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Valproic acid derivatives signal for apoptosis and repair *in vitro*

Manuela G. Neuman [a,*], Radu M. Nanau [a], Tawfeeq Shekh-Ahmad [b], Boris Yagen [b], Meir Bialer [b]

[a] *In Vitro Drug Safety & Biotechnology, Department of Pharmacology and Toxicology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada*
[b] *Department of Pharmaceutics, School of Pharmacy, Faculty of Medicine, Ein Karem, The Hebrew University of Jerusalem, Jerusalem, Israel*

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ABSTRACT

Purpose: To determine the cytotoxicity of valproic acid (VPA) and its derivatives in human hepatoblastoma (HepG2) cells, and to study the possible toxicity of these compounds in human lymphocytes from patients with known hypersensitivity syndrome reactions (HSRs) to other medication.

Methods: Cells were exposed to physiological doses of VPA, valnoctamide (VCD) and its one carbon homologue *sec*-Butyl-propyl-acetamide (SPD) for 2 h and for 24 h. Cell viability was measured using succinate dehydrogenase activity for hepatocytes and lymphocyte toxicity assay (LTA) for lymphocytes. Cytokines and apoptosis [cytokeratine 18 (cCK18-M30)] markers were quantitated by ELISA.

Results: VCD and SPD presented lower cytotoxicity compared to VPA in cultured HepG2 cells. SPD led to cytotoxicity in lymphocytes. VPA and its derivatives increased the release of interferon (IFN)-γ and tumor necrosis factor (TNF)-α in media, but had no influence on the release of either interleukin (IL)-1 or IL-6. Significant increases in the release of IFN-γ and TNF-α were observed in lymphocytes exposed to high doses of VPA, and this increased further with exposure time.

Significance: HepG2 cells exposed to VCD and SPD experienced lower direct cytotoxicity than those treated with VPA. Lymphocytes from patients that experienced HSR to other medication have shown cytotoxicity to VPA and its VPA derivatives-induced. High levels of pro-inflammatory cytokines were released in the cell culture media, suggesting that inflammation plays a key role in VPA-derivatives induced lymphocyte toxicity.

Introduction

Valproic acid (VPA) (2-n-propylpentanoic acid) is a drug traditionally used for the treatment of seizures, and in psychiatric care for the treatment of mania in bipolar patients [1,2]. More recently, it has shown anti-tumorigenic properties in various cancers [3], neuroprotective potential in Alzheimer's patients [4], as well as varying results in schizophrenia [5]. Since its approval in France in 1967, VPA became one of the most widely used anticonvulsants in the world, with a relatively good safety profile and low cost [6–10]. A recent pediatric randomized controlled trial showed that intravenous VPA was associated with fewer adverse drug reactions (ADR) than phenobarbital [11]. Similar results were observed in a separate trial comparing intravenous VPA with diazepam in an adult population [12]. Intravenous VPA and intravenous phenytoin had similar efficacy and safety profiles in a randomized study of 100 SE patients [13]. Moreover, VPA was used also when infection was identified as the primary etiology for status epilepticus (SE) in several studies [14–16]. Intravenous VPA was devoid of ADRs in another study [17].

Immune-mediated drug hypersensitivity reactions (IDHRs) are uncommon adverse events, which are restricted to a subset of vulnerable patients and pharmaceutical products including VPA [18]. Nevertheless, such reactions, and the fear of them as serious and potentially life-threatening consequences of therapy, have a huge impact on clinical practice and the public health. ADRs can limit the use of VPA and its derivatives. Based on a recent systematic review, the most common ADRs of VPA include hepatotoxicity, mitochondrial toxicity, hyperammonemic encephalopathy, hypersensitivity syndrome reactions (HSRs), neurological manifestations, metabolic and endocrine adverse events, and teratogenicity [18]. From the morphological point of view, VPA hepatotoxicity in humans presents microvesicular steatosis, hepatocellular necrosis, and cholestatic lesions. Biochemically hepatotoxicity is manifesting by elevated aminotransferase levels in the presence or absence of bilirubinemia [19–21].

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) [21]. *In vitro*, in human hepatocytes, Neuman et al. had previously demonstrated that VPA metabolites are more toxic than the parent compound, especially

Abbreviations: ADR, adverse drug reaction; CRP, C reactive protein; HSR, hypersensitivity syndrome reaction; IFN, interferon; IL, interleukin; SPD, *sec*-Butyl-propylacetamide; TNF, tumor necrosis factor; VCD, valnoctamide; VPA, valproic acid; SD, standard deviation; SE, status epilepticus.

* Corresponding author at: *In Vitro* Drug Safety & Biotechnology, Department of Pharmacology and Toxicology, Faculty of Medicine, University of Toronto, MaRS Discovery District, 101 College Street, Suite 300, Lab 351, South Tower, Toronto, Ontario M5G 1L7, Canada. Fax: +1 416 6738181.
E-mail address: manuela.neuman@utoronto.ca (M.G. Neuman).

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in instances when cytochrome p450 drug metabolizing enzymes were induced [21].

VPA toxicity is manifested in two different ways: a direct toxicity, which is dose- and frequency-dependent, often asymptomatic, and resolves with dose adjustments or treatment [22], and indirect toxicity involving metabolic idiosyncrasy (IDHR) [18,20,22]. Immunogenetic sensitivity and congenital metabolic errors constitute risk factors for VPA toxicity. Among anticonvulsants, VPA is considered to possess the highest ability to induce mitochondrial toxicity [23–25].

The main treatment-limiting ADRs of VPA are hepatotoxicity and teratogenicity [26]. For these reasons, new derivatives of VPA are synthesized [27]. The chemical structures of these compounds are shown in Fig. 1.

Valnoctamide (valmethamide or 2-ethyl-3-methyl pentanamide, VCD) is a drug that has been available clinically for many decades and is still used as a mild tranquilizer. Valnoctamide has superior anticonvulsant effects to VPA without the risk of teratogenicity. This positive feature is due to the absence of the carboxylic acid moiety and the branching of the parent compound, which have been shown to be the molecular features of VPA that modulate this ADR [2,21]. However, VCD is an inhibitor of the enzyme epoxide hydrolase. Therefore, there is a possibility that its pharmacokinetics and pharmacodynamics, including toxicologic potential and epoxide hydrolase inhibition, are stereoselective.

sec-Butyl-propylacetamide (SPD), the one carbon homologue of VCD, showed anticonvulsant activity in rodent seizure and epilepsy models, including rat and guinea pig models of SE, while it had neuroprotective properties in an organotypic hippocampal slice model of toxic cell death [27].

Using VPA and its derivatives we aimed to assess: 1) the direct VPA-toxicity in the human hepatocellular carcinoma cell line HepG2 and 2) the possible sensitivity of normal human lymphocytes taken from individuals with different immune and genetic backgrounds to the same drugs. For this experiment we used the same doses we employed in our previous experiment with VPA and VPA metabolites [21]. In addition, we wanted to compare the toxicity of parent compounds and reactive drug metabolites. We tested the hypothesis that the possible cytotoxicity is a combination of the direct effects of VPA, as well as being indirectly mediated by elevated levels of pro-inflammatory cytokines. Therefore, in addition to cytotoxicity, we also measured the levels of interleukins (IL)-1 and IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and C reactive protein (CRP) released into the cell culture media by hepatocytes and lymphocytes treated with VPA derivatives.

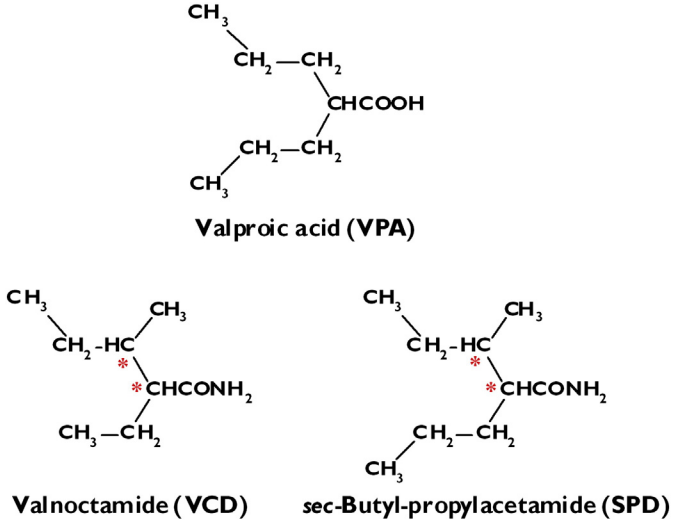

Fig. 1. Chemical structures of valproic acid (VPA), valnoctamide (VCD) and *sec*-Butyl-propylacetamide (SPD). An asterisk shows a chiral center.

In the second set of experiments we employed lymphocytes of patients known to experience a clinical HSR. Since HSRs are dose- and frequency-independent but host-dependent, the dose used to induce the toxicity is less important. However we employed the dose that was reported in blood taken from healthy volunteers and from epilepsy patients that were given the drugs in clinical trials.

Materials and methods

Drugs and chemicals

Plain essential medium (α-MEM), Hanks' balanced sodium solution and calcium chloride were obtained from Gibco (Burlington, ON, Canada). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diophenyl tetrazolium bromide] tetrazolium salt used for the cytoviability assays, Glucose-6-phosphate dehydrogenase enzyme and HEPES buffer were obtained from Sigma Chemical Company (St. Louis, MO, USA). Ficoll [density 1007 g/mL at +20 °C (Ficoll-Paque™ Plus), sterile, endotoxin tested (<0.12 EU/mL)] was obtained from GE Healthcare Bio-Sources AB (Uppsala, Sweden). Dimethylsulfoxide (DMSO) was obtained from Fisher Scientific (Toronto, ON, Canada). Phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} was used to wash the cells. M. Bialer provided VPA and its derivatives. SPD was synthesized according to the method described by Kaufmann et al. [26]. All plastic ware for cell cultures was purchased from Falcon (Becton Dickinson, Oxnard, CA). All of the remaining reagents were of analytical grade, obtained from Sigma Chemical Company (St. Louis, MO, USA).

HepG2 cells

Morphological and functional characteristics of the HepG2 cells and the methodology used in this experiment were previously described [21]. HepG2 cells were shown to retain morphological features of hepatocytes by light and electron microscopy. Demonstrating glucose-6-phosphatase activity, transferrin and albumin secretion, as well as small but sustained inductility of 7-ethoxycoumarin O-diethylase (CYP2B1) activity and p-nitroso-dimethyl-aminine dimethylase (CYP2E1) activity proved the functionality of the parenchymal cells [28].

Cells were seeded in 96-well plates directly to a density of 1×10^6 cells/mL. The cell counts were monitored using a Coulter counter (Coulter Electronics Inc., Hialeah, FL). Cells in long-term cultures were grown in α-MEM supplemented with 10% vol/vol fetal bovine serum. For the cytotoxicity experiments, plated cells were incubated with 50 μg/mL and 100 μg/mL VPA. The entire procedure was conducted under aseptic conditions. Cells were maintained in a humidified environment, under conditions of 37 °C, with 95% air and 5% CO_2. For each treatment, six wells per plate in 2 different plates were quantitated. The results were reported as percent toxicity or apoptosis *versus* control, with non-treated cells taken as 0% toxicity. The standard curve was comprised of six replicates from each of the two plates. The sensitivity of the assay was determined by assaying non-treated cells at time zero, then calculating the mean signal and standard deviation (SD). Details of the individual experiments are provided in the figure legends.

After 24 h incubation with VPA or its derivatives, media was removed and was stored at −80 °C until cytokine analysis was performed. One hundred microliters of MTT solution at a concentration of 5 mg/mL was added to each well. Following a 1 h incubation period, 100 μL isopropanol was used to stop the reaction. Cell viability was assessed spectrophotometrically, using a PowerWave$_X$ microplate reader (Bio-Tek Instruments Inc., Winooski, VT). The plate reader was connected to a computer using SOFTMAX software 2.3 for Windows (Molecular Devices Corporation, Menlo Park, CA). This allowed us to template the plate according to

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