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Amidic derivatives of valproic acid, valpromide and valnoctamide, inhibit HSV-1 infection in oligodendrocytes

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is a ubiquitous infectious agent that can establish latency in neurons, and in some cases, viral retrograde transport results in infection of the central nervous system (CNS). Several antivirals have been identified with the ability to inhibit HSV-1 replication in human cells to a greater or lesser degree, most of which are nucleoside analogues that unfortunately exhibit teratogenic potential, embryotoxicity, carcinogenic or antiproliferative activities and resistances in immunocompromised patients, specially. In the present study, we assessed two amidic derivatives of valproic acid (VPA) – valpromide (VPD) and valnoctamide (VCD) – which are already used in clinic treatments, as feasible HSV-1 antivirals in glial cells. Both VPD and VCD have exhibited increased efficacy in bipolar disorders and as anticonvulsant drugs compared to VPA, while being less teratogenic and hepatotoxic. Cytotoxicity assays carried out in our laboratory showed that VPD and VCD were not toxic in a human oligodendroglioma cell line (HOG), at least at the concentrations established for human treatments. Infectivity assays showed a significant inhibition of HSV-1 infection in HOG cells after VPD and VCD treatment, being more pronounced in VPD-treated cells, comparable to the effects obtained with acyclovir. Furthermore, the same antiherpetic effects of VPD were observed in other oligodendrocytic cell lines and rat primary oligodendrocytes (OPCs), confirming the results obtained in HOG cells. Altogether, our results allow us to propose VPD as a potential antiherpetic drug that is able to act directly on oligodendrocytes of the CNS.

1. Introduction

Herpes simplex virus type 1 (HSV-1) is a ubiquitous infectious agent that can establish latency in the peripheral nervous system (PNS), where the majority of individuals do not develop any symptoms (Finger-Jardim et al., 2017). HSV-1 seroprevalence in the trigeminal ganglion of the PNS affects around 90% of adults in industrialized countries (Wutzler et al., 2000). The most common symptoms are the occasional appearance (in an oral herpes infection) of painful blisters or open ulcers in/around the mouth known as cold sores (World Health Organization). Retrograde virion transport can result in infection of the central nervous system (CNS) (Whitley, 2006), which can cause diseases with high rates of mortality including encephalitis, although in some cases, it can initiate latent infection (Whitley, 2015). In this context, it is feasible that HSV-1 plays some role in the development of certain chronic neurodegenerative and demyelinating diseases, such as Alzheimer's or Multiple Sclerosis (MS) (Hogestyn et al., 2018; Lycke, 2017), so a safe, effective antiviral therapy may prove useful for maintenance of a healthy CNS.

Several antivirals have been identified with the ability to inhibit the replication of HSV-1 in human cells, and some of them, such as valproic acid (VPA), are still under study (Frouco et al., 2017). VPA is a branched short-chain fatty acid (Shekh-Ahmad et al., 2014) that has been used as an anticonvulsant or for the clinical treatment of bipolar disorders. In fact, it is prescribed as an effective anticonvulsant alternative to lithium therapy, approved by the FDA since 1995 (Lopez-Munoz et al., 2018). Various mechanisms of action for VPA have been proposed, such as alteration of lipid metabolism, modulation of gamma-aminobutyric acid (GABA) (Terbach and Williams, 2009), and glutamatergic pathway alteration, as well as inhibition of voltage-gated Na^+ and T-type Ca^{2+} channels in cellular membranes, glycogen synthase kinase 3 (GSK3), protein kinase A (PKA) (Brown et al., 1985), and histone deacetylases (HDACs). As a matter of fact, due to this inhibitory effect, VPA is clinically prescribed for some neurodegenerative diseases

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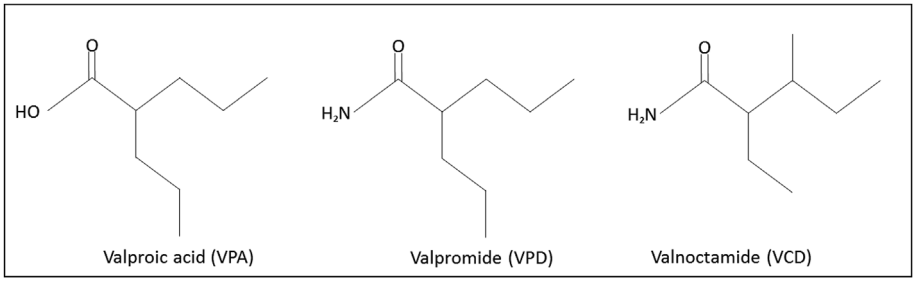

Fig. 1. Structure of valproic acid and its amidic derivatives. Valpromide (VPD) and valnoctamide (VCD) are amide derivatives of VPA with differences in the acidic core.

(Terbach and Williams, 2009), or as an adjuvant in cancer or HIV therapy (Ghodke-Puranik et al., 2013). Nevertheless, different studies have described hepatotoxic and teratogenic properties of this drug. The hepatotoxicity seems to be associated with the parental compound valproate (Isoherranen et al., 2003), specifically attributed to the presence of metabolites carrying a terminal double bond. These valproate metabolites might form an intermediate thioester with the acyl-coenzyme A, decreasing the free form of this molecule in liver, thus triggering hepatotoxicity (Kudin et al., 2017). For teratogenic effects, three structural requirements seem to be necessary: (1) a carboxylic acid in their structure; (2) carbon branching at C-2 with two lateral chains containing each at least three carbon atoms; and (3) a hydrogen atom at C-2 (Luis et al., 2007).

Valpromide (VPD) and valnoctamide (VCD) are amide derivatives of VPA and differences in their acidic core molecule (Fig. 1) cause a lack of some of the toxic and teratogenic effects mentioned above.

In humans, VPD acts as a prodrug, which acquires its active acidic form through metabolic hydrolysis (Isoherranen et al., 2003; Kudin et al., 2017). After administration, VPD undergoes a rapid biotransformation, resulting in a shorter half-life compared to VPA; for example, only one percent of VPD is detected in plasma after oral administration (Bialer, 1991). Nowadays, this VPA derivative is prescribed in primary generalized epilepsies: in convulsive, non-convulsive and myoclonic seizure, bipolar disorders, manic episodes, and when lithium is contraindicated or not-tolerated (Vademecum, 2018). In fact, VPD shows a more potent clinical effect as an anticonvulsant, up to three times more potent than VPA (Bialer, 1991), and crosses the blood-brain barrier easily. In addition, once in the CNS, VPD exhibits, to a certain degree, additional activity as an antipsychotic (Fond et al., 2018). However, VPD is contraindicated in women of childbearing age and in pregnant women when alternative anticonvulsant therapies are available, due to its ability to cross the placenta after transformation into valnoctamic acid (Tomson et al., 2016).

VCD is a constitutional isomer of VPD (Fig. 1), and several mouse studies have shown an absence of the teratogenicity associated with VPA. Moreover, used in different disorders, VCD seems to be more potent than VPA (Isoherranen et al., 2003). As with VPA, the clinical differences between VPD and VCD might also be due to different pharmacokinetics: VCD does not act as prodrug, and is not metabolized to VPA, and thus exhibits different pharmacological activity (Bialer, 1991). VCD was prescribed in the past as a hypnotic sedative in France, commercialized as Nirvanil®. At present, VCD is involved in some phase III clinical trials, and is also being studied as a possible treatment for bipolar disorders (Modi et al., 2014).

In this study, amide derivatives of VPA were tested for their ability to prevent replication of HSV-1 *in vitro*. VPD and VCD were shown to be non-cytotoxic at the concentrations established for human treatments and the antiviral effects of both were characterized in HSV-1-infected human oligodendroglioma (HOG) cells. The activity of VPD was further confirmed in rat oligodendrocyte precursor cells (OPCs), showing it to be a potential antiherpetic drug. In case of confirming HSV-1 as involved agent for MS, VPD would be included in the therapeutic arsenal for the disease.

2. Results

2.1. VPD and VCD are non-toxic in HOG cells at clinically established concentrations

To study the toxicity of VPD and VCD, HOG cells were cultured for 24 h in serum-free differentiation medium (DM) in the presence of different concentrations of each VPA derivative. Cell viability was higher in VPD-treated cells compared to VCD-treated ones (Fig. 2). HOG cells treated with 0.5 mM VPD had greater than 80% viability, whereas cells treated with 0.5 mM VCD in the same conditions had approximately 50% viability ($p < 0.05$). These highest non-toxic concentrations of the VPA derivatives were used in the rest of the assays carried out in this study.

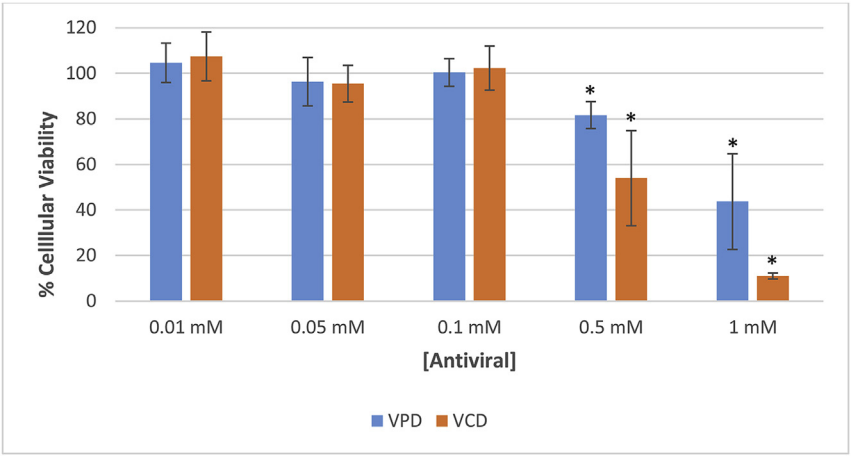

Fig. 2. Viability of HOG cells exposed to VPD and VCD. Human oligodendroglioma cells (HOG) cultured in differentiation medium (DM), were incubated with different concentrations beteween 0.01 mM and 1 mM of VPD or VCD for 24 h. Cell viability was measured by MTT tetrazolium salt assay, and calculated as the percentage of viability compared to the untreated cells; columns represent the mean viability ± S.D. ($n = 4$), after exposure to the drugs; *$p < 0.05$.

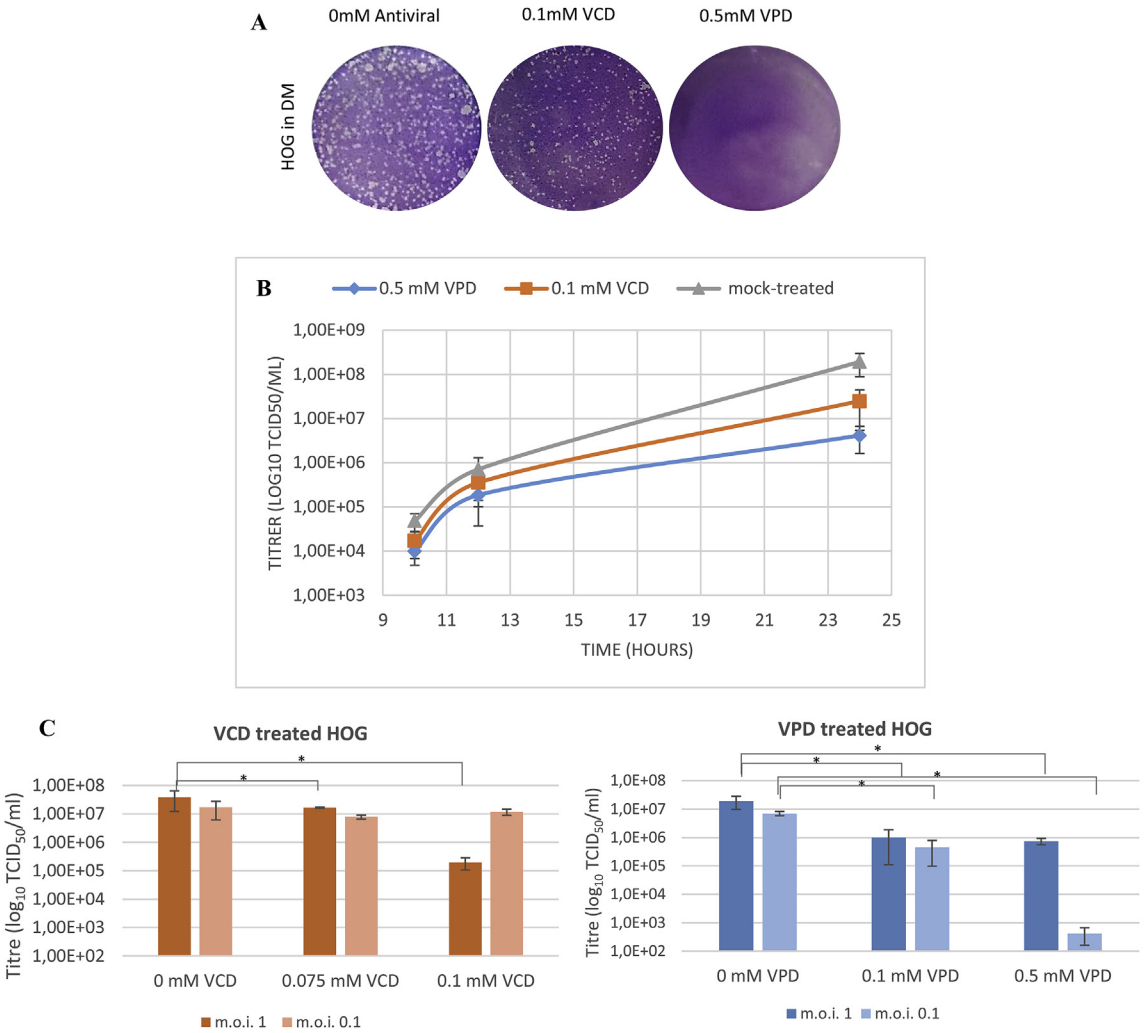

Fig. 3. Effect of VPD and VCD on HSV-1 infection of HOG cells. (A) Plaque assay of human oligodendroglioma cells (HOG) cultured in differentiation medium (DM), treated or mock-treated with VCD (0.1 mM) or VPD (0.5 mM) for 24 h, and infected with HSV-1. **(B)** HOG cells cultured in DM were pre-treated with VPD (0.5 mM) or VCD (0.1 mM) for 30 min and infected with HSV-1 at an m.o.i. of 1, then HOG cells were exposed to these concentrations of VPD and VCD for 10, 12 and 24 h. Viral titres ($TCID_{50}$/ml) were determined at 10, 12 and 24 h post-infection (h p.i.); values are reported as the mean ± S.D. (n = 3). **(C)** Quantification of viral production at 16 h p.i. in HOG cells treated or mock-treated with different VPD (0.1 mM and 0.5 mM) and VCD (0.075 mM and 0.1 mM) concentrations; values are reported as the mean ± S.D. (n = 4), as compared to controls (*p < 0.05).

2.2. Effect of VPD and VCD on HSV-1 infection of HOG cells

To study the effect of VPD and VCD on HSV-1 infection, HOG cells cultured under differentiation conditions were plated in a 6-well tissue culture dish and infected or mock-infected with HSV-1. Before viral infection, HOG cells were treated or mock-treated with VPD (0.5 mM) or VCD (0.1 mM) for 30 min at 37 °C. There was a drastic reduction in the number of lysis plaques in VPD-treated cells compared to untreated controls, almost completely inhibiting the infection. Whereas VCD treatment had a much smaller effect on HSV-1 infection (Fig. 3A), with a decrease of lysis by 50%.

Subsequently, the kinetic of infection was assessed in HOG cells after 24 h treatment with VPD (0.5 mM) or VCD (0.1 mM), and the infection with HSV-1 at an m.o.i. of 1. Virus production at 10, 12 and 24 h post-infection (h p.i.) was determined by endpoint dilution assay, showing a decrease in viral production in cells treated with both drugs (Fig. 3B). The decrease in viral production was observed as early as 10 h p.i. and was still noticeable at 24 h p.i. The number of infectious viral particles in VCD and VPD-treated cells decrease by 1 and 2 orders of magnitude ($p < 0.05$ at 24 h p.i.), respectively, compared to the mock-treated cells (Fig. 3B).

HSV-1 production was also determined at 16 h p.i. in HOG cells treated with different concentrations of VCD and VPD (Fig. 3C). Virus yield was significantly lower ($p < 0.05$) in cells treated with VPD at 0.1 or 0.5 mM and VCD at 0.075 or 0.1 mM. Nevertheless, VPD, once again, exerted a greater antiviral effect than VCD; in fact, the infection was drastically inhibited, with a reduction of four orders of magnitude observed in cells treated with VPD (Fig. 3C). The data on viral production correspond to intracellular virus from cell lysate.

2.3. Effect of VPD on HSV-1 infection of oligodendrocytic KG-1C and oli-neu cells

To assess whether the antiviral effect of VPD in oligodendrocytes was similar in other cell types, human KG-1C and murine Oli-neu cells were treated with these antivirals and infected with HSV-1. Oligodendrocytic cultures were treated with 0.5 mM of VPD and infected with K26-GFP virus. GFP was analyzed by flow cytometry as described in material and methods.

Flow cytometry assay showed a significant decrease of GFP signal (K26-GFP) on treated cells (n = 3) compared to mock-treated cells. The infection decreased by 77% in Oli-neu cells and 75% in KG-1C cells

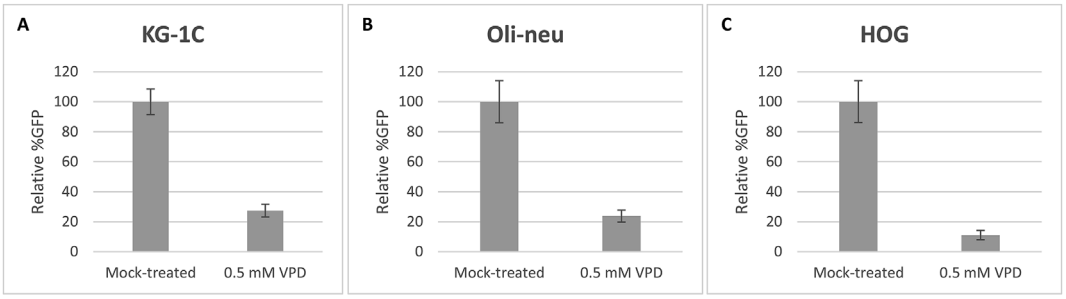

Fig. 4. Effect of VPD on HSV-1 infection. Flow cytometry analysis of HSV-1 K26-GFP-infected – KG-1C **(A)**, Oli-neu **(B)** and HOG **(C)** cells at an m.o.i. of 1, treated or mock-treated with VPD 0.5 mM. Grey bars show the relative infection percentages of cells treated with VPD compared to mock-treated controls (n = 3).

(Fig. 4).

2.4. Comparative effect of acyclovir and VPD on infected HOG cells

To compare the antiherpetic effect of VPD with other well-characterized antiherpetics, HOG cells were treated or mock-treated with VPD and the nucleoside analogue acyclovir (ACV) and infected with K26-GFP virus. The infection of cultures was drastically reduced after treatment with VPD and ACV, alone or combined (Fig. 5). In mock-treated cells, percent of K26-GFP infected cells was about 50%. This percentage decreased to 5% and 1% in VPD or ACV treated cultures, respectively.

2.5. Effect of VPD on rat oligodendrocyte precursor cells (OPCs) infected with HSV-1

To assess whether the results obtained in HOG cells could be extended to primary cultures, we analyzed the effect of VPD in rat oligodendrocyte precursor cells (OPCs). For this aim, we isolated OPCs from postnatal rat cerebral cortex by modifying a previous protocol (Medina-Rodriguez et al., 2013) to use labeled-specific antibodies coupled to magnetic beads (MACS, Miltenyi Biotech). After purification of OPCs, cytotoxicity assay was performed on cultures treated with 0.5 and 0.1 mM of VPD. No cytotoxicity was observed in OPCs exposed to the drug used at these concentrations (Fig. 6A).

Later, OPCs were seeded on round glass coverslips in a 24-well plate, treated with 0.5 mM VPD (with a pre-treatment of 30 min), and infected with HSV-1 K26-GFP at an m.o.i. of 2. At 16 h p.i., cells were washed, fixed and processed for immunofluorescence assay using an antibody against human Olig2, a transcription factor used as an oligodendrocyte lineage marker. Immunofluorescence microscopy images showed a decrease in infected (GFP-positive) OPCs that were treated with VPD, compared to mock-treated ones (Fig. 6C). Quantification of double-staining ($GFP^+/Olig2^+$) in mock-treated cells revealed 50% of OPCs were infected by HSV-1, compared with a 12% infection rate in VPD-treated OPCs (Fig. 6C). Three fields of view corresponding to both treated and mock-treated points of the experiment were analyzed (between 200 and 250 cells per field).

Finally, we studied the effect of VPD on HSV-1 production in infected OPCs. At 16 h p.i., OPCs treated with VPD showed a decrease in viral production of around 1 order of magnitude, compared to mock-treated cells (Fig. 6B).

2.6. Effect of VPD on virus entry

To study the effect of VPD on virus entry, we infected HOG cells with R120vGF virus (Bello-Morales et al., 2014), an EGFP expressing recombinant HSV lacking ICP4. After entry into cells, R120vGF expresses EGFP and immediate early proteins, but is unable to complete the viral cycle due to the absence of ICP4. HOG cells were grown in DM. Subsequently, cultures were infected with R120vGF virus at an m.o.i. of 5 and analyzed at 16 h p.i.

Cytometry data showed a decrease of GFP (16% vs 9%) in VPD treated HOG cells infected with R120vGF virus compared to mock-treated cultures (n = 4) (Fig. 7A and B). Furthermore, the effect of VPD on viral expression of ICP4, polymerase and gC genes was analyzed by RTq-PCR. All three analyzed viral mRNAs: ICP4 (an immediately early gene), viral polymerase (early gene) and gC (late gene) decreased in treated HOG cells compared to mock-treated ones (Fig. 7C). VPD was maintained throughout the infection.

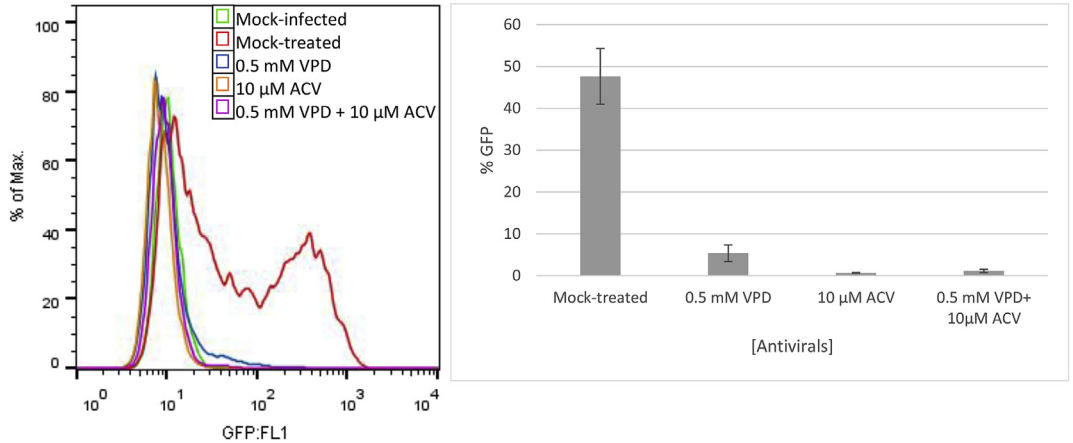

Fig. 5. Antiviral effect of ACV and VPD on infected HOG cells. Cytometry assay of HOG cells infected with HSV-1 K26-GFP at an m.o.i. of 1, treated or mock-treated with VPD and ACV. Grey bars represent the percentage of GFP-signal reported as the mean ± S.D. (n = 3).

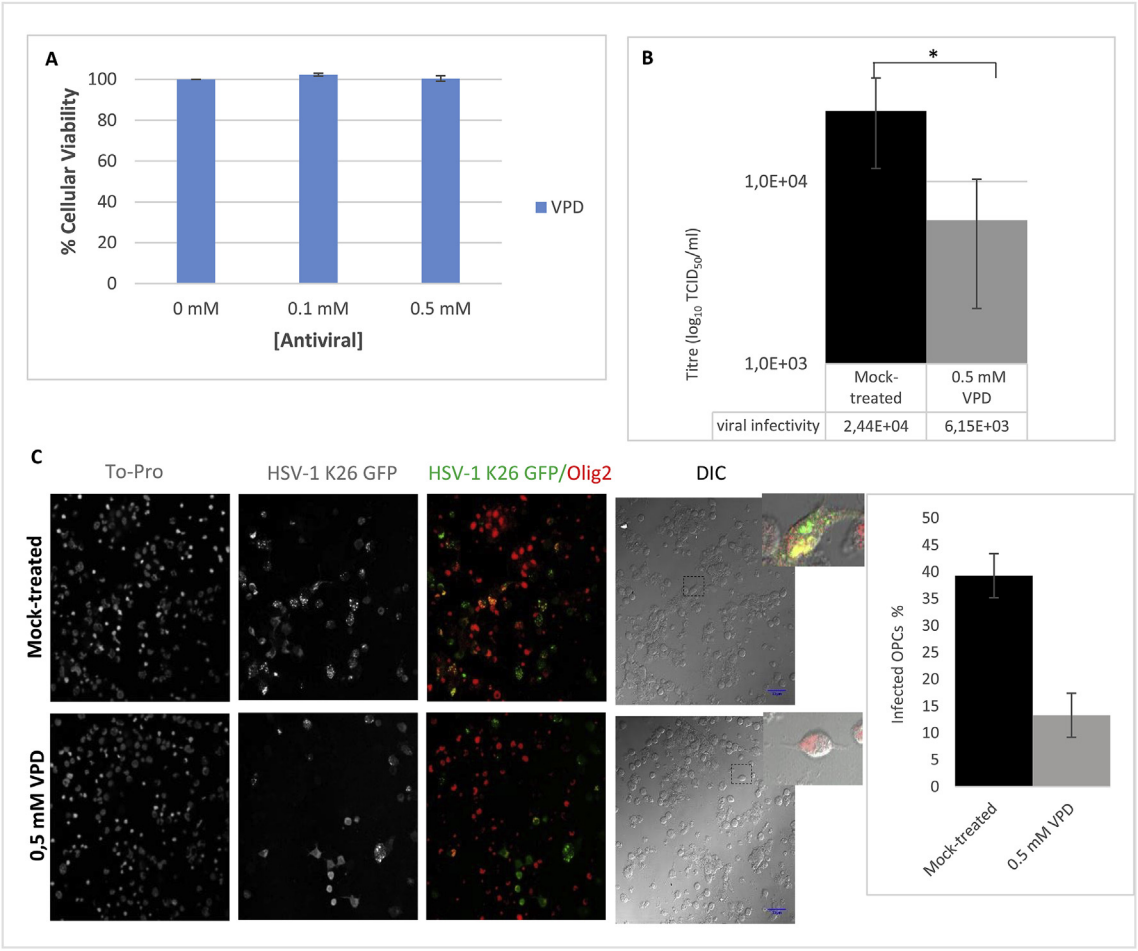

Fig. 6. Effect of VPD in rat oligodendrocyte primary cells (OPCs). (A) OPCs cultured in differentiation Neurobasal Medium were incubated with 0.1 or 0.5 mM of VPD for 24 h. Cell viability was measured by MTT tetrazolium salt assay and calculated as the percentage of viability compared to mock-treated cells. Bars represent the mean viability ± S.D. (n = 4), after exposure to the drug; **(B)** OPCs treated or mock-treated with 0.5 mM VPD were infected with HSV-1 at an m.o.i. of 1, and viral titres ($TCID_{50}$/ml) were determined at 16 h p.i. Grey bar shows the viral yield in treated HOG cells, compared with untreated cells (black bar). Values are reported as the mean ± S.D. (n = 3; *p < 0.05). **(C)** OPCs cultured in Neurobasal Medium were pre-treated with 0.5 mM of VPD for 30 min and infected with HSV-1 K26GFP at an m.o.i. of 2. VPD was maintained and at 16 h post-infection (h p.i.) stained with an Olig2 antibody. **(D)** Yellow signal reveals colocalization of OPCs (red) with HSV-1 infection (green).

3. Materials and methods

3.1. Cell cultures

The HOG cell line was established from a surgically removed human oligodendroglioma (Post and Dawson, 1992), and kindly provided by Dr. A. T. Campagnoni (University of California, Los Angeles, USA). Cells were cultured in growth medium (GM) containing low-glucose DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 µg/ml) at 37 °C in a humidified atmosphere of 5% CO_2. To induce differentiation, cells were cultured in serum-free differentiation medium (DM), containing low-glucose DMEM supplemented with penicillin (50 U/ml), and streptomycin (50 µg/ml) and 50 µg/ml apo-transferrin (T203), 0.5 mg/l insulin (I9278), 30 nM triiodothyronine (T3), 30 nM sodium selenite and 16.1 mg/l putrescine (P5780). Cells cultured in this medium were also treated with 0.5 mM dbcAMP (D0627) and IBMX at a final concentration of 0.5 mM (reagents from Sigma Chemical Co.).

The oligodendrocytic line Oli-neu (Jung et al., 1995) is a murine oligodendrocyte precursor cell line kindly provided by Dr. Hugo Cabedo (Universidad Miguel Hernández, Murcia) with the permission of Prof. J. Trotter (University of Heidelberg, Germany). The cell line KG-1C is a human immature cell line consisting of undifferentiated glial cells (Bello-Morales et al., 2005). These cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS) in a 5% CO2 atmosphere at 37 °C.

3.2. Primary cultures

OPCs were obtained from postnatal P7 rats. Murines were euthanised and carefully dissected in accordance with Spanish (RD233/88) and European (2010/63/EU) regulations. Brain cortices were dissociated triturated and mixed with different dissociation solutions (Medina-Rodriguez et al., 2017) with DNAse on a heater shaker (Dincman et al., 2012). The suspension was slowly decanted through a strainer, then the collected cells were incubated with an O4 hybridoma antibody and rat anti-mouse O4 IgM MicroBeads (MACs Antibody, Miltenyi Biotec). The OPCs were purified by magnetic cell sorting (MACS® Column, Miltenyi Biotec), and cultured for 24 h in MACs Neurobasal Medium (Gibco) supplemented with 2 mM L-glutamine containing 25 ng/ml human growth factor, 25 ng/ml recombinant human platelet derived growth factor-AA (PDGF-AA) and 0.5% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

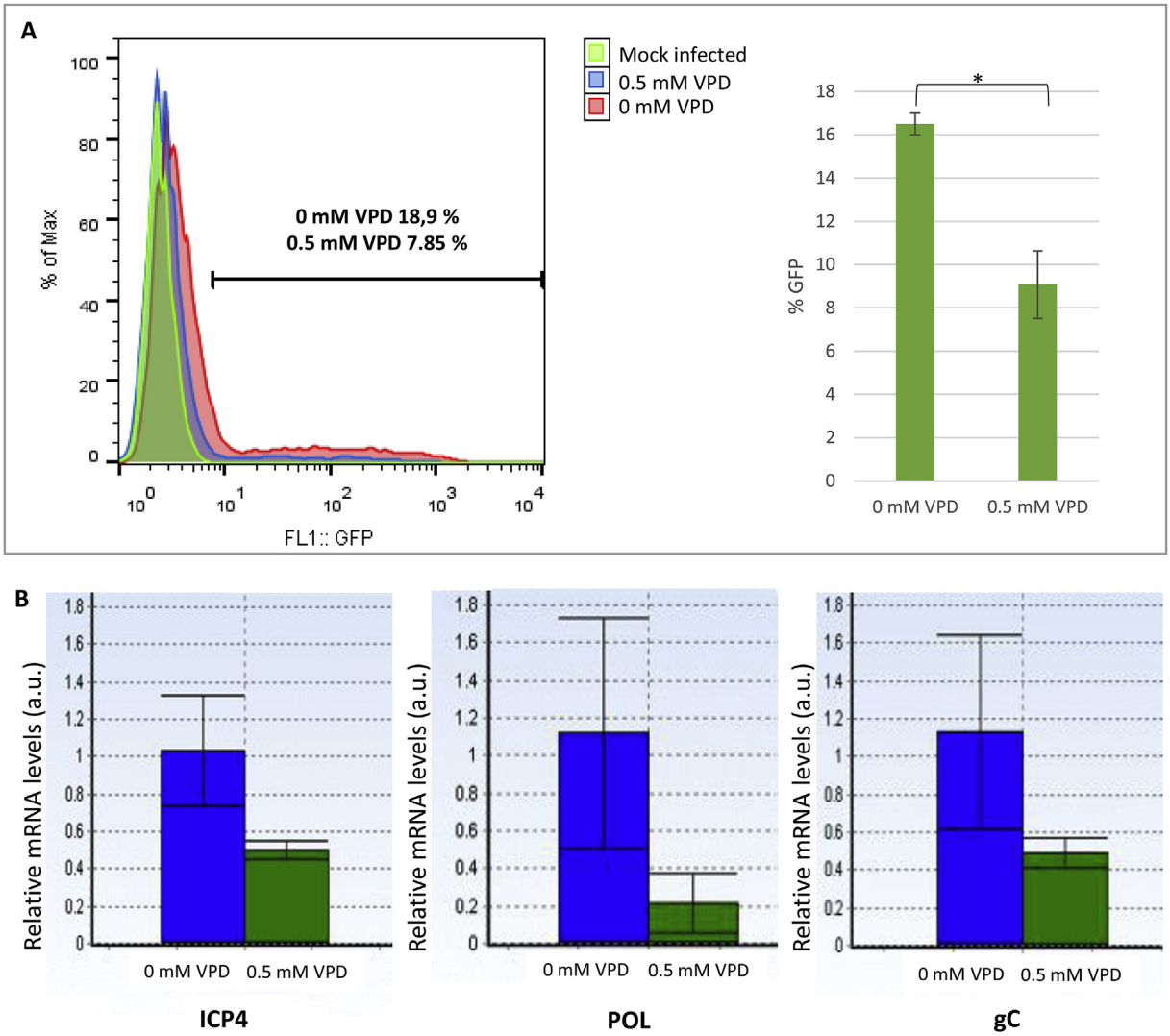

Fig. 7. Effect of VPD on viral entry and gene expression. (A) Flow cytometry analysis of R120vGF virus-infected HOG cells at an m.o.i. of 5, treated or mock-treated with 0.5 mM of VPD. Green bars show the percentage of GFP-signal. Values are reported as the mean ± S.D. ($n = 4$; *$p < 0.05$). **(B)** RT-qPCR of mRNAs. Bars represent relative mRNA levels corresponding to ICP4 (extracted at 3 h p.i.), polymerase (extracted at 6 h p.i.) and glycoprotein C (extracted at 8.5 h p.i.) from HSV-1-infected HOG cells –treated or mock-treated with VPD. (a.u., arbitrary units).

3.3. Viruses

Wild type HSV-1 (F strain) (GenBank accession number for the DNA genome sequence is GU734771). HSV-1 K26-GFP was obtained by fusing GFP to the HSV-1 capsid protein VP26 (Desai and Person, 1998), and was a kind gift from Dr. Desai (Johns Hopkins University, Baltimore, USA). K26-GFP and wild type HSV-1 viruses were propagated and titrated on Vero cells. The R120vGF, an EGFP-expressing recombinant virus, was propagated in E5 Vero cells expressing the HSV-1 ICP4 protein (Bello-Morales et al., 2014; DeLuca et al., 1985).

3.4. Antibodies and reagents

VPD (V3640), VCD (V4765), ACV (PHR1254), low-glucose DMEM and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Olig 2 was from Merck (AB9610) and Alexa Fluor 555-conjugated secondary antibodies were from Molecular Probes (Eugene, OR, USA).

Mowiol was from Calbiochem (Merck Chemicals, Germany).

3.5. Cell viability assay

The cytotoxic effects of VPD and VCD in OPCs and HOG cells were analyzed by the MTT method (Roche-11465007001). Non-confluent monolayers of OPCs and HOG cells plated in 96-well tissue culture dishes and cultured in Neurobasal Medium and GM were incubated for 24 h with different concentrations between 0.01 mM and 1 mM of VPD and VCD. Then, cells were incubated with a final concentration of 0.5 mg/ml of MTT in a humidified atmosphere for 4 h, at which point formazan crystals were solubilized in 10% SDS in 0.01 M HCl. The resulting coloured solution was quantified using a scanning multiwell spectrophotometer (ELISA reader), measuring the absorbance of formazan at 595 nm.

3.6. Viral infections

For plaque assays, confluent monolayers of HOG cells cultured in DM were plated in 6-well tissue culture dishes and exposed for 30 min to VPD (0.5 mM) or VCD (0.1 mM), and infected with serial dilutions of HSV-1. After viral adsorption, cells were washed and overlaid with carboxymethylcellulose (CMC) in the presence of VPD (0.5 mM) or VCD (0.1 mM). The CMC solution was prepared in distilled water at 2% (w/

v) and stirred at room temperature for 1 h, and the CMC overlay (1% final concentration) was prepared by mixing equal volumes of 2% CMC and 2x DM. Two ml of CMC overlay containing VPA amide derivatives was added to each well, and plates were incubated for 48 h at 37 °C in a humidified 5% CO_2 incubator. Subsequently, the CMC overlay was aspirated and cells were washed with PBS, and plaques were visualized by staining with crystal violet.

For quantification of viral infection, 6×10^5 HOG cells/well were grown in 6-well tissue culture dishes for 24 h in GM, then rinsed and cultured in DM for 24 h, and infected with the corresponding virus. Viral titres were quantified in Vero cells by endpoint dilution assay to determine the 50% tissue culture infective dose ($TCID_{50}$), calculated after observation of cytopathic effect using the method of Reed and Muench.

For viral quantification in OPCs, 4.2×10^4 cells/well were plated in 96-well tissue culture dishes in Neurobasal Medium for 24 h, then mock-infected or infected as described for HOG cells.

To determine the kinetics of virus production, first HOG cells cultured in DM were exposed to VPD or VCD for 30 min pre-treatment and infected, then HOG cells were exposed for 24 h to VPD and VCD. Cells treated and mock-treated with the VPA derivatives were infected with wild type HSV-1 at an m.o.i. of 1, and viral titres at 10, 12 and 24 h p.i. were determined in Vero cells.

3.7. Immunofluorescence microscopy

OPCs were plated on round coverslips pre-coated with laminin (1.5×10^5 cells/well). Cells were infected with HSV-1 K26-GFP at an m.o.i. of 2 for 16 h and treated or mock-treated with 0.5 mM VPD, then fixed in 4% paraformaldehyde for 20 min and rinsed with PBS. The cells were then permeabilized with 0.2% Triton X-100, rinsed with PBS, and incubated with 3% bovine serum albumin in PBS for 20 min, as described previously (Bello-Morales et al., 2016).

For double-label immunofluorescence analysis, cells were incubated for 30 min at 37 °C in a humidified 5% CO_2 incubator with the appropriate primary antibodies, rinsed several times, and incubated at room temperature for 30 min with the relevant fluorescent secondary antibodies. Labeling specificity was assessed by incubation with control primary antibody Olig2 or omission of primary antibody. After thorough washing in PBS, coverslips were mounted in Mowiol, and images were obtained using an LSM510 system (Zeiss) coupled to an inverted Axiovert 200 microscope.

To quantify fluorescence, confocal images were analyzed by FIJI-ImageJ software, and data were subjected to Student's t-test using the SPSS program.

3.8. Flow cytometry analysis

For flow cytometry analysis, HOG cells were cultured in DM and treated or mock-treated with 0.5 mM of VPD and infected with R120vGF virus at an m.o.i. of 2, as described above for viral entry assays. KG-1G and Oli-neu cells were treated, or mock-treated, with 0.5 mM of VPD and, subsequently, infected with K26-GFP virus at an m.o.i. of 1. To compare the antiherpetic effect of VPD with others nucleoside analogues drugs, HOG cells were treated with 0.5 mM of VPD and/or 10 μM of ACV (Chemaly et al., 2019) and infected with K26-GFP virus at an m.o.i. of 1. Then, cultures were dissociated by 1 min incubation with 0.05% trypsin/0.1% EDTA (Invitrogen) at room temperature, washed and fixed in 4% paraformaldehyde for 15 min. Subsequently, cells were rinsed and resuspended in PBS. Analysis was performed using a FACSalibour Flow Cytometer (BD Biosciences).

3.9. Real-time quantitative RT-PCR assay

Total RNA from quadruplicate samples of HOG cells cultured in 60-mm dishes under differentiation conditions and infected at an m.o.i. of 1 with HSV-1 at different h p.i. -as described above-, was extracted using RNeasy Qiagene Mini kit (Qiagen, Valencia, CA, USA). Later, RNA was quantified in a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and their integrity tested using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All the samples showed 260/280 ratio values around 2, which correspond to pure RNA. The high integrity of RNA samples was analyzed by the Integrity Number (D/R-IN), with values between 9.0 and 10.0. Genomic DNA contamination was assessed by amplification of representative samples without retrotranscriptase (RT).

Real-time quantitative RT-PCR assay was performed as previously described (Crespillo et al., 2016), with the primer sequences ICP4 (RNA extracted 3 h p.i.), viral polymerase (extracted 6 h p.i.) and gC (8.5 h p.i.), and analyzed by the Genomics Core Facility at Centro de Biología Molecular Severo Ochoa (CSIC-UAM).

In order to consider the most suitable genes for the normalization, the stability of three candidates –GAPDH, 18 S and Ubiquitin (UBQ)– was assayed by using the NormFinder algorithm. Given its exceptionally high stability, GAPDH was chosen as the most appropriate.

4. Discussion

VCD and VPD are drugs lacking any teratogenic and cytotoxic effect, in contrast to the parent compound VPA (Samur et al., 2018). The detrimental effects of VPA could be due to a free carboxyl group in its chemical structure causing an inhibition of HDAC (Nakashima et al., 2015). VPD and VCD both lack the free carboxyl and subsequent HDAC inhibitory activity, which might be critical at a CNS level (Younus and Reddy, 2017). Currently, VPD is being used as anticonvulsant and VCD is under phase II and III clinical trials (Bialer and White, 2010; Bialer et al., 2017).

In this study, we demonstrated that VPD functions as an anti-HSV-1 drug in oligodendrocytes, suggesting its antiherpetic use as a possible alternative to traditional nucleoside analogues. Nucleoside analogues have potential teratogenic, embryotoxic, carcinogenic and antiproliferative activities; for example, some studies have shown that acyclovir or gancyclovir can cross the placenta, and therefore may affect embryogenesis (Ahrens et al., 2013). Regarding gancyclovir, it has been shown that in some cases it can trigger encephalopathy and ataxia, in addition to myelosuppression in immunosuppressed patients (Mercorelli et al., 2011; Whitley, 2012). Regarding acyclovir, studies in rats revealed some teratogenic effects after administration of high doses during organogenesis (Ahrens et al., 2013). In fact, there is an increasing number of cases of patients –immunocompromised, particularly - resistant to acyclovir (Jiang et al., 2016). This data highlights the desirability of alternatives to nucleoside analogues, and VPD and VCD may be appropriate candidates for future anti-HSV-1 research.

Cytotoxicity assays demonstrated that in the presence of 0.5 mM and 0.1 mM of VPD or VCD, respectively, oligodendrocyte cultures maintained more than 80% viability. At these concentrations, both compounds showed an antiviral effect against HSV-1, although to a different extent. The increased effect of VPD may be due to differences in the pharmacodynamics and pharmacokinetics of these two compounds (Lin et al., 2018). It has been shown that VPD acts as a prodrug and is partially metabolized to valnoctamic acid, whereas VCD is not hydrolysed to its corresponding acid. Previous studies, some of which were carried out in our laboratory, have shown that the parent compound VPA may also act as an antiviral against HSV-1, both in HOG cells (Crespillo et al., 2016) and *in vivo*. Furthermore, in our hand, the inhibitory effect of VPD was extensible to other oligodendrocytes cultures infected with HSV-1, such as the human KG-1C or the murine Oli-neu cell lines, being, this antiherpetic effect comparable to that produced by ACV in HOG cells.

In order to extend the results obtained in HOG, KG-1C and Oli-neu cells to a more physiological model, we carried out experiments in primary cells, using rat OPCs, as in previous studies (Bello-Morales

et al., 2014, 2016; Hare et al., 2016). These experiments in primary cultures were performed only with VPD, due to its greater antiviral activity found in the previous experiments.

The specific antiviral mechanism of the VPA derivatives is still the object of study, though the kinetics of viral production in VPD- and VCD-treated HOG cells showed that they have an effect starting from the first viral cycle, and that decreased production is maintained at different times post-infection. Some studies have suggested that VPD and the related valnoctamic acid have an important role in virus entry (Crespillo et al., 2016). HSV-1 and other herpesviruses have the ability to infect several hosts and cell types by using different adsorption and entry strategies (Bello-Morales et al., 2018), an ability that may be due to the number of glycoproteins involved and the existence of multiple alternative cell receptors (Karasneh and Shukla, 2011). In an early stage of cytomegalovirus infection, the cellular protein HSPGs is recognized by the viral glycoprotein gB, inducing viral attachment. VPD and VCD seem to affect the recognition of this protein (Ornaghi et al., 2016), and an analogous mechanism would be feasible for anti-HSV-1 activity. Furthermore, another common effect of VPA is the disruption of lipid synthesis (Chang et al., 2015), which could affect morphogenesis of the viral lipid envelope (Vazquez-Calvo et al., 2011). Taken into account the lipid nature of VPA and its derivatives, as well as a feasible effect on the stability of lipid membranes, we evaluated the effect of VPD with the viral entry. In this sense, the infection with the GFP-recombinant R120vGF virus, decreased in VPD treated cultures. Flow cytometry analysis supports this suggestion of a possible role of VPD in the viral entry. In addition, the study of immediate early, early and late genes expression by RT-PCR in VPD-treated cells corroborated these results. Obviously, a decrease in immediate early genes transcription leads to the reduction in the detection of early and late viral genes, as well.

Further studies, even with murine models, are needed to unravel the final mechanism responsible for the antiherpetic effects observed for VPD and VCD. Taking into account the probable implication of HSV-1 in the etiology, or development, of the MS disease, the possibility of characterizing new safe and effective antiherpetics capable of successfully reaching the CNS seems to be very attractive. In summary, we propose VPD and VCD as suitable alternatives to nucleoside analogues as antiherpetic drugs in the CNS, with the greater antiviral activity of VPD making it the better candidate.

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