situation, various case studies of VPA-induced hepatotoxicity have not reported abnormal 4-ene-VPA levels (Eadie *et al.*, 1990; Fisher *et al.*, 1992; Kondo *et al.*, 1992; Kuhara *et al.,*1990; McLaughlin *et al.*, 2000; Paganini *et al.*, 1987; Siemes *et al.*, 1993; Tennison *et al.*, 1988). Likewise, in our own clinical study, we did not observe an association between the levels of 4-ene-VPA and 15-F_{2t}-isoprostane in the urine of children receiving chronic VPA therapy (Chapter 4). However, in contrast to rats, mechanistic studies are still lacking in a human model to investigate the role of the *in situ* generated 4-ene-VPA in VPA-induced hepatotoxicity. From an *in vitro* reaction phenotyping study (Chapter 5), we have identified the hepatic CYP enzyme responsible for the formation of 4-ene-VPA in human liver microsomes. Based on the results of this study, one would be able to design chemical inhibition experiments to further investigate the role of 4-ene-VPA in VPA-induced hepatotoxicity in a human model (e.g. cultured human hepatocytes).

The exogenously added (*E*)-2,4-diene-VPA was much more toxic than VPA in SCRH (Chapter 2 & 3), thus it was of interest to determine whether the *in situ* generated (*E*)-2,4-diene-VPA was associated with the observed VPA-induced toxicities in SCRH. Unfortunately, the levels of (*E*)-2,4-diene-VPA, its major thio-conjugate, 5-GS-3-ene-VPA (Kassahun *et al.,*1991; Kassahun *et al.,*1994), or the (*E*)-2,4-diene-VPA-glucuronide were well below the limit of quantitation in SCRH treated with VPA; thus the role of *in situ* generated (*E*)-2,4-diene-VPA in the VPA-induced hepatotoxicity remains inconclusive. A rationale for the lack of detection of (*E*)-2,4-diene-VPA following VPA administration in SCRH could be that the metabolite underwent covalent binding as fast as it was generated *in situ*. In order to test this hypothesis, one could measure all known GSH conjugates of (*E*)-2,4-diene-VPA (Kassahun *et al.,*1991; Kassahun *et al.,* 1994) which could then serve as surrogates for the (*E*)-2,4-diene-VPA. The extent of protein binding of (*E*)-2,4-diene-VPA can also be determined in SCRH using the radio-

labelled derivatives of VPA. However, if the *in situ* generated (*E*)-2,4-diene-VPA were responsible for the VPA-induced toxicities in SCRH, the use of 1-ABT or KTZ, which would have attenuated the production of (*E*)-2,4-diene-VPA, should have reduced the toxicities associated with VPA. None of the toxicity markers altered by VPA (i.e. mitochondrial dysfunction, cytotoxicity, oxidative stress, and glutathione depletion) were attenuated by 1-ABT or KTZ treatment, and this would suggest that (*E*)-2,4-diene-VPA was unlikely associated with the VPA-induced toxicities in SCRH.

On the other hand, the lack of detection of (*E*)-2,4-diene-VPA can also be attributed to the inadequate generation of the metabolite in an *in vitro* model with reduced metabolic capacity. Hepatocytes tend to lose phase I metabolic capacity, which is needed for the generation of (*E*)-2,4-diene-VPA from VPA, during isolation and while in culture (as reviewed in the introduction to the thesis). As evident by our ability to detect 4-ene-VPA, a quantitatively minor metabolite from the administration of VPA (Abbott and Anari, 1999), our *in vitro* model of SCRH would appear to be metabolically competent. However, compared to 4-ene-VPA, even smaller amounts of administered VPA are converted to (*E*)-2,4-diene-VPA, the detection of which has proved difficult in some *in vivo* models (e.g. Tong *et al.,*2003). If reduced metabolism is the cause of the lack of detection of (*E*)-2,4-diene-VPA in SCRH, further optimization of the model (i.e. with respect to medium composition, cell density, or extracellular matrix compositions, as reviewed in the introduction of this thesis), may improve the metabolic capacity and allow one to detect trace metabolites like (*E*)-2,4-diene-VPA.

In contrast to the *in vitro* situation, we were able to detect (*E*)-2,4-diene-VPA in the urine of children receiving steady-state VPA therapy. However, a correlation between the levels of (*E*)-2,4-diene-VPA and 15-F_{2t}-isoprostane was not obtained in this patient population (Chapter 4). On the other hand, we did obtain a weak, but inverse correlation between the levels of *N*-acetylcysteine (NAC) metabolites of VPA and that of 15-F_{2t}-isoprostane (Chapter 4). As these

NAC metabolites are generated from the (*E*)-2,4-diene-VPA, the inverse correlation would rather suggest that the (*E*)-2,4-diene-VPA was unlikely involved in the induction of lipid peroxidation in children receiving VPA therapy. In support of our observation, none of the human subjects with documented VPA-induced hepatotoxicity had abnormal levels of (*E*)-2,4-diene-VPA in a series of case studies published by Siemes *et al.* (1993). Likewise, Loscher *et al.* (1993) also did not observe a correlation between the plasma levels of (*E*)-2,4-diene-VPA and the extent of hepatic steatosis in rats. These observations, in conjunction with our findings in SCRH, would support the contention that *in situ* generated (*E*)-2,4-diene-VPA is unlikely involved in the VPA-induced hepatotoxicity in rats and humans.

In addition to the chemical inhibition approach to study the role of biotransformation in VPA-induced hepatotoxicity, we also tested α-F-VPA, which is resistant to bioactivation, in SCRH. α-F-VPA was less toxic than VPA with respect to mitochondrial dysfunction, cytotoxicity, oxidative stress, and total cellular GSH depletion in SCRH (Chapters 2 & 3). Consistent with these observations, the α-fluorinated analogues of 4-ene-VPA or VPA are less effective inducers of hepatic steatosis (Tang *et al.,*1995) or lipid peroxidation (Tong *et al.,*2005b) in rats, respectively, when compared to their non-fluorinated counterparts. The substitution of a fluorine atom at the α position blocks the formation of (*E*)-2,4-diene-VPA (Tang *et al.,*1995) and limits the generation of VPA-glucuronide (Tang *et al.*, 1997) or VPA-CoA (Grillo *et al.*, 2001), thus a limitation of α-F-VPA is that it is resistant to bioactivation by multiple biotransformation pathways. To address the multiple effects of α-F-VPA on normal VPA metabolism, different fluorinated analogues of VPA have been synthesized in our laboratory with the aim to selectively block only the formation of (*E*)-2,4-diene-VPA, while retaining the ability to form VPA-CoA esters or VPA-glucuronide. The testing of these analogues, already underway in SCRH, might further elucidate the biotransformation pathways responsible for the hepatotoxicity of VPA.

The lack of toxicity of α-F-VPA, which is resistant to the formation of the glucuronide, (*E*)-2,4-diene-, and CoA ester, in conjunction with the observed lack of toxicity of the *in situ* generated VPA-glucuronide or (*E*)-2,4-diene-VPA in VPA-treated SCRH (Chapters 2 & 3), would suggest that the formation of VPA-CoA is possibly associated with the VPA-induced hepatotoxicity. In support of this view, the depletion of CoA from the administration of VPA is considered a potential mechanism of VPA-induced hepatotoxicity (reviewed in the Introduction to the thesis). As well, the VPA-CoA is more toxic than VPA as an inhibitor of mitochondrial ß-oxidation or oxidative phosphorylation in various rat preparations (Ito *et al.*, 1990; Aires *et al.*, 2008).

To further explore the role of VPA-CoA, or the formation thereof, in the VPA-induced hepatotoxicity, the effects of the synthesized VPA-CoA can be compared to that of VPA on the already established toxicity markers in SCRH. As well, one could also inhibit the formation of VPA-CoA from VPA using a chemical modulator such as trimethylacetic acid (Li *et al.,* 2003). If the "CoA" hypothesis were valid, the co-administration of a CoA inhibitor such as trimethylacetic acid should attenuate the VPA-associated toxicities in SCRH. Furthermore, a VPA analogue that is selectively resistant to the formation of VPA-CoA can also be synthesized and its toxic effects in SCRH compared to VPA. The results of these future experiments, in conjunction with that obtained from the testing of fluorinated analogues that are selectively resistant to the formation of the (*E*)-2,4-diene- metabolite, should allow one to determine the relative contributions of the individual VPA biotransformation pathways in the VPA-induced hepatotoxicity. On the other hand, as suggested by the reactivity of (*E*)-2,4-diene-VPA-CoA toward protein binding or GSH conjugation in various experimental models (Kassahun *et al.,*1991), the acyl CoA esters of VPA metabolites could also contribute to the hepatotoxicity of VPA. To further explore this hypothesis, the reactivity and toxicity of the other CoA esters of VPA metabolites should be characterized.

6.4 Mechanisms of Valproic Acid-Associated Glutathione Depletion and Its Role in the Induction of Mitochondrial Dysfunction, Cytotoxicity, and Oxidative Stress in Sandwich-Cultured Rat Hepatocytes

A specific objective for the present thesis was to study the role of GSH homeostasis in VPA-induced hepatotoxicity. To do so, the time-dependent effects of VPA on markers of mitochondrial dysfunction, cytotoxicity, and oxidative stress were characterized in relation to the depletion of glutathione. The effects of exogenously administered glutathione on the attenuation of VPA-induced toxicities were also examined in SCRH. Only a few mechanistic studies have investigated the role of abnormal glutathione homeostasis in the VPA-induced hepatotoxicity in rat hepatocytes (Jurima-Romet *et al.,* 1996; Tong *et al.*, 2005a). Unlike our investigation in SCRH, most of these studies only employed a limited number of toxicity markers and did not characterize the temporal relationships between abnormal glutathione homeostasis and the induction of toxicity. From our experiments, we found that total cellular GSH depletion is unlikely a factor in VPA-induced mitochondrial dysfunction, while hepatic glutathione serves a protective role against VPA-induced oxidative stress in SCRH (Chapter 3). It was also reasoned that the depletion of total cellular GSH may play a partial, or synergistic role in VPA-induced cytotoxicity, although further experiments are needed to confirm this hypothesis.

The mechanism(s) by which VPA depletes total cellular GSH in SCRH is unclear. Based on our chemical inhibition experiments, CYP-mediated oxidative metabolism of VPA and the UGT-mediated glucuronidation of VPA do not appear to play a significant role (Chapter 3). From the results of the α-F-VPA experiment, it can be proposed that the formation of VPA-CoA may be involved, but further mechanistic experiments, as discussed above, are needed to address the hypothesis. Based on the observations that the VPA-induced oxidative stress preceded the depletion of total cellular GSH (Chapter 3), and that the extent of oxidative stress in SCRH treated with VPA or the individual VPA metabolites correlated strongly with the extent of total cellular GSH depletion (Chapter 3), the induction of oxidative stress from VPA treatment may

also contribute to the depletion of total cellular GSH in SCRH. To explore the oxidative stress hypothesis, SCRH can be exposed to VPA in the presence of absence of antioxidants (e.g. vitamin C or vitamin E) and levels of GSH in SCRH can be quantified. If the VPA-induced oxidative stress were to play a role in the depletion of total cellular GSH, then the antioxidant treatments should attenuate the effects of VPA on the depletion total cellular GSH.

Alternatively, the effects of VPA on the enzymes responsible for the synthesis or degradation of GSH could also contribute to the depletion of total cellular GSH in SCRH. As evident in rats, VPA reduces the activities of hepatic glutathione reductase and increases activities of hepatic glutathione-S-transferase (Cotariu *et al.,*1990; Seckin *et al.*, 1999), which can lead to decreased formation or increased degradation of GSH, respectively. The effects of VPA on the other regulatory enzymes of GSH biosynthesis (e.g. γ-glutamylcysteine synthetase, GSH synthetase, glutathione peroxidase) should be investigated to further support this hypothesis.

A limitation of the present investigation is that we only characterized the total cellular glutathione in our experiments in SCRH. A better approach would have been to quantify the levels of reduced (GSH) as well as oxidized glutathione (GSSG). This would allow us to determine the cellular redox potential (GSH:GSSG), which may be used as an indicator of oxidative stress induction (Han *et al.*, 2006) to support our DCF data. Furthermore, the levels of mitochondrial GSH may also be a better indicator than total cellular GSH for the assessment of VPA-induced hepatotoxicity. Because the organelle does not contain the enzymes required for GSH synthesis, mitochondria are more susceptible to the toxic effects of drugs once the limited pool of mitochondrial GSH is depleted (Kretzschmar, 1996). An example would be the comparison between 4-ene-VPA and α-F-4-ene-VPA (Tang *et al.,*1995). Both these agents are capable of depleting hepatic total cellular GSH in rats, but the difference observed with these two agents, with respect to the induction of hepatic steatosis, could only be attributed to the

selective ability of 4-ene-VPA to deplete mitochondrial GSH (Tang *et al.,*1995). Thus, further studies on the toxicity of VPA or VPA metabolites would better characterize the mitochondrial GSH, the GSH:GSSH ratio, in addition to the measurement of total cellular GSH.

6.5 Contribution of Human Hepatic CYP Enzymes to the Oxidative Metabolism of VPA

CYP-mediated oxidative metabolism of VPA is a relatively minor, but toxicologically significant biotransformation pathway as it is responsible for the production of the putative hepatotoxin, 4-ene-VPA (Abbott and Anari, 1999). CYP enzymes also catalyze the formation of 3-OH-, 4-OH-, and 5-OH-VPA (Abbott and Anari, 1999), although the 3-OH-VPA is also a product of the mitochondrial β-oxidation pathway (Li *et al.*, 1991). Little is known of the human CYP enzymes responsible for the terminal desaturation or hydroxylation of VPA and even less is known of the relative contributions of the individual CYP enzymes for these reactions in the human liver. To address these knowledge gaps, we conducted an *in vitro* reaction phenotyping study using various complementary approaches: individual cDNA-expressed CYP enzymes, chemical inhibitors for the CYP enzymes, CYP-specific inhibitory monoclonal antibodies (MAb), human liver microsomes obtained from nine individual donors, and correlational analysis. Our results indicated that the CYP2C9 was a major catalyst (i.e. 75 – 80 % of the reactions) for the terminal desaturation, 4-hydroxylation, and 5-hydroxylation of VPA in human liver microsomes obtained from individuals with the CYP2C9*1/*1 genotype, whereas CYP2A6 and CYP2B6 catalyzed the remainder of the reactions. On the other hand, CYP2A6 was capable of forming the 3-OH-VPA, but only to a partial extent (i.e. ~ 50 %) in human liver microsomes.

Our experiments with the cDNA expressed enzymes confirmed previous observations that CYP2A6, CYP2B6, and CYP2C9 are active catalysts for the terminal desaturation of VPA and that CYP2C9 is capable of forming the 4-OH and 5-OH-VPA (Ho *et al.*, 2003; Sadeque *et al.*, 1997; Anari *et al.*, 2000). A novel finding in our study is the catalytic activities of various

other CYP enzymes in the formation of 3-OH-, 4-OH-, and 5-OH-VPA. The use of CYP-specific inhibitory MAbs allowed us to calculate the relative contributions of the individual CYP enzymes toward the oxidative metabolism of VPA in human liver microsomes. The chemical inhibition experiments and the correlational analyses supported the data obtained with the MAbs, and the use of the various complementary approaches has ensured the robustness of the observations obtained in this investigation. Modest contributions of CYP2A6 and CYP2C9, as determined by the extent of inhibition by coumarin or sulfaphenazole, respectively, to the terminal desaturation of VPA in human liver microsomes have also been reported by Sadeque *et al.* (1997). However, that study only employed human liver microsomes obtained from two individuals and the relative contributions of the CYP enzymes toward the hydroxylation of VPA were systematically determined (Sadeque *et al.,*1997). As well, it was not clear whether the chemical inhibitors used by Sadeque *et al.* (1997) were in fact inhibitory or specific toward their respective enzymes.

As discussed previously, our finding that the human hepatic CYP2C9 is the major catalyst for the formation of 4-ene-VPA would allow us to set up further mechanistic experiments in cultured human hepatocytes to investigate the role of *in situ* generated 4-ene-VPA in the VPA-induced hepatotoxicity. Our results were obtained using human liver microsomes from donors with the CYP2C9*1/*1 genotype. As determined by Ho *et al.* (2003), other mutant variants of CYP2C9 have reduced catalytic activity toward the metabolism of VPA, thus one might expect the relative contributions of the individual human CYP enzymes toward the oxidative metabolism of VPA to differ in individuals possessing these other CYP2C9 alleles. Further experiments should be conducted to address this hypothesis.

6.6 Overall Significance and Contribution of the Thesis

The present thesis has provided a comprehensive analysis of the toxicities of several synthesized VPA metabolites in a novel model of sandwich-cultured rat hepatocytes. Our finding that (*E*)-2,4-diene-VPA is more potent and toxic than VPA with respect to markers of mitochondrial dysfunction, cytotoxicity, oxidative stress, and glutathione depletion further supports the hypothesis that VPA is bioactivated into toxic and reactive metabolites. As well, these findings also provide a foundation for further mechanistic studies on the toxicities of VPA metabolites.

Our novel finding that CYP-mediated oxidative metabolism of VPA to 4-ene-VPA or (*E*)-2,4-diene-VPA is unlikely involved in VPA-induced toxicities in SCRH would suggest that other biotransformation pathways or metabolites of VPA are responsible for the VPA-induced hepatotoxicity. More experiments are needed to determine if the UGT-mediated glucuronidation of VPA to VPA-glucuronide is involved in the VPA-induced toxicities in SCRH. Based on our experiments with α-F-VPA, another good candidate for further investigation appears to be the acyl-CoA ester of VPA.

The results of our clinical study suggest that all of theVPA metabolites examined are unlikely associated with lipid peroxidation observed in pediatric patients on VPA therapy. Although the VPA-glucuronide and NAC metabolites were found to be statistically significant predictors of lipid peroxidation in this population, these associations are likely too weak to be clinically relevant.

This thesis is also the first to examine the relationships between VPA-induced mitochondrial dysfunction, cytotoxicity, oxidative stress, and glutathione depletion in a single setting using SCRH. As such, we are able to characterize the temporal relationships between the various altered-markers of toxicity associated with VPA treatment. Our findings from the GSH

supplementation experiments also support the associations between depletion of glutathione and induction of oxidative stress or cytotoxicity in SCRH treated with VPA.

Finally, we have determined the relative contribution of individual CYP enzymes on the oxidative metabolism of VPA in a panel of human liver microsomes. This information facilitates the design of mechanistic experiments that investigate the role of CYP2C9-mediated oxidative metabolism in VPA-induced hepatotoxicity in a human model (e.g. cultured human hepatocytes). Our findings also provide a mechanistic basis for further experiments investigating drug-drug interactions with VPA in humans that are potentially mediated by CYP2C9.

6.7 References

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7 Appendices

7.1 Appendix A: additional data not shown in the text

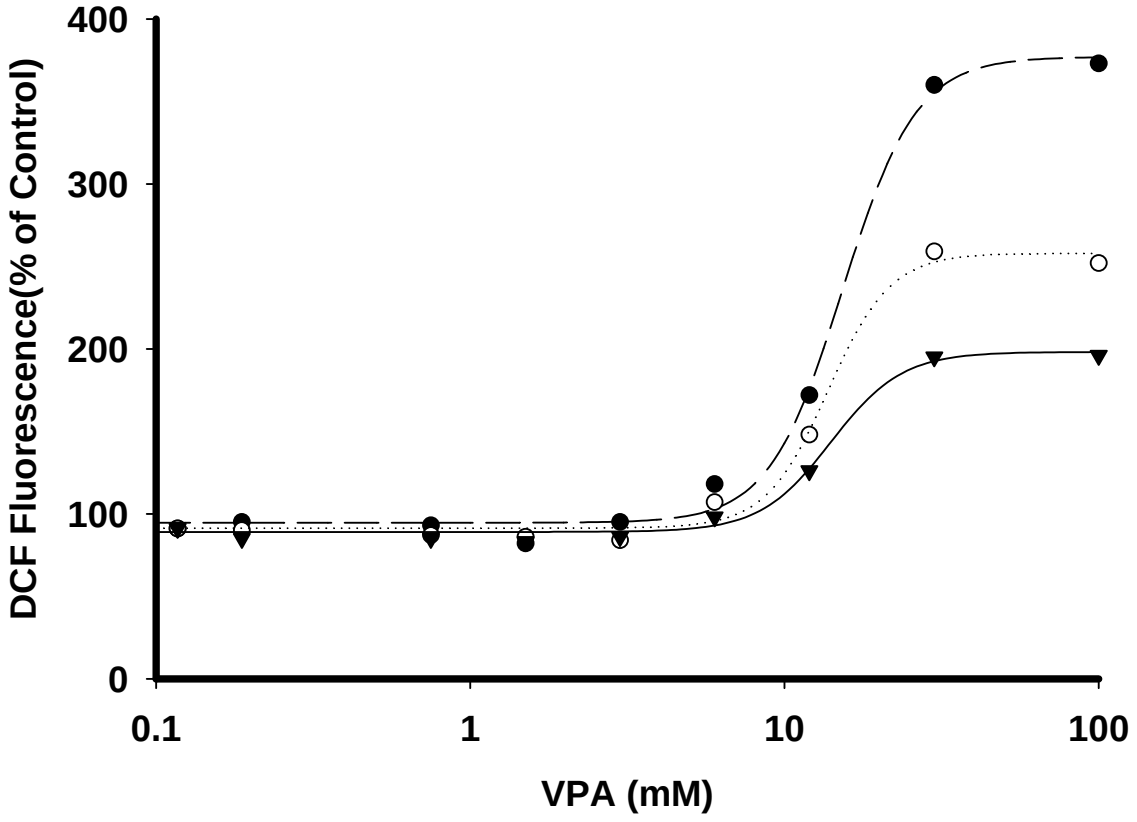

Figure A-1: Effects of varying DCFDA concentrations on VPA-induced DCF fluorescence. Sandwich cultured rat hepatocytes were exposed to the culture medium (vehicle) or VPA (0 – 100 mM) for 24 hours. The DCF assay was conducted as described in the Experimental Section in Chapter 2. Data are expressed as percentages of the vehicle control, based on duplicate determinations from one experiment. DCFDA concentrations: 10 µM (▼), 5 µM (○), and 2 µM (●).

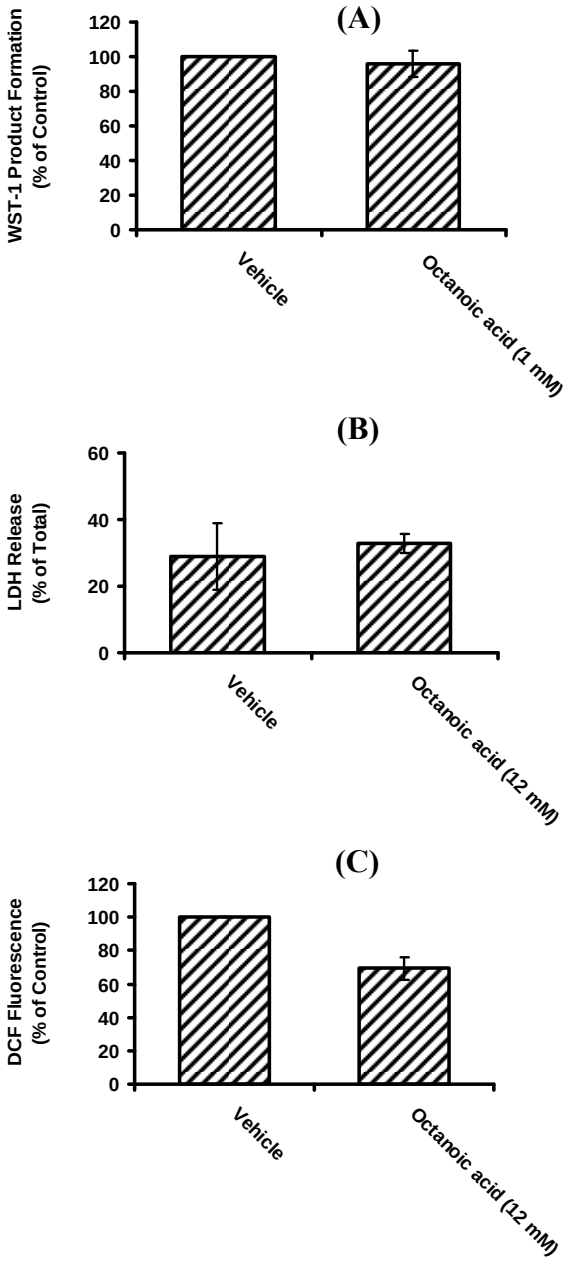

Figure A-2: Effects of octanoic acid on VPA-associated (A) WST-1 product formation (N = 4), (B) LDH release (N = 3), and (C) DCF formation (N = 3) in SCRH. Rat hepatocytes were treated with the vehicle (culture medium) or octanoic acid for 24 hours. The concentrations of octanoic acid were 1 mM for the WST-1 assay and 12 mM for the LDH and DCF assays, respectively. The individual assays were conducted as described in the Experimental Section in Chapter 2. Data are presented as the mean ± SEM of 3 – 4 individual experiments. The vehicle control values were 2.2 x 10^{-3} ± 0.6 ± 10^{-3} (mean ± SEM) absorption unit / min for the WST-1 marker; 0.046 ± 0.009 (supernatant activity) and 0.151 ± 0.014 (total activity) absorption unit / min for the LDH marker; and 1.03 x 10^{-3} ± 0.38 x 10^{-3} µmole DCF / min for the DCF marker.

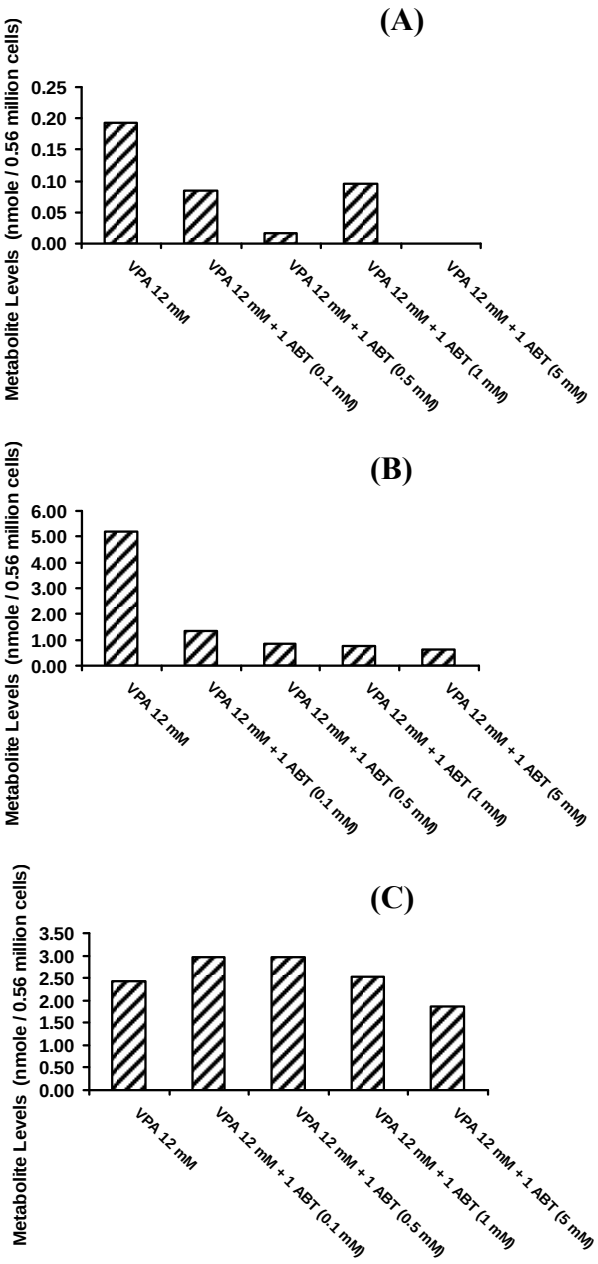

Figure A-3: Concentration-(in)dependent effects of 1-aminobenzotriazole (1-ABT) on the formation of (A) 4-ene-VPA, (B) 4-OH-VPA, and (C) (*E*)-2-ene-VPA in SCRH. Rat hepatocytes were pre-treated with the vehicle (culture medium) or 1-ABT (0 – 5 mM) for 60 minutes prior to treatments with the vehicle (culture medium), VPA (12 mM), or VPA and 1-ABT for 24 hours. Levels of VPA metabolites were determined by a GC/MS assay, as described in the Experimental Section in Chapter 2. The results presented are based on a single experiment. The 1-ABT concentration of 0.5 mM was used for all subsequent experiments because it maximally reduced the levels of 4-ene-VPA and 4-OH-VPA, which are surrogate products of the oxidative metabolism of VPA, but did not affect the levels of (*E*)-2-ene-VPA, a surrogate product of the ß-oxidation of VPA. 1-ABT, at a concentration of 0.5 mM, also did not induce hepatocyte toxicity (Figure 2-5).

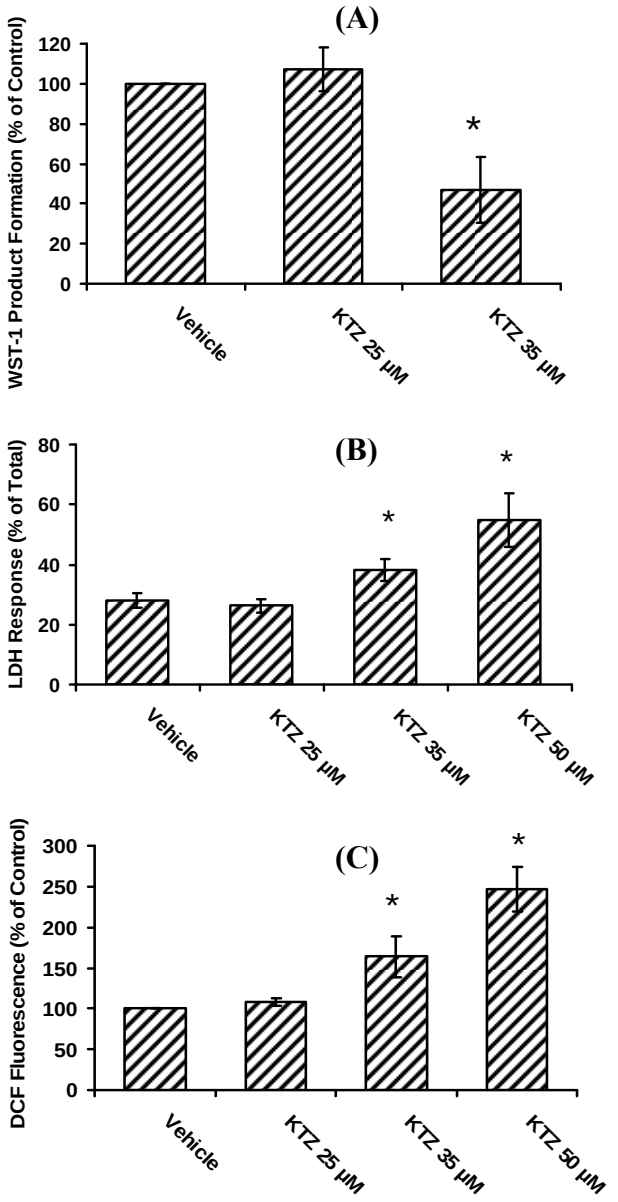

Figure A-4: Effects of ketoconazole (KTZ) on (A) WST-1 product formation (N = 4), (B) LDH release (N = 5), and (C) DCF formation (N = 5) in SCRH. Rat hepatocytes were treated with the vehicle (culture medium containing 0.5 % MeOH v/v) or KTZ (0 – 50 µM) for 24.5 hours. The individual assays were conducted as described in the Experimental Section in Chapter 2. Data are presented as the mean ± SEM of 4 - 5 individual experiments.*p < 0.05 vs. the vehicle control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were 4.8 x 10^{-3} ± 1.5 x 10^{-3} (mean ± SEM) absorption unit / min for the WST-1 marker; 0.034 ± 0.005 (supernatant activity) and 0.121 ± 0.013 (total activity) absorption unit / min for the LDH marker; and 1.45 x 10^{-3} ± 0.17 x 10^{-3} µmole DCF / min for the DCF marker. The KTZ concentration of 25 µM was used for all subsequent experiments due to its lack of toxicity to SCRH.

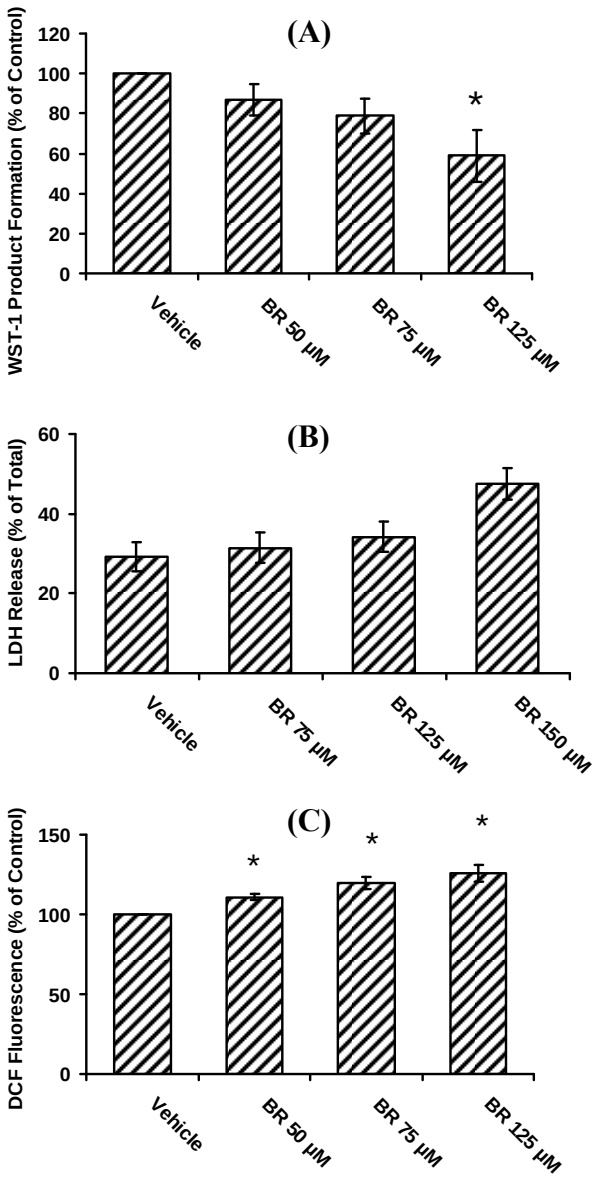

Figure A-5: Effects of (-)-borneol (BR) on (A) WST-1 product formation (N = 5), (B) LDH release (N = 4), and (C) DCF formation (N = 7) in SCRH. Rat hepatocytes were treated with the vehicle (culture medium containing 0.5 % DMSO v/v) or BR (0 – 150 µM) for 24.5 hours. The individual assays were conducted as described in the Experimental Section in Chapter 2. Data are presented as the mean ± SEM of 4 - 7 individual experiments.*p < 0.05 vs. the vehicle control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The vehicle control values were $3.3 \times 10^{-3} \pm 0.7 \times 10^{-3}$ (mean ± SEM) absorption unit / min for the WST-1 marker; 0.040 ± 0.004 (supernatant activity) and 0.138 ± 0.008 (total activity) absorption unit / min for the LDH marker; and $1.53 \times 10^{-3} \pm 0.18 \times 10^{-3}$ µmole DCF / min for the DCF marker. BR, at concentrations of 50 µM and 125 µM, were used for the WST-1 and LDH markers, respectively.

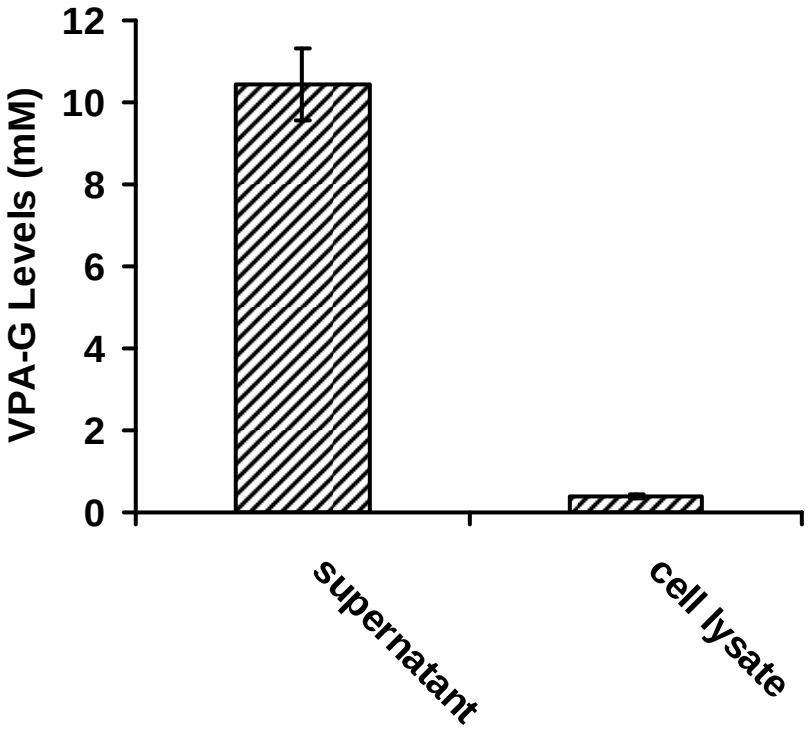

Figure A-6: Concentrations of VPA-glucuronide (VPA-G) found in the culture supernatant and cell lysates of hepatocytes exposed to VPA-glucuronide. Sandwich-cultured Rat hepatocytes were exposed to 12 mM of VPA-glucuronide for 24 hours and washed with warm (37 ºC) phosphate buffered saline (1 mL x 3 times) prior to being harvested. Cells were scraped from the culture plates in 1.0 mL cold buffer (phosphate buffered saline containing 1 mM EDTA, 4 ºC). Levels of VPA-G were determined by an LC/MS/MS assay described in the Experimental Section in Chapter 2. Data are presented as the mean ± SEM of 4 individual experiments. The presence of VPA-glucuronide in cell lysates suggested that exogenously-administered VPA-glucuronide was able to gain entry into the hepatocytes. Further experiments are needed to confirm this observation.

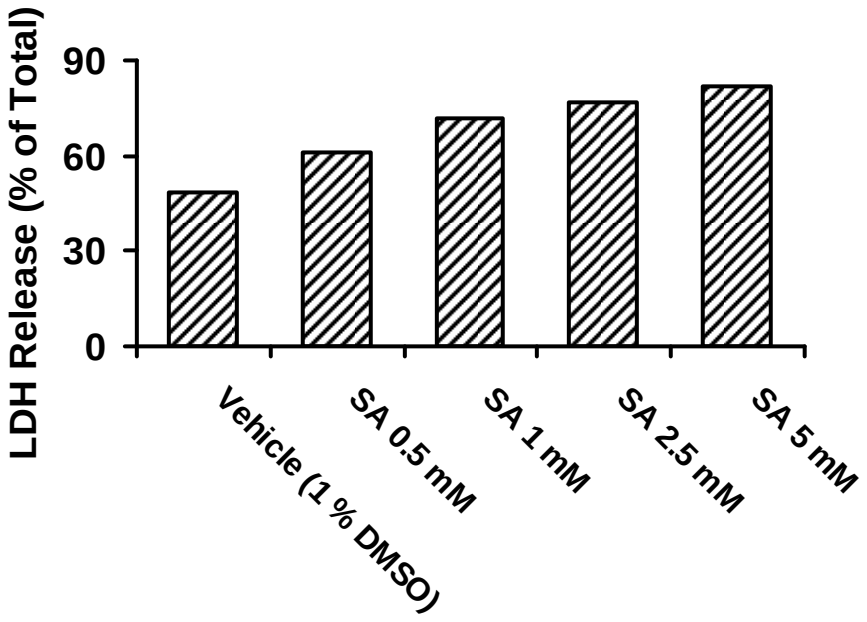

Figure A-7: Effects of salicylamide (SA) on LDH release in SCRH. Rat hepatocytes were treated with the vehicle (culture medium containing 1 % DMSO v/v) or SA (0 – 5 mM) for 25 hours. The LDH assay was conducted as described in the Experimental Section in Chapter 2. Data are presented as the average of duplicate determinations from a single experiment. The vehicle control values were 0.046 (supernatant activity) and 0.074 (total activity) absorption unit / min for the LDH marker. A trend toward an elevation of LDH release was observed for all concentrations of SA tested.

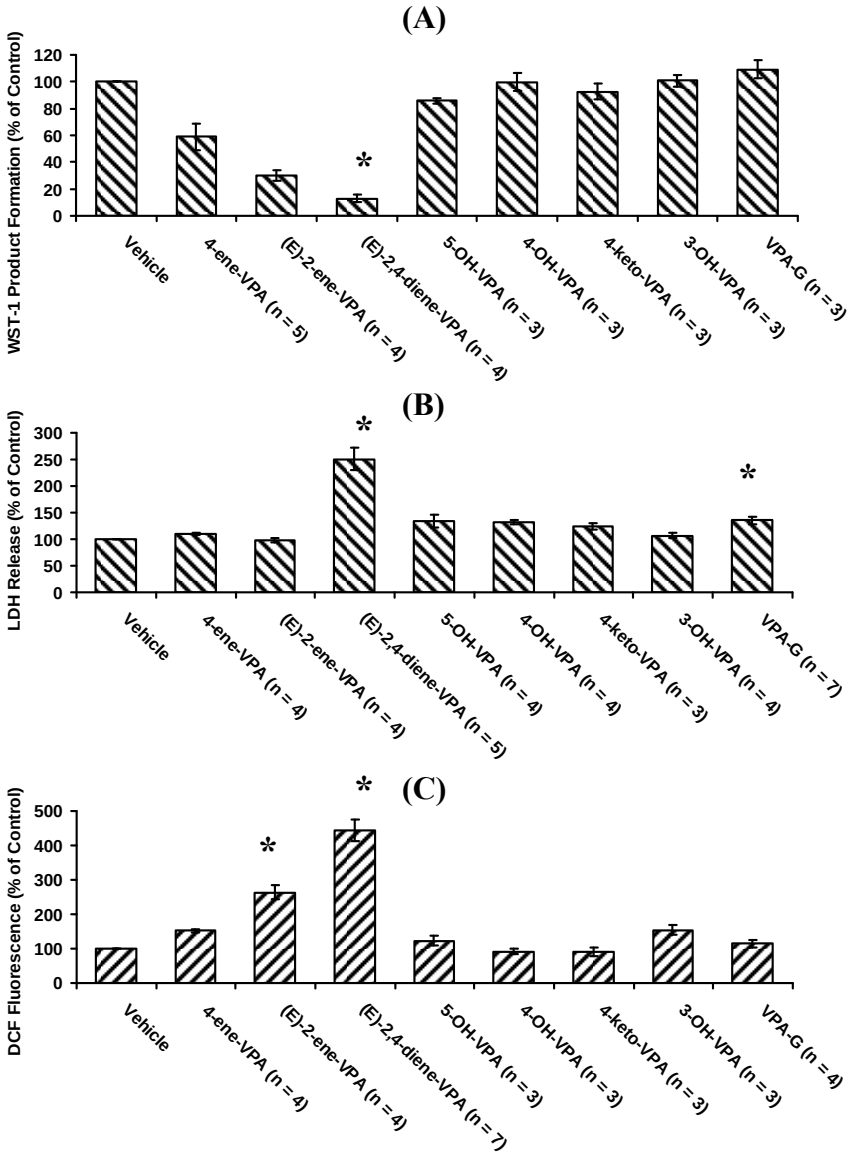

Figure A-8: Effects of exogenously-administered VPA metabolites on the (A) WST-1, (B) LDH, and (C) DCF markers relative to the vehicle-treated control in SCRH. Rat hepatocytes were exposed to the vehicle (culture medium) or each metabolite for 24 hours. The concentrations of exposure were 1 mM (WST-1 assay), and 12 mM (DCF and LDH assays). The individual assays were conducted as described in the Experimental Section in Chapter 2. Data are expressed as a percentage of the vehicle control (mean ± SEM) with the sample sizes indicated in brackets above. *p < 0.05 vs. the vehicle control based on Kruskal Wallis one-way ANOVA followed by the Dunn's post hoc analysis. The vehicle control values were $3.7 \times 10^{-3} \pm 0.4 \times 10^{-3}$ (mean ± SEM) absorbance units / min for the WST-1 marker; 31 ± 2 % (mean ± SEM) total LDH activity for the LDH marker; and $1.4 \times 10^{-3} \pm 0.2 \times 10^{-3}$ µmole DCF / min for the DCF marker.

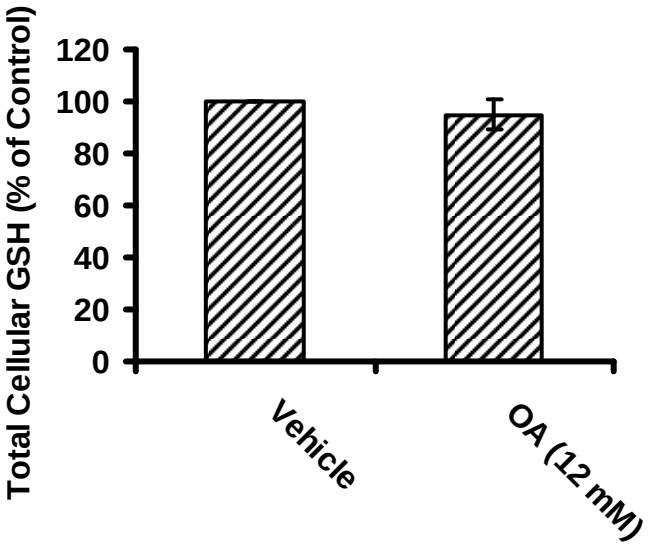

Figure A-9: Effects of octanoic acid (OA) on levels of total cellular GSH in SCRH. Rat hepatocytes were treated with the vehicle (culture medium) or 12 mM OA for 24 hours. The GSH assay was conducted as described in the Experimental Section in Chapter 3. Data are presented as the mean ± SEM of 5 individual experiments. The total GSH levels in the vehicle control were 9.28 ± 0.18 µM (mean ± SEM).

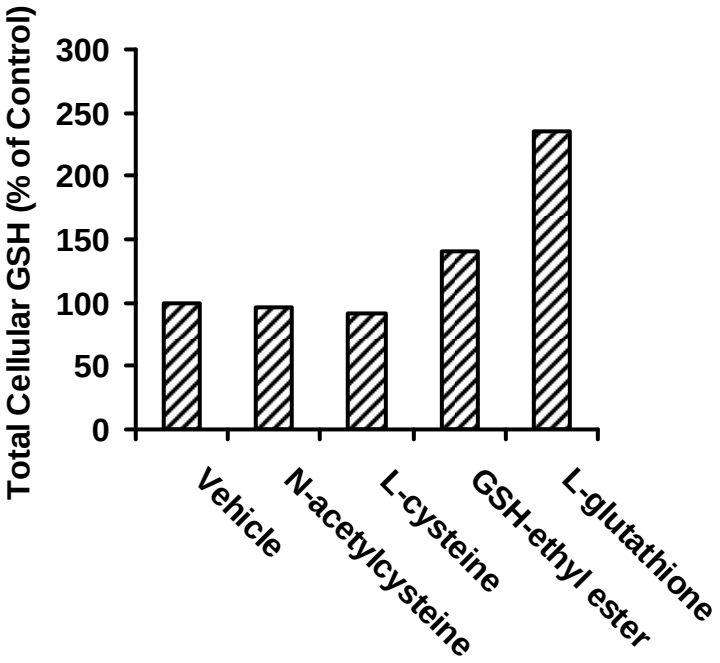

Figure A-10: Effects of N-acetylcysteine, L-cysteine, GSH-ethyl ester, and L-glutathione on levels of total cellular GSH in SCRH. Rat hepatocytes were treated with the vehicle (culture medium) or 2.5 mM of each agent for 24 hours. The GSH assay was conducted as described in the Experimental Section in Chapter 3. Based on data obtained from one experiment. The total GSH levels in the vehicle-treated cells were 18 µM.

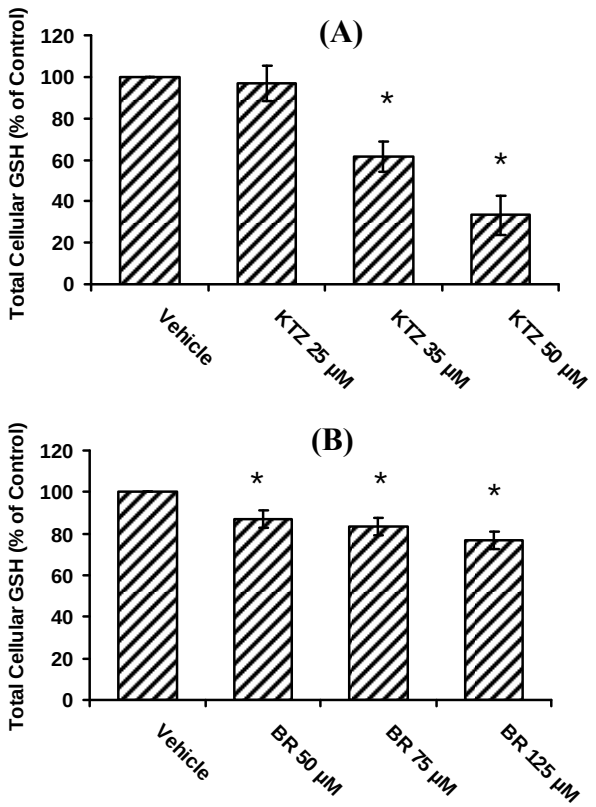

Figure A-11: Effects of (A) ketoconazole (KTZ, N = 5) or (B) (-)-borneol (BR, N = 8) on the depletion of total cellular GSH in SCRH. Rat hepatocytes were treated with the vehicle (culture medium containing 0.5 % MeOH v/v for KTZ or 0.5 % DMSO v/v for BR), KTZ (0 – 50 µM), or BR (0 – 125 µM) for 24.5 hours. The total cellular GSH assay was conducted as described in the Experimental Section in Chapter 3. Data are presented as the mean ± SEM of 5 - 8 individual experiments.*$p < 0.05$ vs. the vehicle control, based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total GSH levels in the vehicle-treated cells were (A) 9.2 ± 0.7 µM (mean ± SEM) and (B) 11.8 ± 1.1 µM.

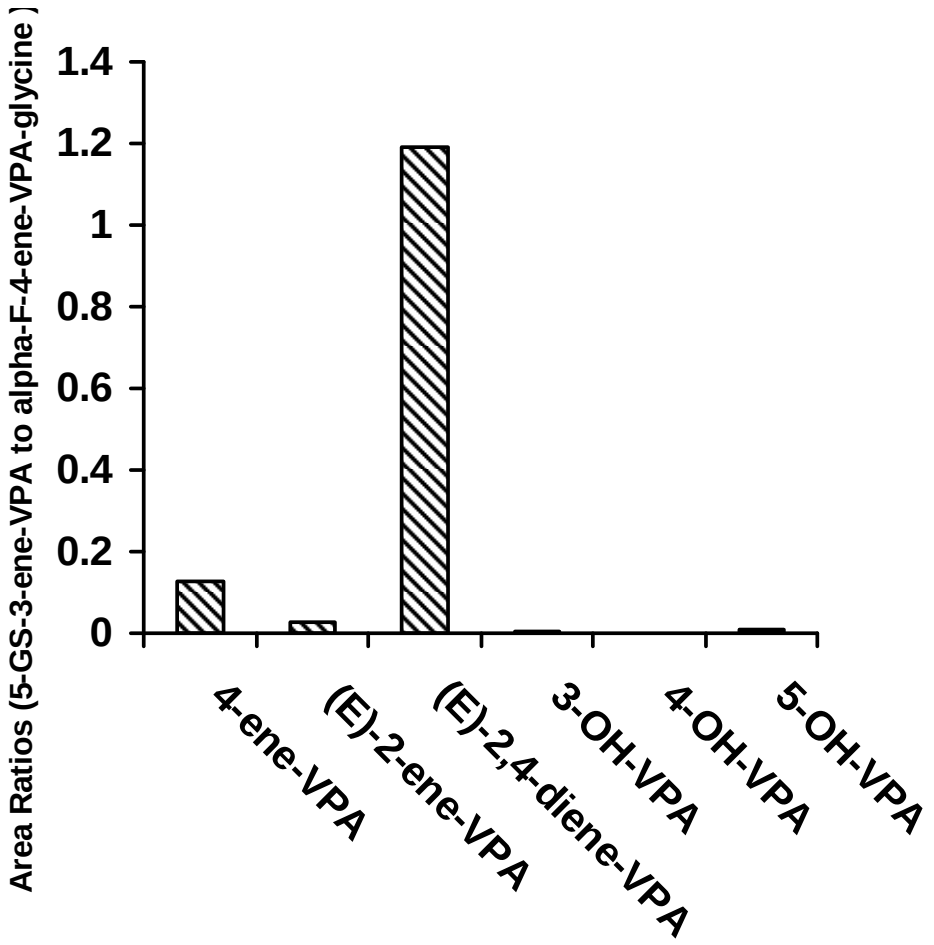

Figure A-12: Relative amounts of 5-GS-3-ene-VPA generated from exogenously administered VPA metabolites in SCRH. Rat hepatocytes were exposed to 12 mM of 4-ene-VPA, (*E*)-2-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, or 5-OH-VPA for 24 hours. A volume of the culture supernatant was collected and added α-F-4-ene-VPA-glycine, the internal standard, to a final concentration of 50 µg/mL. 10 µL of the mixture was injected directly into a Waters Acquity™ Ultra Performance LC fitted with an Acquity UPLC™ BEH C_{18} column (100 x 2.1 mm, I.D., 1.7 µm). The mobile phase, delivered at a flow rate of 0.2 mL/min, consisted of a mixture of acetonitrile and water, containing 0.2 % formic acid. The percentage of acetonitrile was increased from 25 % to 90 % over 5.5 minutes and sharply reduced and held at 25 % for 3.5 minutes prior to the next injection. Detection was achieved with a Waters Micromass Quattro Premier™ Tandem Mass Spectrometer (Micromass Ltd., Montreal, Canada) using the positive electrospray mode. Ions of interest were detected under multiple reaction monitoring of the transition from m/z 448 → 216 for 5-GS-3-ene-VPA and m/z 289 → 160 for α-F-4-ene-VPA Glycine. The instrument had been optimally tuned at the following conditions: source temperature, 150 ºC; capillary voltage, 3.5 kV; cone voltage, 15 V, and multiplier voltage, 650 V. The area ratios of 5-GS-3-ene-VPA to that of α-F-4-ene-VPA Glycine were calculated based on duplicate determinations from a single experiment.

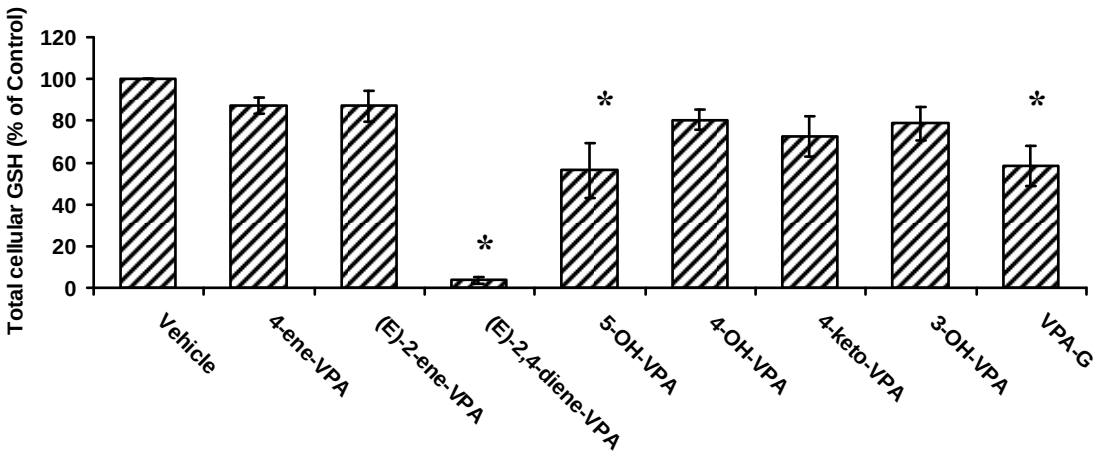

Figure A-13: Effects of exogenously administered VPA metabolites on levels of total cellular GSH in SCRH. Rat hepatocytes were exposed to the vehicle (culture medium) or 12 mM of each metabolite for 24 hours. The total cellular GSH assay was conducted as described in the Experimental Section in Chapter 3. Data are expressed as a percentage of the vehicle control (mean ± SEM, n = 3). *$p < 0.05$ vs. the vehicle control based on one-way ANOVA followed by the Student-Newman-Keuls post hoc analysis. The total cellular GSH levels in the vehicle-treated cells were 7.50 ± 0.90 μM (mean ± SEM).

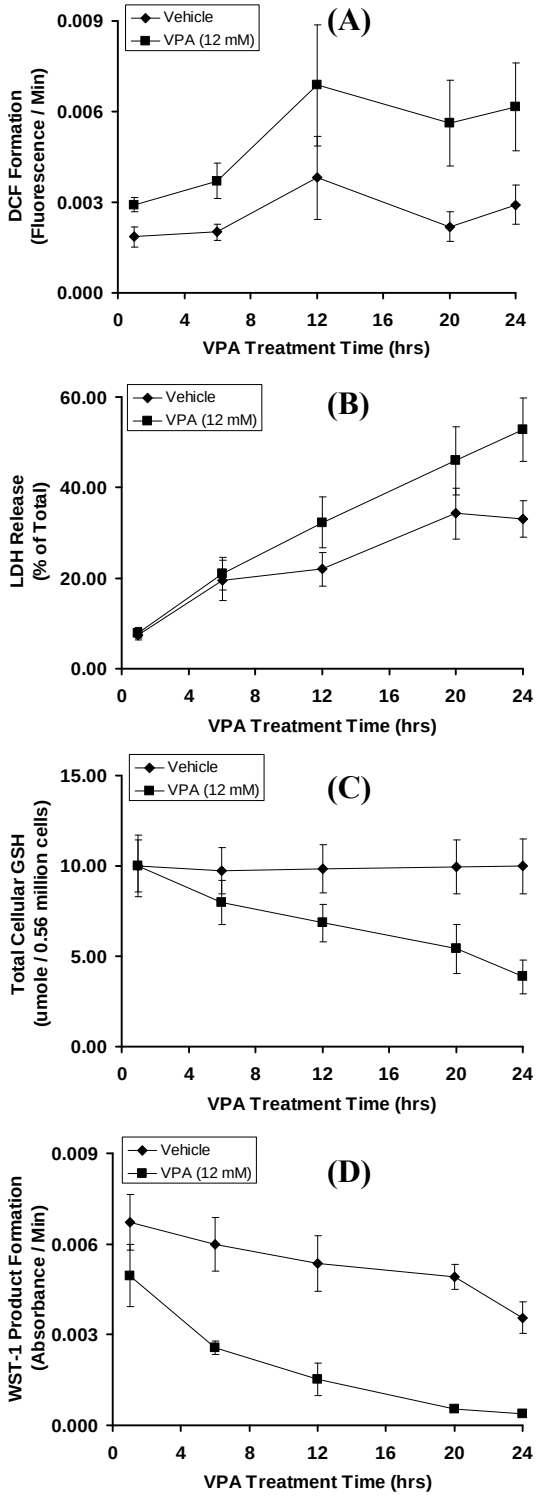

Figure A-14: Effects of vehicle or VPA on (A) mitochondrial dysfunction (WST-1), (B) cytotoxicity (LDH), (C) oxidative stress (DCF), and (D) GSH depletion in SCRH at different exposure times. The experimental conditions are detailed in Figure 3-3.

7.2 Appendix A: Animal Care Certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A08-0347

Investigator or Course Director: Frank S. Abbott

Department: Pharmaceutical Sciences

Animals:

Rats Sprague-Dawley 172

Start Date: October 1, 2005 **Approval Date:** October 14, 2008

Funding Sources:

Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Valproic acid analogues: Glutathione-dependent metabolism and mechanism of toxicity
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Valproic acid analogues: Glutathione metabolism and mechanisms of toxicity
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

7.3 Appendix B: Biohazard Approval Certificate

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: **H05-0167**

INVESTIGATOR OR COURSE DIRECTOR: **Abbott, Frank S.**

DEPARTMENT: **Pharmaceutical Sciences**

PROJECT OR COURSE TITLE: **Valproic Acid Analogues: Gluthathione-Dependent Metabolism and Mechanism of Toxicity**

APPROVAL DATE: **08-12-16**

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: **Canadian Institutes of Health Research (CIHR)**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

7.4 Appendix C: Clinical Research Ethics Certificate

The University of British Columbia
Office of Research Services,
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

Certificate of Full Board Approval

Clinical Research Ethics Board Official Notification

PRINCIPAL INVESTIGATOR	DEPARTMENT	NUMBER
Farrell, K.	Paediatrics	C05-0346

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT

Children's & Women's Health Centre

CO-INVESTIGATORS:

Abbott, Frank, Pharmaceutical Sciences; Chang, Thomas, Pharmaceutical Sciences

SPONSORING AGENCIES

Canadian Institutes of Health Research

TITLE:

Valproic Acid Analogues: Glutathione Dependent Metabolism and Mechanisms of Toxicity

APPROVAL DATE	TERM (YEARS)	DOCUMENTS INCLUDED IN THIS APPROVAL:
13 September 2005	1	Protocol version date 5 July 2005; Subject Consent Form (Subjects on VPA only) version date 5 July 2005; Subject Consent Form (Subjects on anticonvulsants only, or VPA & anticonvulsants) version date 5 July 2005; Subject Consent Form (Controls) version date 5 July 2005; Assent Form (Cases) version date 5 July 2005; Assent Form (Controls) version date 5 July 2005; Information Letter for Parents of Subjects version date 5 July 2005; Information Letter for Parents of Controls version date 5 July 2005

CERTIFICATION:

In respect of clinical trials:

1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.

2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.

3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The documentation included for the above-named project has been reviewed by the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.

The CREB approval for this study expires one year from the approval date.

Approval of the Clinical Research Ethics Board by one of:
Dr. Gail Bellward, Chair
Dr. James McCormack, Associate Chair