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# Role of Oxidative Metabolism in the Effect of Valproic Acid on Markers of Cell Viability, Necrosis, and Oxidative Stress in Sandwich-Cultured Rat Hepatocytes

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**Valproic acid (VPA) is a drug known for idiosyncratic hepatotoxicity and is associated with oxidative stress. It is metabolized extensively with at least one pathway leading to reactive metabolites. The primary aim of the present study was to determine whether oxidative metabolites of VPA generated *in situ* contribute to the toxicity of the parent drug in sandwich-cultured rat hepatocytes. Concentration-response experiments with VPA produced median effective concentration values (mean ± SEM) of 1.1 ± 0.4, 12.2 ± 1.4, and 12.3 ± 1.9mM in the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1; cell viability), lactate dehydrogenase (LDH; necrosis), and 2′,7′-dichlorofluorescein (DCF; oxidative stress) assays, respectively. At equimolar concentrations, only the unsaturated metabolites of VPA gave responses comparable to VPA, with 2,4-diene-VPA calculated to be 3-, 6-, and 10-fold more potent than VPA in the WST-1, LDH, and DCF assays, respectively. In support of a role for reactive metabolites, 2-fluoro-2-propylpentanoic acid, which is relatively resistant to biotransformation to form a 2,4-diene metabolite, yielded little or no toxicity when compared with the nonhepatotoxic octanoic acid or the vehicle-treated control. By comparison, attenuating the *in situ* formation of 2-propylpent-4-enoic acid (4-ene-VPA), 3-hydroxy-2-propylpentanoic acid, 4-hydroxy-2-propylpentanoic acid, and 5-hydroxy-2-propylpentanoic acid by an inhibitor of cytochrome P450 (1-aminobenzotriazole) did not alter the effects of VPA on the WST-1, LDH, or DCF assay. Overall, VPA toxicity in sandwich-cultured rat hepatocytes is independent of the *in situ* formation of cytochrome P450–dependent oxidative metabolites, including 4-ene-VPA. However, the data obtained from structural analogues of VPA suggest that biotransformation does appear to play a role in VPA toxicity in rat hepatocytes.**

***Key Words:*** **cytotoxicity; hepatocytes; metabolism; oxidative stress; reactive metabolites; valproic acid.**

Drug-induced liver injury is a leading cause of acute liver failure (Russmann *et al.*, 2009), and it is one of the most common reasons for withdrawing a drug from the market (Senior, 2007). Except for accidental or intentional overdose, most drug-induced hepatotoxicities in humans are idiosyncratic in nature (Park *et al.*, 2005), including the one associated with valproic acid (VPA; also known as 2-propylpentanoic acid or dipropylacetic acid) (Zaccara *et al.*, 2007). This drug has a therapeutic role in various seizure disorders, migraine headache, nerve pain, and bipolar disorder (Johannessen and Johannessen, 2003). The mechanism responsible for VPA hepatotoxicity is not known, but it has been postulated to involve reactive metabolites of VPA (Tang, 2007) and oxidative stress (Chang and Abbott, 2006).

VPA undergoes extensive hepatic biotransformation (Abbott and Anari, 1999). In addition to undergoing glucuronidation and β-oxidation, VPA is also metabolized by cytochrome P450–catalyzed terminal desaturation to form 2-propylpent-4-enoic acid (4-ene-VPA), which is subsequently converted in a β-oxidation reaction to produce the electrophilic (*E*)-2-propylpent-2,4-dienoic acid ((*E*)-2,4-diene-VPA) (Fig. 1). That a metabolite of VPA might be responsible for VPA hepatotoxicity was first hypothesized following an analysis of fatal cases of hepatic injury in patients prescribed VPA therapy (Zimmerman and Ishak, 1982). Since then, various clinical reports have noted an abnormal pattern of VPA metabolite profile in specific cases of VPA hepatotoxicity in patients (e.g., McLaughlin *et al.*, 2000); however, no definitive conclusion could be drawn from those individual case reports. *In vivo*, the direct administration of synthetic 4-ene-VPA or (*E*)-2,4-diene-VPA (100 mg/kg/day for several days) to rats has been shown to result in hepatic microvesicular steatosis (Granneman *et al.*, 1984; Kesterson *et al.*, 1984; Loscher *et al.*, 1993; Tang *et al.*, 1995). However, the degree of liver injury resulting from 4-ene-VPA is not comparable from one study to another using apparently similar experimental conditions (Loscher *et al.*, 1993; Tang *et al.*, 1995). In rats administered VPA, urinary levels of total 4-ene-VPA (i.e., free and conjugated forms), but not those of (*E*)-2,4-diene-VPA, are correlated with serum levels of α-glutathione *S*-transferase (Lee *et al.*, 2009), which is an *in vivo* marker of hepatotoxicity (Clarke *et al.*, 1997).

*In vitro*, 4-ene-VPA (0.5mM) was reported to be cytotoxic in isolated rat hepatocytes in one study (Kingsley *et al*., 1983), but not in another study at concentrations up to 20mM, unless the cells were pretreated with buthionine sulfoximine to deplete glutathione (Jurima-Romet *et al*., 1996). Additionally, 4-ene-VPA has been reported as being less toxic (Jurima-Romet *et al*., 1996), equally toxic (Kingsley *et al*., 1983), or more toxic than VPA (Kesterson *et al*., 1984), which reflects the different experimental models and toxicity markers employed by the investigators in those studies. To date, there has been no experimental evidence indicating that 4-ene-VPA or (*E*)-2,4-diene-VPA is responsible for the hepatotoxic actions of the parent drug. Moreover, the studies described above were performed with the synthetic form of the 4-ene-VPA and (*E*)-2,4-diene-VPA metabolites and at concentrations/doses considerably greater than the levels that would normally be attained endogenously after administration of the parent drug. It is now recognized that a synthetic metabolite may not necessarily exhibit the same pharmacokinetic and toxicity profile as the corresponding metabolite generated endogenously or *in situ* from the parent drug (Prueksaritanont *et al*., 2006). Therefore, it remains to be determined whether the process of biotransformation *per se* is responsible for the hepatotoxic effects following VPA administration and which of the *in situ*–generated metabolites of VPA contributes to the hepatotoxicity.

The present study was conducted in sandwich-cultured rat hepatocytes to gain insight into the chemical mechanism of VPA hepatotoxicity. As indicated in a recent review article, this hepatocyte culture model is known to "maintain cell viability, polarized architecture, and liver-specific function" (Swift *et al*., 2010). Specific experiments were designed (1) to compare directly the toxicity of a panel of oxidative metabolites of VPA (i.e., (*E*)-2-propylpent-2-enoic acid [(*E*)-2-ene-VPA], 2-propylpent-3-enoic acid [3-ene-VPA], 4-ene-VPA, (*E*)-2,4-diene-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]) with that of the parent drug; (2) to determine whether the *in situ*–generated 4-ene-VPA and other primary metabolites (i.e., 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA) produced by cytochrome P450–mediated enzymatic reactions contribute to VPA toxicity; and (3) to investigate the effects of structural analogs of VPA (i.e., 2-fluoro-2-propylpentanoic acid [alpha-F-VPA] and octanoic acid; Fig. 2) known to be compromised in specific biotransformation pathways. The results are discussed in the context of the role of reactive metabolites in the development of toxicity in hepatocytes treated with VPA.

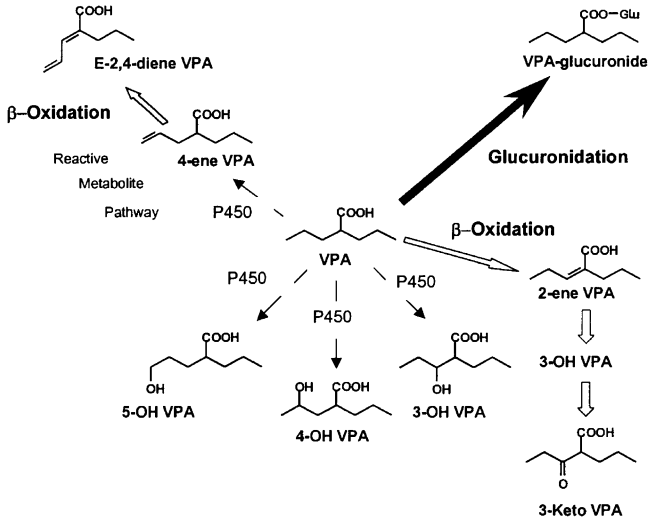


**FIG. 1.** A simplified scheme illustrating the major biotransformation pathways of VPA (Chang and Abbott, 2006).

## MATERIALS AND METHODS

***Chemicals and reagents.*** Sodium valproate, 1-aminobenzotriazole, *tert*-butylhydroperoxide, dimethylsulfoxide, Triton X-100, and fetal bovine serum were purchased from Sigma-Aldrich Chemical Co. (Oakville, Ontario, Canada). Matrigel and Hepato-Stim medium were bought from BD Biosciences (Mississauga, Ontario, Canada). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA), HepatoZYME-SFM medium, penicillin-streptomycin, and L-glutamine were purchased from Invitrogen (Burlington, Ontario, Canada). Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) reagent were obtained from Roche Applied Sciences (Mississauga, Ontario, Canada).

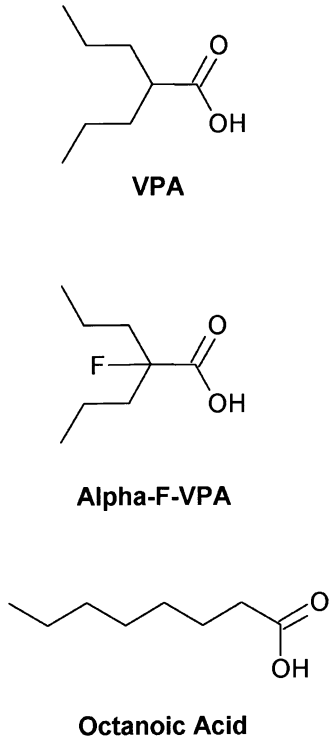


**FIG. 2.** Chemical structures of VPA, alpha-F-VPA, and octanoic acid.

Ammonium acetate (High Performance Liquid Chromatography grade), anhydrous ethyl ether (certified ACS grade), EDTA, *o*-phosphoric acid, *n*-hexanes, methanol, and sodium hydroxide were bought from Fisher Scientific (Ottawa, Ontario, Canada). 2′,7′-Dichlorofluorescein (DCF) was purchased from PolySciences, Inc. (Warrington, PA).

***Synthesis of VPA metabolites and analogues.*** The following VPA metabolites and analogues were synthesized as described previously: 4-ene-VPA and (*E*)-2,4-diene-VPA (Kassahun *et al*., 1991); (*E*)-2-ene-VPA, 3-ene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, and 4-keto-VPA (Zheng, 1993); and alpha-F-VPA (Tang *et al*., 1997). Purity was > 99% as determined by liquid chromatography and mass spectrometry.

***Animals.*** Adult male Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. (Senneville, Quebec, Canada) and housed in a temperature-controlled (22°C) room with 12 h dark-light cycles. The animals were provided *ad libitum* with food (Labdiet 5001 Rodent Diet, PMI Feeds Inc., Richmond, IN) and water. All animal experiments were approved by the Animal Care Committee at the University of British Columbia.

***Isolation and culturing of rat hepatocytes.*** Hepatocytes were isolated by a two-step collagenase method (Seglen, 1993) as described previously (Tong *et al*., 2005a). Cell viability was > 85%, as assessed by trypan blue exclusion (Jauregui *et al*., 1981). Cells were suspended at a concentration of 0.7 million cells per milliliter in Hepato-Stim medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2mM), and heat-inactivated fetal bovine serum (10% vol/vol). Isolated hepatocytes were seeded at a density of 0.56 million cells per well in 12-well plates (Costar 3513, Corning Inc.) coated with Matrigel and allowed to attach for 4 h in a 37°C, 5% $CO_2$ incubator. Subsequently, the medium was aspirated and replaced with 0.8 ml of HepatoZYME-SFM medium supplemented with Matrigel (250 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2mM). The plates were then placed in a 37°C, 5% $CO_2$ incubator for 20 h to allow for the formation of the sandwich culture (Swift *et al*., 2010).

***Treatment of cultured rat hepatocytes.*** At 24 h after plating of hepatocytes, culture medium was aspirated and replaced with fresh HepatoZYME-SFM medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2mM). Hepatocytes were treated with VPA, (*E*)-2-ene-VPA, 3-ene-VPA, 4-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 4-keto-VPA, alpha-F-VPA, octanoic acid, or *tert*-butylhydroperoxide for 24 h at the concentrations indicated in each figure legend. In another experiment, hepatocytes were pretreated with 1-aminobenzotriazole (0.5mM) or culture medium (vehicle control) 30 min prior to the administration of VPA (1mM in the WST-1 assay and 12mM in the LDH and DCF assays) or culture medium (vehicle control). At 24 h thereafter, cytotoxicity assays were performed. A previous study showed that treatment of rat hepatocytes with VPA for 24 h leads to maximal or near-maximal effects in various indices of hepatocyte toxicity (Takeuchi *et al*., 1988).

***WST-1 assay.*** Hepatocyte viability was assessed by the WST-1 assay (Rousar *et al*., 2009). At the end of the drug treatment period, hepatocytes were washed with warm (37°C) and sterile PBS (pH 7.4) and incubated at 37°C with 1 ml of the supplemented culture medium containing 50 μl of the stock WST-1 solution. The reduction of the WST-1 reagent to its formazan product was monitored spectrophotometrically at regular intervals at a wavelength of 450 nm in a Labsystems Multiskan Ascent multiwell plate reader (Thermo Electron Corp., Burlington, Ontario, Canada). The rate of WST-1 product (formazan) formation was calculated as a percentage of the rate in the vehicle-treated control cells. The blank consisted of the culture medium and an equal amount of the tetrazolium dye.

***LDH assay.*** Hepatocyte necrosis was measured by the cellular release of LDH (Bajt *et al*., 2004). The LDH assay was performed essentially according to the instructions in the LDH Cytotoxicity Detection Kit (Roche Applied Sciences). At the end of the drug treatment period, an aliquot of the culture supernatant was immediately stored on ice. Subsequently, hepatocytes were washed with warm PBS (pH 7.4) and incubated with 1.25 ml of lysis buffer (2% vol/vol Triton X-100 and 20mM EDTA in PBS, pH 7.4). The enzymatic reaction was initiated by the addition of 100 μl of the reaction mixture (provided in the kit). Reaction product was monitored spectrophotometrically at regular intervals at a wavelength of 492 nm in a Labsystems Multiskan Ascent multiwell plate reader (Thermo Electron Corp.). LDH release in the supernatant was calculated as a percentage of the total cellular LDH content (i.e., sum of the LDH levels in the supernatant and in cell lysate).

***DCF assay.*** The cellular conversion of DCFDA to DCF was used as a general index of oxidative stress (Halliwell and Whiteman, 2004). At the end of the drug treatment period, hepatocytes were washed with sterile PBS (pH 7.4) and incubated with 1 ml of the supplemented culture medium containing DCFDA. Preliminary experiments indicated that a suitable concentration was 2μM. The fluorescence emission of DCF was monitored at regular intervals at an excitation wavelength of 485 nm (band width of 20 nm) and an emission wavelength of 530 nm (band width of 25 nm) in a Cytofluor Series 4000 multiwell fluorescence plate reader (Applied Biosystems, Bedford, CA). The blank samples contained culture medium, but without cells. The amount of DCF formed was calculated based on a calibration curve constructed using authentic DCF standard. The rate of DCF formation was calculated as a percentage of the DCF formation in the vehicle-treated control group.

***Quantification of VPA metabolites.*** Levels of 4-ene-VPA, 4-OH-VPA, 5-OH-VPA, 3-OH-VPA, (*E*)-2-ene-VPA, 3-ene-VPA, (*E*,*E*)-2,3-diene-VPA, (*E*,*Z*)-2,3-diene-VPA, and (*E*)-2,4-diene-VPA were quantified by gas chromatography-mass spectrometry (Tong *et al*., 2003). VPA-1-*O*-acyl glucuronide was quantified by liquid chromatography-tandem mass spectrometry as described previously (Tong *et al*., 2005b), except that the dwell time was 0.05 s, cone voltage was 20 V, and collision energy was 10 eV.

***Statistical analysis.*** Data were analyzed by one-way ANOVA (SigmaStat for Windows, Version 3.5, Systat Software, Inc., Chicago, IL) and, when appropriate, was followed by the Student-Newman-Keuls multiple comparison test. In cases where assumptions for a parametric test were violated, the data were analyzed by a nonparametric test (Kruskal-Wallis one-way ANOVA by ranks). effective concentration ($EC_{50}$) values were generated by sigmoidal curve fitting using the four-parameter Hill equation: $y = y_0 + [ax^b/(c^b + x^b)]$ (SigmaPlot 2001 for Windows, version 7.0, SPSS, Inc., Chicago, IL).

## RESULTS

### *Effects of VPA and tert-Butylhydroperoxide*

An initial experiment in sandwich-cultured rat hepatocytes was performed to assess the effects of VPA on WST-1 product formation, LDH release, and DCF levels and to verify the effects of a known hepatotoxicant, *tert*-butylhydroperoxide (Cervinkova *et al*., 2009; Nieminen *et al*., 1995; Martin *et al*., 2001), on these markers. The results indicate that VPA and *tert*-butylhydroperoxide decreased WST-1 product formation (Fig. 3A) and increased LDH release (Fig. 3B) and DCF levels (Fig. 3C).

### *Concentration-Response Relationship in the Effects of VPA*

To further characterize the effects of VPA on markers of cytotoxicity and oxidative stress, concentration-response experiments were performed. Sandwich-cultured rat hepatocytes were treated with varying concentrations of the drug for 24 h. As evident by the concentration-response curves, VPA decreased WST-1 product formation, elevated LDH release, and increased

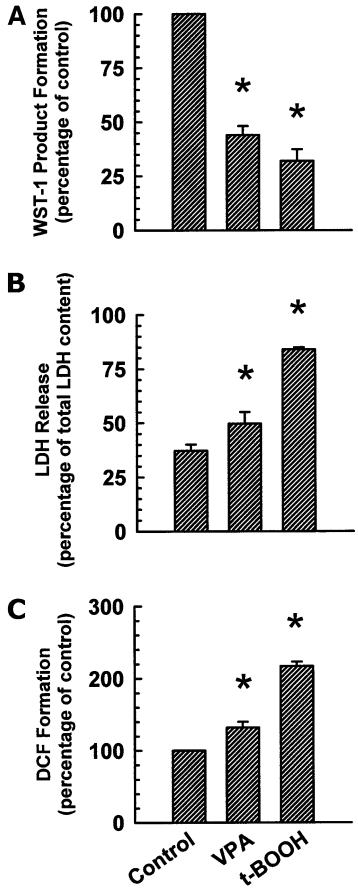


**FIG. 3.** Effects of VPA and *tert*-butylhydroperoxide on markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. Freshly isolated hepatocytes were cultured and treated with VPA (1mM in the WST-1 assay and 6mM in the LDH and DCF assays), *tert*-butylhydroperoxide (*t*-BOOH, 5mM), or culture medium (vehicle control) for 24 h in a 37°C, 5% $CO_2$ incubator. WST-1 (A), LDH (B), and DCF (C) assays were performed as described under ''Materials and Methods'' section. Data are expressed as mean ± SEM for three or four independent experiments. *Significantly different from the vehicle-treated control group, $p < 0.05$.

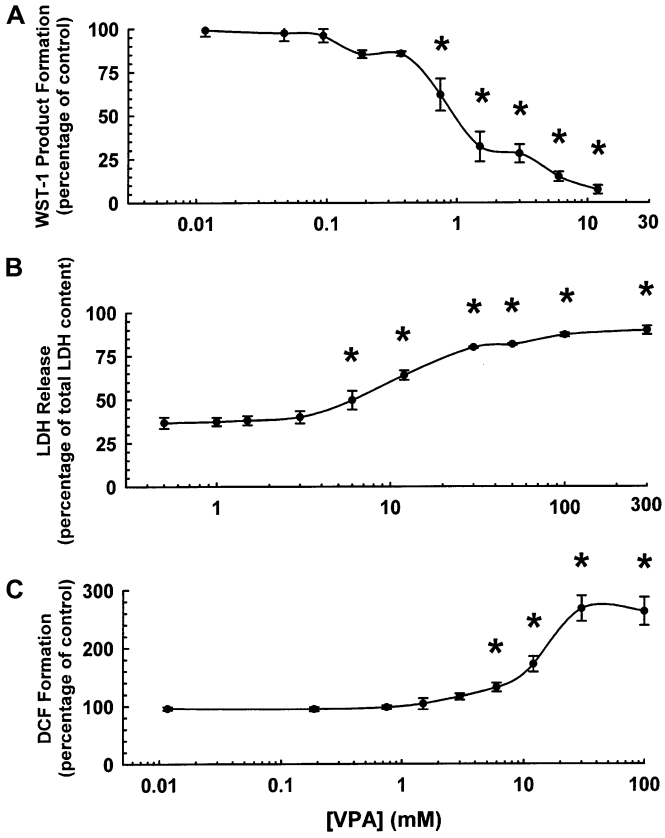


**FIG. 4.** Dose-response relationship in the effects of VPA on markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. Freshly isolated hepatocytes were cultured and treated with varying concentrations of VPA or culture medium (vehicle) for 24 h in a 37°C, 5% $CO_2$ incubator. WST-1 (A), LDH (B), and DCF (C) assays were performed as described under ''Materials and Methods'' section. Data are expressed as mean ± SEM for four independent experiments. *Significantly different from the vehicle-treated control group, $p < 0.05$.

DCF levels over the range of 0.75–12mM (Fig. 4A), 6–300mM (Fig. 4B), and 6–100mM (Fig. 4C), respectively. The experimentally derived $EC_{50}$ values were 1.1 ± 0.4mM (mean ± SEM) in the WST-1 assay, 12.2 ± 1.4mM in the LDH assay, and 12.3 ± 1.9mM in the DCF assay.

### *Effects of Individual Synthetic Metabolites of VPA*

To compare the relative toxicity of individual synthetic metabolites of VPA, sandwich-cultured rat hepatocytes were treated with an equimolar concentration (1mM in the WST-1 assay and 12mM in the LDH and DCF assays) of (*E*)-2-ene-VPA, 3-ene-VPA, 4-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, or 4-keto-VPA for 24 h. The concentrations were chosen to reflect the $EC_{50}$ values in the effect of VPA in each of the assays. Among the individual synthetic metabolites investigated, only (*E*)-2-ene-VPA, 4-ene-VPA, (*E*)-2,4-diene-VPA, and VPA reduced WST-1 product formation, and the magnitude of the effect was greater for (*E*)-2-ene-VPA and (*E*)-2,4-diene-VPA than for VPA (Fig. 5A). In contrast, only (*E*)-2,4-diene-VPA and VPA increased LDH release, and the magnitude of the effect of the former was greater than that of the latter (Fig. 5B). By comparison, only (*E*)-2-ene-VPA, 3-ene-VPA, (*E*)-2,4-diene-VPA, and VPA increased DCF levels, and the magnitude of the effect of (*E*)-2,4-diene-VPA was greater than that of VPA (Fig. 5C).

### *Dose-Response Relationship in the Effects of Synthetic 2,4-Diene-VPA*

As demonstrated in Figures 5A–C, (*E*)-2,4-diene-VPA was the only synthetic metabolite among those investigated that consistently produced greater toxicity than VPA in sandwich-cultured rat hepatocytes. Therefore, we further characterized the effects of synthetic (*E*)-2,4-diene-VPA, with the aim of identifying the minimum toxic concentration of this metabolite in each of the toxicity markers employed in the present study. Concentration-response experiments showed that the toxicity

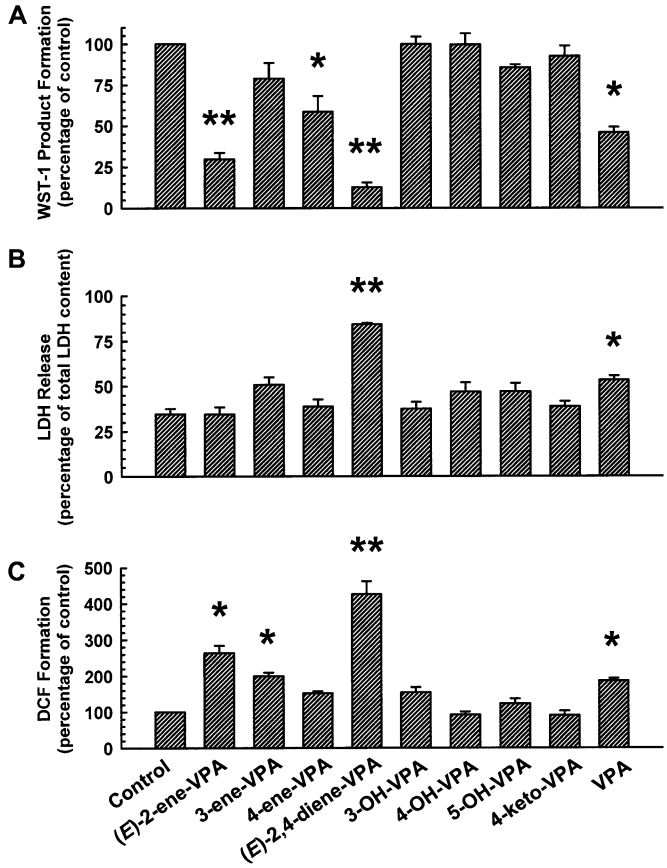


**FIG. 5.** Effect of individual synthetic metabolites of VPA on markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. Freshly isolated hepatocytes were cultured and treated with (*E*)-2-ene-VPA, 3-ene-VPA, 4-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, 4-keto-VPA, or VPA at a concentration of 1mM (WST-1 assay) or 12mM (LDH and DCF assays) for 24 h in a 37°C, 5% $CO_2$ incubator. Control cells were treated with culture medium (vehicle) for the same length of time. WST-1 (A), LDH (B), and DCF (C) assays were performed as described under ''Materials and Methods'' section. Data are expressed as mean ± SEM for three to five independent experiments. *Significantly different from the vehicle-treated control group, $p < 0.05$. **Significantly different from the vehicle-treated and VPA-treated groups, $p < 0.05$.

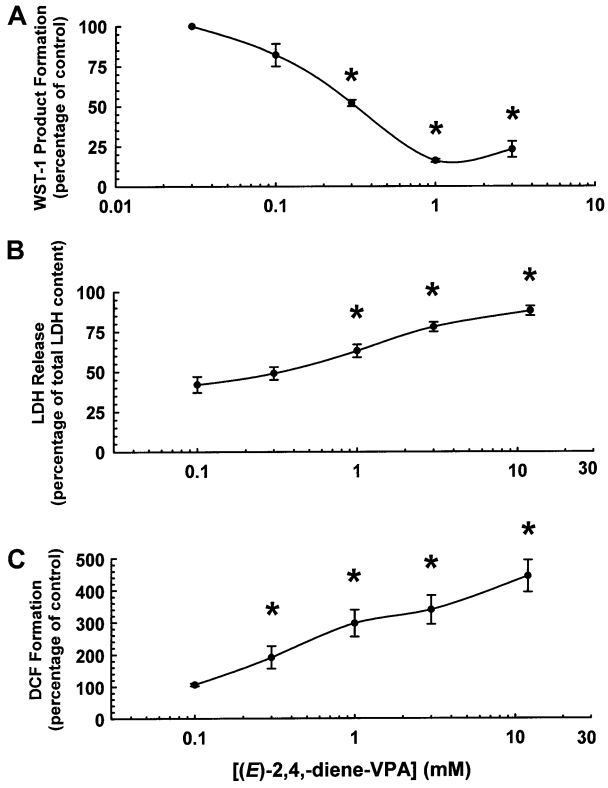


**FIG. 6.** Dose-response relationship in the effects of synthetic (*E*)-2,4-diene-VPA on markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. Freshly isolated hepatocytes were cultured and treated with varying concentrations of (*E*)-2,4-diene-VPA or culture medium (vehicle) for 24 h in a 37°C, 5% $CO_2$ incubator. WST-1 (A), LDH (B), and DCF (C) assays were performed as described under ''Materials and Methods'' section. Data are expressed as mean ± SEM for three independent experiments. *Significantly different from the vehicle-treated control group, $p < 0.05$.

of this metabolite became statistically significant at concentrations $\geq$ 0.3mM (Fig. 6A), 1mM (Fig. 6B), and 0.3mM (Fig. 6C) in the WST-1, LDH, and DCF assays, respectively. By comparison, the effects of VPA became statistically significant at concentrations $\geq$ 0.75mM (Fig. 4A), 6mM (Fig. 4B), and 3mM (Fig. 4C) in the WST-1, LDH, and DCF assays, respectively. Therefore, (*E*)-2,4-diene-VPA was 3-, 6-, and 10-fold more potent than VPA in the WST-1, LDH, and DCF assays, respectively, as judged by the minimum toxic concentrations.

### *Effects of Inhibiting the* In Situ *Formation of Cytochrome P450–Dependent Oxidative Metabolites of VPA*

Biotransformation studies have shown that VPA is metabolized to various cytochrome P450–dependent oxidative metabolites, including 4-ene-VPA (Abbott and Anari, 1999), which subsequently undergoes β-oxidation to produce (*E*)-2,4-diene-VPA (Fig. 1). Therefore, we determined whether the *in situ*–generated 4-ene-VPA and other primary metabolites (i.e., 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA) of cytochrome P450–dependent enzymatic reactions contributed to the development of toxicity in hepatocytes treated with VPA. Our experimental strategy involved the use of 1-aminobenzotriazole, which is a broad spectrum, mechanism-based inactivator of cytochrome P450 enzymes (Ortiz de Montellano and Mathews, 1981). This chemical has been shown to inhibit the *in vivo* formation of cytochrome P450–dependent oxidative metabolites of VPA in rats dosed with VPA (Tong *et al.*, 2003). Preliminary experiments with sandwich-cultured rat hepatocytes indicated that at a concentration of 0.5mM, 1-aminobenzotriazole maximally inhibited the oxidative metabolism of VPA and it did not produce cytotoxicity, as assessed by the WST-1 and LDH assays, or oxidative stress, as indicated by the DCF assay (data not shown). Consequently, this concentration of

1-aminobenzotriazole was used in our inhibition experiment. As shown in Figures 7A–C, treatment of hepatocytes with VPA alone produced the expected effects in the WST-1, LDH, and DCF assays. However, 1-aminobenzotriazole did not attenuate the magnitude of the various toxicity markers in VPA-treated hepatocytes. Control analysis indicated that 1-aminobenzotriazole suppressed the *in situ* formation of 4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA (Table 1). Overall, based on the results obtained from the inhibition experiments with 1-aminobenzotriazole (Figs. 7A–C), the *in situ*–generated 4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA did not contribute to the toxicity in cultured rat hepatocytes treated with VPA.

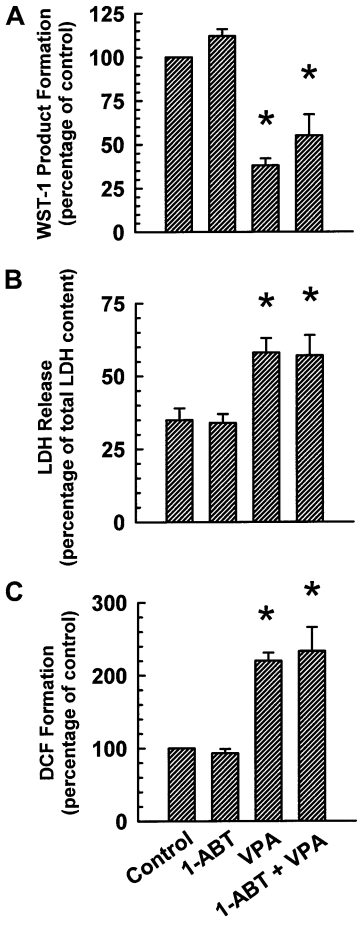


**FIG. 7.** Effects of 1-aminobenzotriazole on markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes treated with VPA. Freshly isolated hepatocytes were cultured and pretreated with 1-aminobenzotriazole (1-ABT, 0.5mM) or culture medium (vehicle) 30 min prior to the administration of VPA (1mM in the WST-1 assay and 12mM in the LDH and DCF assays) or culture medium (vehicle). Cells were incubated for 24 h in a 37°C, 5% $CO_2$ incubator. WST-1 (A), LDH (B), and DCF (C) assays were performed as described under ''Materials and Methods'' section. Data are expressed as mean ± SEM for four independent experiments in the WST-1 assay, seven in the LDH assay, and five in the DCF assay. *Significantly different from the vehicle-treated control group, $p < 0.05$.

### *Comparative Effects of Alpha-F-VPA, Octanoic Acid, and VPA*

To gain further insight into the chemical mechanism of VPA toxicity, we employed an experimental strategy that involved the investigation of structural analogues of VPA (i.e., alpha-F-VPA and octanoic acid, Fig. 2). Previous studies have shown that alpha-F-VPA is resistant to the enzymatic generation of the corresponding (*E*)-2,4-diene (Tang *et al.*, 1997), glucuronide (Tang *et al.*, 1997), and Coenzyme A (CoA) esters (Grillo *et al.*, 2001). By comparison, octanoic acid, which is an eight-carbon straight chain fatty acid, is not expected to undergo biotransformation to form a 4-ene or 2,4-diene metabolite, as predicted on the basis of its chemical structure. Therefore, we compared the effects of an equimolar concentration of alpha-F-VPA, octanoic acid, and VPA on markers of cytotoxicity and oxidative stress in sandwich-cultured rat hepatocytes. The results show that alpha-F-VPA and octanoic acid had little or no effect on WST-1 product formation (Fig. 8A), LDH release (Fig. 8B), or DCF levels (Fig. 8C) when compared with the vehicle-treated control group. In agreement with the findings from a previous experiment (e.g., Figs. 3A–C), VPA decreased WST-1 product formation (Fig. 8A), whereas it increased LDH release (Fig. 8B) and DCF levels (Fig. 8C).

## DISCUSSION

### *Relative Toxicity of Synthetic, Oxidative Metabolites of VPA*

The present study provides the first comprehensive characterization of the cytotoxic potential of a panel of specific

**TABLE 1**
**Effect of 1-Aminobenzotriazole on VPA Metabolite Levels in Sandwich-Cultured Rat Hepatocytes Treated with VPA**

| Metabolite | Control | 1-Aminobenzotriazole |
|---|---|---|
| | Concentration (μM) | |
| 4-ene-VPA | 0.11 ± 0.04 | None detected* |
| 4-OH-VPA | 3.78 ± 0.69 | 0.45 ± 0.11* |
| 5-OH-VPA | 3.09 ± 0.64 | 0.31 ± 0.14* |
| 3-OH-VPA | 0.32 ± 0.05 | 15 ± 0.02* |
| (*E*)-2-ene-VPA | 2.76 ± 0.46 | 3.03 ± 0.44 |
| 3-ene-VPA | 0.82 ± 0.23 | 0.80 ± 0.22 |
| (*E*,*E*)-2,3-diene-VPA | 3.65 ± 1.23 | 3.72 ± 1.21 |
| (*E*,*Z*)-2,3-diene-VPA | 0.38 ± 0.09 | 0.42 ± 0.08 |
| (*E*)-2,4-diene-VPA | None detected | None detected |
| VPA-1-*O*-acyl glucuronide | 94 ± 26 | 98 ± 27 |

*Note.* Sandwich-cultured rat hepatocytes were pretreated with 1-aminobenzotriazole (0.5mM) or culture medium (vehicle control) for 30 min prior to the administration of VPA (12mM) or culture medium (vehicle control). At 24 h thereafter, supernatant was collected. VPA metabolite levels were quantified by gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry as described in the ''Materials and Methods'' section.

*Significantly different from the control group, $p < 0.05$.

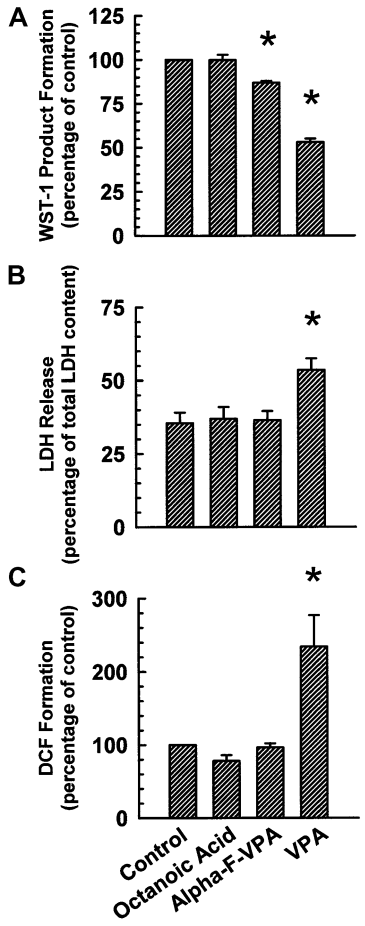


**FIG. 8.** Comparative effects of octanoic acid, alpha-F-VPA, and VPA on markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. Freshly isolated hepatocytes were cultured and treated with an equimolar concentration of octanoic acid, alpha-F-VPA, or VPA (1mM in the WST-1 assay and 12mM in the LDH and DCF assays). Control cells were treated with culture medium (vehicle). All cells were incubated for 24 h in a 37°C, 5% $CO_2$ incubator. WST-1 (A), LDH (B), and DCF (C) assays were performed as described under ''Materials and Methods'' section. Data are expressed as mean ± SEM for three or four independent experiments. *Significantly different from the vehicle-treated control group, $p < 0.05$.

metabolites of VPA in a single experimental model. Among the individual metabolites investigated, only (*E*)-2,4-diene-VPA was consistently more toxic than VPA, as assessed by markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. Consistent with this finding, this metabolite was also more effective than VPA in the development of hepatic steatosis and inhibition of β-oxidation in rats (Kesterson *et al*., 1984). Interestingly, elevated levels of thio-conjugated (*E*)-2,4-diene-VPA have been reported in urine of patients who developed hepatotoxicity (Kassahun *et al*., 1991). As determined in our cell culture experiments, 4-ene-VPA did not increase LDH release or DCF levels when compared with the control group, whereas it did compromise cell viability as measured by the WST-1 assay and to a similar extent as the parent drug. The lack of an effect of 4-ene-VPA on LDH release is in contrast to the increase reported in an earlier study in cultured rat hepatocytes (Kingsley *et al*., 1983). However, it is in agreement with a subsequent study that showed a lack of an effect by the metabolite unless the hepatocytes were depleted of glutathione (Jurima-Romet *et al*., 1996). By comparison, (*E*)-2-ene-VPA, which is a β-oxidation metabolite of VPA (Abbott and Anari, 1999), decreased WST-1 product formation and increased DCF levels, suggesting that they compromised cell viability and produced oxidative stress. The lack of an effect on LDH release by (*E*)-2-ene-VPA is in agreement with a previous finding in cultured rat hepatocytes (Jurima-Romet *et al*., 1996). It is also consistent with the observation that the administration of 2-ene-VPA to rats does not produce hepatic steatosis (Kesterson *et al*., 1984; Loscher *et al*., 1992). Overall, our analysis of a panel of specific metabolites of VPA identified (*E*)-2,4-diene-VPA as one that was consistently more toxic than VPA in sandwich-cultured rat hepatocytes.

### *Assessment of the Role of* In Situ*–Generated Cytochrome P450–Dependent Metabolites of VPA in VPA Hepatocyte Toxicity*

As shown in the present study, 1-aminobenzotriazole was capable of inhibiting the *in situ* formation of 4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA in sandwich-cultured rat hepatocytes treated with VPA, in agreement with our previous *in vivo* findings in rats (Tong *et al*., 2003). However, the decrease in the *in situ* formation of these metabolites was not accompanied by any changes in WST-1 product formation, LDH release, or DCF levels, suggesting that these oxidative metabolites of VPA did not significantly contribute to the development of cytotoxicity or oxidative stress in hepatocytes treated with the parent drug. In a previous study, 1-aminobenzotriazole pretreatment to rats administered VPA also did not attenuate the plasma levels of 15-$F_{2t}$-isoprostane (Tong *et al*., 2003), which is a marker of lipid peroxidation (Halliwell and Whiteman, 2004). Based on the published literature (Tang *et al*., 1997) and data from the dose-response experiment shown in the present study, (*E*)-2,4-diene-VPA would be another candidate metabolite that might be involved in VPA hepatocyte toxicity. In principle, the application of a general cytochrome P450 inhibitor such as 1-aminobenzotriazole is expected to compromise the enzymatic formation of (*E*)-2,4-diene-VPA in hepatocytes treated with the parent drug. This metabolite could be formed either from (*E*)-2-ene-VPA in a reaction that is cytochrome P450 dependent or from 4-ene-VPA, the formation of which is also cytochrome P450 dependent (Fig. 1). It is unlikely that (*E*)-2,4-diene-VPA itself would be able to contribute to the cytotoxicity and oxidative stress by the parent drug because according to our dose-response data, the minimum toxic concentrations of this metabolite were 0.3, 1, and 0.3mM in the WST-1, LDH, and DCF assays, respectively. As shown in the present study, (*E*)-2,4-diene-VPA was not detectable in our sandwich-cultured rat

hepatocytes treated with VPA. Overall, the results from our chemical inhibition experiments suggest that the *in situ*–generated 4-ene-VPA, 3-OH-VPA, 4-OH-VPA, and 5-OH-VPA did not contribute in a significant way to the cytotoxicity or oxidative stress in sandwich-cultured rat hepatocytes treated with the parent drug.

### *Potential Role of Biotransformation in VPA Hepatocyte Toxicity*

We employed structural analogues of VPA as a means to gain additional insight into the potential role of biotransformation in the development of toxicity in rat hepatocytes treated with VPA. As shown in the present study, treatment of sandwich-cultured rat hepatocytes with alpha-F-VPA or octanoic acid produced little or no cytotoxicity or oxidative stress. In previous *in vivo* studies, treatment of rats with alpha-F-VPA or alpha-F-4-ene-VPA was not associated with steatosis (Tang *et al*., 1995) or lipid peroxidation as assessed by the 15-$F_{2t}$-isoprostane assay (Tong *et al*., 2005b), in contrast to their nonfluorinated counterparts. The lack of toxicity observed with the alpha-fluorinated analogues of VPA was originally attributed to their inability to form the toxic (*E*)-2,4-diene metabolite (Tang *et al*., 1995). However, subsequent studies indicated that they were also considerably less able to form a CoA ester (Grillo *et al*., 2001) or a glucuronide (Tang *et al*., 1997). Thus, our experimental findings with alpha-F-VPA and octanoic acid imply that biotransformation appears to play a role in VPA toxicity in rat hepatocytes in culture and that the CoA ester or glucuronide of VPA could be candidate metabolites that contribute to the toxicity of the parent drug. Indeed, CoA esters of carboxylic acids are electrophilic reactive species, as demonstrated by the reactivity of (*E*)-2,4-diene-VPA-CoA toward glutathione and hepatic proteins (Tang and Abbott, 1996). Depletion of CoA from VPA treatment has been associated with impaired mitochondrial β-oxidation or decreased oxidative phosphorylation in various experimental models (Silva *et al*., 2008). That the glucuronidation pathway of VPA may also contribute to VPA hepatotoxicity is suggested by our previous finding of an association between the formation of VPA-1-*O*-acyl glucuronide and the development of lipid peroxidation in rats (Tong *et al*., 2005b). Acyl glucuronides of certain drugs have been shown to possess toxicity, including diclofenac 1-*O*-β-acyl glucuronide, which is known to bind to cellular proteins and suspected to be responsible for the hepatotoxicity of the parent drug (Tang, 2007). Further mechanistic experiments are warranted to determine whether the formation of VPA-CoA ester or VPA glucuronide is responsible for the hepatotoxicity of the parent drug.

### *Summary and Conclusion*

Among a panel of synthetic metabolites of VPA (i.e., (*E*)-2-ene-VPA, 3-ene-VPA, 4-ene-VPA, (*E*)-2,4-diene-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA, and 4-keto-VPA), only (*E*)-2,4-diene-VPA was consistently more potent and toxic than VPA, as evaluated by markers of cell viability, necrosis, and oxidative stress in sandwich-cultured rat hepatocytes. *In situ*–generated cytochrome P450–dependent oxidative metabolites, including 4-ene-VPA, did not contribute to hepatocyte toxicity of the parent drug, according to our chemical inhibition experiment. However, it appears that biotransformation does play a role, as inferred by the lack of or substantially decreased toxicity of structural analogues of VPA (i.e., alpha-F-VPA and octanoic acid) that are compromised in specific biotransformation pathways.

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## REFERENCES

Abbott, F. S., and Anari, M. R. (1999). Chemistry and biotransformation. In *Milestones in Drug Therapy. Valproate* (W. Loscher, Ed.), pp. 47–75. Birkhauser Verlag, Basel, Switzerland.

Bajt, M. L., Knight, T. R., Lemasters, J. J., and Jaeschke, H. (2004). Acetaminophen-induced oxidant stress and cell injury in cultured mouse hepatocytes: protection by *N*-acetylcysteine. *Toxicol. Sci.* **80,** 343–349.

Cervinkova, Z., Krivakova, P., Labajova, A., Rousar, T., Lotkova, H., Kucera, O., Endlicher, R., Cervinka, M., and Drahota, Z. (2009). Mechanisms participating in oxidative damage of isolated rat hepatocytes. *Arch. Toxicol.* **83,** 363–372.

Chang, T. K. H., and Abbott, F. S. (2006). Oxidative stress as a mechanism of valproic acid-associated hepatotoxicity. *Drug Metab. Rev.* **38,** 627–639.

Clarke, H., Egan, D. A., Heffernan, M., Doyle, S., Byrne, C., Kilty, C., and Ryan, M. P. (1997). α-Glutathione S-transferase (α-GST) release, an early indicator of carbon tetrachloride hepatotoxicity in the rat. *Hum. Exp. Toxicol.* **16,** 154–157.

Granneman, G. R., Wang, S., Kesterson, J. W., and Machinist, J. M. (1984). The hepatotoxicity of valproic acid and its metabolites in rats. II. Intermediary and valproic acid metabolism. *Hepatology* **4,** 1153–1158.

Grillo, M. P., Chiellini, G., Tonelli, M., and Benet, L. Z. (2001). Effect of α-fluorination of valproic acid on valproyl-*S*-acyl-CoA formation in vivo in rats. *Drug Metab. Dispos.* **29,** 1210–1215.

Halliwell, B., and Whiteman, M. (2004). Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? *Br. J. Pharmacol.* **142,** 231–255.

Jauregui, H. O., Hayner, N. T., Driscoll, J. L., Williams-Holland, R., Lipsky, M. H., and Galletti, P. M. (1981). Trypan blue dye uptake and

lactate dehydrogenase in adult rat hepatocytes—freshly isolated cells, cell suspensions, and primary monolayer cultures. *In Vitro* **17,** 1100–1110.

Johannessen, C. U., and Johannessen, S. I. (2003). Valproate: past, present and future. *CNS Drug Rev.* **9,** 199–216.

Jurima-Romet, M., Abbott, F. S., Tang, W., Huang, H. S., and Whitehouse, L. W. (1996). Cytotoxicity of unsaturated metabolites of valproic acid and protection by vitamins C and E in glutathione-depleted rat hepatocytes. *Toxicology* **112,** 69–85.

Kassahun, K., Farrell, K., and Abbott, F. S. (1991). Identification and characterization of the glutathione and *N*-acetylcysteine conjugates of (*E*)-2-propyl-2,4-pentadienoic acid, a toxic metabolite of valproic acid, in rats and humans. *Drug Metab. Dispos.* **19,** 525–535.

Kesterson, J. W., Granneman, G. R., and Machinist, J. M. (1984). The hepatotoxicity of valproic acid and its metabolites in rats. I. Toxicologic, biochemical and histopathologic studies. *Hepatology* **4,** 1143–1152.

Kingsley, E., Gray, P., Tolman, K. G., and Tweedale, R. (1983). The toxicity of metabolites of sodium valproate in cultured hepatocytes. *J. Clin. Pharmacol.* **23,** 178–185.

Lee, M. S., Lee, Y. J., Kim, B. J., Shin, K. Y., Chung, B. C., Baek, D. J., and Jung, B. H. (2009). The relationship between glucuronide conjugate levels and hepatotoxicity after oral administration of valproic acid. *Arch. Pharm. Res.* **32,** 1029–1035.

Loscher, W., Nau, H., Wahnschaffe, U., Honack, D., Rundfeldt, C., Wittfoht, W., and Bojic, U. (1993). Effects of valproate and E-2-en-valproate on functional and morphological parameters of rat liver. II. Influence of phenobarbital comedication. *Epilepsy Res.* **15,** 113–131.

Loscher, W., Wahnschaffe, U., Honack, D., Wittfoht, W., and Nau, H. (1992). Effects of valproate and E-2-valproate on functional and morphological parameters of rat liver. I. Biochemical, histopathological and pharmacokinetic studies. *Epilepsy Res.* **13,** 187–198.

Martin, C., Martinez, R., Navarro, R., Ruiz-Sanz, J. I., Lacort, M., and Ruiz-Larrea, M. B. (2001). *tert*-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals. *Biochem. Pharmacol.* **62,** 705–712.

McLaughlin, D. B., Eadie, M. J., Parker-Scott, S. L., Addison, R. S., Henderson, R. D., Hooper, W. D., and Dickinson, R. G. (2000). Valproate metabolism during valproate-associated hepatotoxicity in a surviving adult patient. *Epilepsy Res.* **41,** 259–268.

Nieminen, A. L., Saylor, A. K., Tesfai, S. A., Herman, B., and Lemasters, J. J. (1995). Contribution of the mitochondrial permeability transition to lethal injury after exposure to hepatocyte to *t*-butylhydroperoxide. *Biochem. J.* **307,** 99–106.

Ortiz de Montellano, P. R., and Mathews, J. M. (1981). Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. *Biochem. J.* **195,** 761–764.

Park, B. K., Kitteringham, N. R., Maggs, J. L., Pirmohamed, M., and Williams, D. P. (2005). The role of metabolic activation in drug-induced hepatotoxicity. *Annu. Rev. Pharmacol. Toxicol.* **45,** 177–202.

Prueksaritanont, T., Lin, J. H., and Baillie, T. A. (2006). Complicating factors in safety testing of drug metabolites: kinetic differences between generated and preformed metabolites. *Toxicol. Appl. Pharmacol.* **217,** 143–152.

Rousar, T., Kucera, O., Krivakova, P., Lotkova, H., Kandar, R., Muzakova, V., and Cervinkova, Z. (2009). Evaluation of oxidative status in acetaminophen-treated rat hepatocytes in culture. *Physiol. Res.* **58,** 239–246.

Russmann, S., Kullak-Ublick, G. A., and Grattagliano, I. (2009). Current concepts of mechanisms in drug-induced hepatotoxicity. *Curr. Med. Chem.* **16,** 3041–3053.

Seglen, P. O. (1993). Isolation of hepatocyes by collagenase perfusion. *Methods Toxicol.* **1A,** 231–243.

Senior, J. R. (2007). Drug hepatotoxicity from a regulatory perspective. *Clin. Liver Dis.* **11,** 507–524.

Silva, M. F. B., Aires, C. C. P., Luis, P. B. M., Ruiter, J. P. N., Ijlst, L., Duran, M., Wanders, R. J. A., and Tavares de Almeida, I. (2008). Valproic acid metabolism and its effects on mitochondrial fatty acid oxidation: a review. *J. Inherit. Metab. Dis.* **31,** 205–216.

Swift, B., Pfeifer, N. D., and Brouwer, K. L. R. (2010). Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. *Drug Metab. Rev.* **42,** 446–471.

Takeuchi, T., Sugimoto, T., Nishida, N., and Kobayashi, Y. (1988). Evaluation of the cytotoxicity of sodium valproate on primary cultured rat hepatocytes. *Neuropediatrics* **19,** 158–161.

Tang, W. (2007). Drug metabolite profiling and elucidation of drug-induced hepatotoxicity. *Expert Opin. Drug Metab. Toxicol.* **3,** 407–420.

Tang, W., and Abbott, F. S. (1996). Characterization of thiol-conjugated metabolites of 2-propyl-4-pentenoic acid (4-ene-VPA), a toxic metabolite of valproic acid, by electrospray tandem mass spectrometry. *J. Mass Spectrom.* **31,** 926–936.

Tang, W., Borel, A. G., Fujimiya, T., and Abbott, F. S. (1995). Fluorinated analogues as mechanistic probes in valproic acid hepatotoxicity: hepatic microvesicular steatosis and glutathione status. *Chem. Res. Toxicol.* **8,** 671–682.

Tang, W., Palaty, J., and Abbott, F. S. (1997). Time course of α-fluorinated valproic acid in mouse brain and serum and its effect on synaptosomal γ-aminobutyric acid levels in comparison to valproic acid. *J. Pharmacol. Exp. Ther.* **282,** 1163–1172.

Tong, V., Chang, T. K. H., Chen, J., and Abbott, F. S. (2003). The effect of valproic acid on hepatic and plasma levels of 15-$F_{2t}$-isoprostane in rats. *Free Radic. Biol. Med.* **34,** 1435–1446.

Tong, V., Teng, X. W., Chang, T. K. H., and Abbott, F. S. (2005a). Valproic acid II: effect on oxidative stress, mitochondrial membrane potential, and cytotoxicity in glutathione-depleted rat hepatocytes. *Toxicol. Sci.* **86,** 436–443.

Tong, V., Teng, X. W., Karagiozov, S., Chang, T. K. H., and Abbott, F. S. (2005b). Valproic acid glucuronidation is associated with increases in 15-F2t-isoprostane in rats. *Free Radic. Biol. Med.* **38,** 1471–1483.

Zaccara, G., Franciotta, D., and Perucca, E. (2007). Idiosyncratic adverse reactions to antiepileptic drugs. *Epilepsia* **48,** 1223–1244.

Zheng, J. J. (1993). In *Metabolism and pharmacokinetic studies of valproic acid using stable isotope techniques. MSc Thesis*. The University of British Columbia, Vancouver, Canada.

Zimmerman, H. J., and Ishak, K. G. (1982). Valproate-induced hepatic injury: analyses of 23 fatal cases. *Hepatology* **2,** 591–597.