ORIGINAL ARTICLE

The histone deacetylase inhibitor valproic acid enhances equine herpesvirus type 1 (EHV-1)-mediated oncolysis of human glioma cells

MC White and AR Frampton Jr

Histone deacetylase (HDAC) inhibitors represent a promising new therapy against malignant glioma. When used in conjunction with oncolytic viral vectors, these compounds have been shown to augment virotherapy. In the current study, we examined the antitumor effect of combining the lytic animal virus equine herpesvirus type 1 (EHV-1) with the HDAC inhibitor valproic acid (VPA). Pretreatment of two human glioblastoma cell lines (U251 and SNB19) with VPA resulted in a significant increase in virus entry, replication, cell to cell spread and cell lysis. Overall, these data indicate that VPA significantly improves EHV-1-mediated oncolysis of human glioma cells that are only moderately killed by EHV-1 alone.

Cancer Gene Therapy (2013) **20**, 88–93; doi:10.1038/cgt.2012.89; published online 11 January 2013

Keywords: EHV-1; glioblastoma; valproic acid; virotherapy

INTRODUCTION

Glioblastoma multiforme (GBM) is the most frequently occurring primary brain tumor in humans.¹ These aggressive tumors are highly resistant to standard modes of treatment including surgical resection, radiotherapy and chemotherapy. The median survival rate of GBM patients is 12–18 months with aggressive therapy,^{2–4} and <5% of patients survive out to 5 years.^{5,6} Therefore, new treatment options are desperately needed for GBM patients.

One novel treatment option that has become increasingly popular is the use of viruses for oncolytic therapy. Many viruses have been evaluated for their oncolytic potential against human gliomas, including human herpes simplex virus type 1 (HSV-1),⁷ adenovirus,⁸ vesicular stomatitis virus,⁹ Newcastle disease virus,¹⁰ myxoma virus¹¹ and vaccinia virus.¹²

We recently showed that the animal virus equine herpesvirus type 1 (EHV-1) can effectively enter and kill human glioma cells.¹³ EHV-1 is a member of the alphaherpesvirus family, which includes the human herpes simplex viruses HSV-1 and HSV-2.¹⁴ EHV-1 has many attributes that make it an attractive candidate for oncolytic virotherapy against GBM. Some of these favorable properties include: (1) the virus is well characterized and the genome has been sequenced, thus allowing for the rational design of various recombinant viruses;¹⁵ (2) EHV-1 has a short replication cycle and infection results in the lysis of cells;¹⁶ and (3) humans have no pre-existing antibodies to the virus.¹⁷

In an effort to enhance the oncolytic ability of viral vectors, researchers have examined the effect of delivering chemical adjuvants in combination with these viruses on glioma cells. One group of compounds that has been shown to significantly enhance virotherapy is histone deacetylase (HDAC) inhibitors. HDACs are responsible for the deacetylation of histone proteins on chromosomes, which leads to a selective increase in gene expression.¹⁸ The HDAC inhibitors valproic acid (VPA), trichostatin A and FR901228 have all been shown to enhance oncolysis

mediated by various viral vectors including adenovirus,⁸ adeno-associated virus,¹⁹ vaccinia virus²⁰ and HSV-1.²¹ These HDAC inhibitors augment virotherapy in a variety of ways, including upregulating virus entry receptors,^{22–24} increasing viral replication^{8,25} and inhibiting interferons.^{26,27} Collectively, data obtained from these studies show that HDAC inhibitors alter the microenvironment of the tumor in ways that allow for better virus infection and lysis of cancer cells.

We previously reported that certain human glioma cell lines are more susceptible to EHV-1 than others.¹³ As an ongoing challenge to viral oncolytic therapy is the ability of the virus to kill a large number of the existing tumor cells, the current study evaluated the ability of VPA to enhance the EHV-1-mediated oncolysis of U251 and SNB19 glioma cells, which are two GBM cell lines that are moderately susceptible to EHV-1 infection.¹³ Data collected in this study show that treatment of U251 and SNB19 cells with VPA before EHV-1 infection results in a significant increase in EHV-1 entry, replication, cell to cell spread (CTCS) and cell lysis compared with viral infection alone.

MATERIALS AND METHODS

Cell lines, reagents and virus

Human glioblastoma lines (U251 and SNB19) were purchased from ATCC (Manassas, VA, USA). Rabbit kidney (RK13) cells were kindly provided by Dennis O'Callaghan (Louisiana State University Health Sciences Center, Shreveport, LA, USA). All cell lines were maintained at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. The HDAC inhibitor VPA was purchased from EMD Chemicals (San Diego, CA, USA). The recombinant EHV-1 *LacZ* reporter virus, L11ΔgIΔgE, was described previously.²⁸ L11ΔgIΔgE was derived from the pathogenic RacL11 strain and is deleted for glycoproteins I (gI) and E (gE). In place of these glycoproteins, a *LacZ* reporter cassette has been inserted. This allows for production of β-galactosidase (β-gal) in infected cells, which can be

measured quantitatively with ONPG (ortho-nitrophenyl- β -D-galactoside) and qualitatively with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside).

Cytotoxicity assays

The extent of glioma cell death mediated by EHV-1 alone or in conjunction with VPA was measured using an MTS assay. This assay assesses the metabolic activity of cells by measuring the reduction of the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] to formazan. The MTS assay was performed using the Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Madison, WI, USA). Cells were seeded in a 96-well plate at a cell density of 1×10^4 cells per well in triplicate. For the pretreatment assay, the cells were untreated or treated with 30 mM VPA in 30 μ l of DMEM for 2.5 h. After this time, 70 μ l of maintenance DMEM was added to all wells. At 24 h after addition of inhibitor, the VPA and media were removed from the wells and cells were washed with 100 μ l of DMEM. Cells were then mock-infected or infected with L11 Δ gI Δ gE at multiplicities of infection (MOIs) of 1, 3 and 10. An additional column of cells served as a total cell death control and did not receive any virus. For the co-treatment assay, 30 mM VPA was added at the same time as the virus. Virus was incubated on the cells for 2 h in 30 μ l of DMEM. For the pretreatment assay, the virus and media mixture were then removed and 100 μ l of DMEM was added to each well. For the co-treatment assay, the virus was removed, replaced with VPA media for 22 h and then replaced with DMEM for the remaining 24 h. At 48 h post infection (p.i.), the cells in the total cell death control wells were lysed with 10 μ l lysis buffer (10% Triton X-100) for 5 min. Media was removed from all wells and each well was washed with 100 μ l serum- and phenol red-free DMEM (Lonza, Walkersville, MD, USA). Assay mixture (100 μ l) consisting of five parts phenol red-free DMEM to one part MTS solution from the Promega kit was then added to each well. The plate was incubated for 60 min at 37 °C and 5% CO₂ and then read on a plate reader (Multiskan Ascent photometric plate reader, Thermo Electron Corporation, Vantaa, Finland) at 492 nm. For each well, percent cell survival was calculated by determining the average absorbances of that run's mock-infected wells and total cell death wells and the following formula was applied: $\frac{\text{abs (well)} - \text{abs (total death)}}{\text{abs (mock)} - \text{abs (total death)}} \cdot 100$. Three trials per cell line were performed and the overall average for each cell line was calculated and graphed.

Infectivity assays

Glioma cells were seeded in a 96-well plate at a cell density of 3×10^4 cells per well in triplicate. For the pretreatment assay, the cells were treated with or without VPA as described above. Cells were then mock-infected or infected with L11 Δ gI Δ gE at MOIs of 1, 3 and 10. For the co-treatment assay, VPA was added simultaneously with the virus. At 2 h p.i., 70 μ l of DMEM was added to each well and the cells were incubated for an additional 4 h. The virus and media mixture were then removed and the cells were washed with 150 μ l of phosphate-buffered saline (PBS). ONPG solution (PBS, ONPG, Triton X-100, BME/MgCl₂) was added to each well and the plate was incubated for 30 min. The plate was then read at 405 nm on a plate reader to determine the amount of β -gal produced. Each of these triplicated assays was run three times and the average of the absorbances across all data sets was calculated and graphed.

Viral replication assays

Glioma cells were seeded in a 24-well plate at a cell density of 2×10^5 cells per well, with three replicates of $\pm$ VPA for each of two time points. Cells were treated with or without VPA for 24 h and then infected with L11 Δ gI Δ gE at an MOI of 3. At 2 h p.i., cells were washed three times with media and then 500 μ l of DMEM was added to each well. The three replicates (cells and media) for each time point were harvested 24 and 48 h p.i. The harvested cells and media were centrifuged at 14 000 r.p.m. for 5 min in a Spectrafuge 16M microcentrifuge (Labnet International, Edison, NJ, USA). The supernatants were transferred to new microcentrifuge tubes and the cell pellets were resuspended in 50 μ l of their supernatant. Cell pellets were disrupted to release intracellular virus by three freeze-thaw (–80 to 37 °C) cycles. The disrupted cell pellets were then added back to their supernatants and a 10-fold dilution series was generated. The amount of infectious EHV-1 at each time point was quantified using a standard plaque assay on RK13 cells. A total of 100 μ l of each dilution from each time point was added to monolayers of RK13 cells and incubated for 1.5 h. Following incubation, 1 ml of 1% methylcellulose in DMEM containing 10%

fetal bovine serum was added to each well. Cells and virus were then incubated at 37 °C for 3 days. Cells were fixed, stained with crystal violet and plaques counted to determine the plaque-forming units per ml (PFU per ml) at each time point.

Viral entry kinetics

Glioma cells were seeded in a 96-well plate at a cell density of 3×10^4 cells per well in triplicate. For the pretreatment assay, the cells were untreated or treated with VPA as described in the cytotoxicity assay methods. For the co-treatment assay, VPA was added at the same time as the virus and was kept on the cells for the duration of the assay. After 24 h, the media was removed from the plates and the cells were washed with 100 μ l of cold DMEM. Cold DMEM was again added to the wells and the plates were placed on ice for 5 min. After this time, cells were mock-infected or infected on ice with L11 Δ gI Δ gE at MOIs of 1, 3 or 10 for 2 h. After 2 h, the cells were washed twice with cold DMEM and 100 μ l of warm DMEM (37 °C) was added to the wells, allowing for virus entry. At 5, 15, 30 and 60 min after virus entry, the media was removed from the wells and 30 μ l of a warm low pH glycine buffer was added for 30 s. Glycine buffer was removed and the cells were washed three times with warm DMEM. Next, 100 μ l of warm DMEM was added to the wells and the plate was incubated. At 6 h after virus entry, an ONPG assay was performed as described above. The average of the absorbance values at each of the four time points across all data was calculated and graphed, comparing the untreated cells with the cells treated with VPA.

Cell to cell spread

Cells were seeded in a 12-well plate at a cell density of 3×10^5 cells per well. Cells were treated with or without VPA for 24 h, and then infected with ~ 300 PFU of L11 Δ gI Δ gE and incubated for 2 h. Following incubation, 1 ml of 1% methylcellulose in DMEM containing 10% fetal bovine serum was added to each well. Cells and virus were incubated at 37 °C for 4 days, after which the plate was stained with X-gal as follows. Cells were rinsed with PBS and fixed with a 0.5% glutaraldehyde solution for 10 min. Cells were again rinsed with PBS and X-gal stain was added to each well and incubated at 37 °C for 2 h. X-gal stain was then removed, 1 ml of PBS was added to each well and plaque formation was visualized with an inverted Motic AE21 microscope (British Columbia, Canada). Images were captured using a Moticam 2.0 camera (Motic) at $\times 100$ magnification.

RESULTS

VPA enhances EHV-1-mediated oncolysis of human glioma cells

In order to determine if VPA could synergize with EHV-1 and enhance viral-mediated oncolysis, an MTS cell viability assay was performed as described in the Materials and Methods. Glioma cells were untreated or treated with VPA for 24 h, mock-infected or infected with the EHV-1 *LacZ* reporter virus L11 Δ gI Δ gE²⁸ at various MOIs, and then cell viability was assessed at 48 h p.i. (Figure 1a). A significant increase in cell death was observed in the U251 and SNB19 cell lines that were treated with VPA compared with the untreated cells. At each MOI, cell death was significantly increased by VPA pretreatment. At the highest MOI of 10, VPA pretreatment resulted in 70% cell death in both cell lines versus only 10–30% cell death in the untreated cells that were infected with EHV-1. These results show that VPA pretreatment acts synergistically with EHV-1 to kill GBM cells that are only moderately killed by EHV-1 infection alone. Interestingly, cell killing was enhanced by VPA only when the cells were treated for 24 h before infection. No difference in cell viability was observed when VPA and EHV-1 were added to cells at the same time (Figure 1b). The ability of VPA to enhance cell lysis only when added before EHV-1 infection suggests that treatment of U251 and SNB19 cells with this HDAC inhibitor sensitizes the cells to EHV-1 infection.

VPA enhances EHV-1 infection of human glioma cells

In order to assess whether VPA pretreatment increased EHV-1 infection, U251 and SNB19 cells were untreated or treated with VPA for 24 h and then mock-infected or infected with L11 Δ gI Δ gE

Figure 1. Valproic acid (VPA) enhances equine herpesvirus type 1 (EHV-1)-mediated oncolysis of human glioma cells. (a) U251 (U) and SNB19 (S) cells were treated with (+ V) or without VPA in triplicate for 24 h and then infected at various multiplicities of infection (MOIs) with L11ΔglΔgE. Cell viability was assessed 48 h post infection (p.i.) by MTS assay. (b) VPA and L11ΔglΔgE were co-administered on the glioma cells and cell viability was assessed 48 h p.i. Error bars ± 1 s.d.

at MOIs of 1, 3 and 10 for 6 h. The extent of infection was measured by examining β-gal expression using an ONPG assay as described in the Materials and Methods. As shown in Figure 2a, pretreatment with VPA resulted in a significant increase in infection on the U251 and SNB19 cell lines, most evident at the lower MOIs. However, co-administration of VPA and L11ΔglΔgE on these cells did not result in enhanced infection (Figure 2b). This increase in infection with VPA preincubation correlates well with the increase in cell death observed in the cytotoxicity assays with VPA pretreatment.

VPA increases EHV-1 yield in human glioma cells

To evaluate if VPA pretreatment enhanced EHV-1 yield in the U251 and SNB19 cell lines, cells were pretreated with or without VPA for 24 h and then infected with L11ΔglΔgE at an MOI of 3. Cells were harvested 24 and 48 h p.i. and total virus collected at each time point was titrated using a standard plaque assay on RK13 cells to determine the number of infectious virus particles or PFU per ml (Figure 3). As shown in Figure 3, EHV-1 yield in both the U251 and SNB19 cells was increased significantly in the presence of VPA at 24 and 48 h p.i. The VPA-treated U251 cells showed a 0.7 log increase in viral yield at 24 h p.i. and a full log increase at 48 h p.i. compared with the untreated cells. The SNB19 cells exhibited a stronger enhancement of viral replication with VPA pretreatment, with over a full log increase in EHV-1 yield at both time points.

Figure 2. Valproic acid (VPA) enhances equine herpesvirus type 1 (EHV-1) infection of human glioma cells. U251 (U) and SNB19 (S) cells were treated with (+ V) or without VPA in triplicate for 24 h and then infected at various multiplicities of infection (MOIs) with L11ΔglΔgE (a) or co-treated with VPA and L11ΔglΔgE (b). β-galactosidase (β-gal) production was assessed 6 h post infection (p.i.) by ONPG (ortho-nitrophenyl-β-D-galactoside) assay. Absorbance was measured at 405 nm. Error bars ± 1 s.d.

Figure 3. Valproic acid (VPA) increases equine herpesvirus type 1 (EHV-1) yield in human glioma cells. Glioma cells were treated with or without VPA in triplicate for 24 h and then infected with L11ΔglΔgE at a multiplicity of infection (MOI) of 3. At (a) 24 h and (b) 48 h post infection (p.i.), cells were harvested and the virus titer was determined using standard plaque assays on RK13 cells. Error bars ± 1 s.d.

EHV-1 entry is increased with VPA pretreatment

The ability of VPA pretreatment to increase infection and cell killing of U251 and SNB19 cells led us to investigate potential mechanisms by which VPA sensitizes cells to EHV-1-mediated lysis. One hypothesis is that VPA pretreatment influences an early stage of EHV-1 infection and results in an increased rate of entry of virus particles into GBM cells. In order to assess whether VPA increased virus entry, cells were pretreated with or without VPA for 24 h and

then infected with L11ΔgIΔgE at various MOIs at 4 °C for 2 h. After this attachment period, the temperature was raised to 37 °C to allow for synchronized virus entry. Extracellular virus was neutralized with a low pH glycine buffer at 5, 15, 30 and 60 min after temperature shift. At 6 h after virus entry, the amount of virus that was able to enter cells within the allotted 5, 15, 30 or 60 min was measured by examining β-gal expression using an ONPG assay as described in the Materials and Methods. Figures 4a and b demonstrate that the rate of EHV-1 entry was increased in cells that were treated with VPA 24 h before infection. At an MOI of 10, the absorbance values for the U251 cells pretreated with VPA are roughly twice those of the untreated cells at the same MOI across all time points. The SNB19 cells that had been pretreated with VPA demonstrate a marked increase in absorbance values at all MOIs compared with the untreated cells at all time points. However, co-administration of VPA and L11ΔgIΔgE on both of these cell lines did not result in increased virus entry at any time point (Figures 4a and b).

Figure 4. The rate of equine herpesvirus type 1 (EHV-1) entry is increased with valproic acid (VPA) pretreatment. Glioma cells were pretreated with or without VPA for 24 h or co-treated with VPA in triplicate and then infected with L11ΔgIΔgE at various multiplicities of infection (MOIs) for 2 h at 4 °C. After 2 h, the cells were washed twice with Dulbecco's modified Eagle's medium (DMEM) and the temperature was raised to 37 °C to allow for synchronized virus entry. At 5, 15, 30 and 60 min after virus entry, extracellular virus was neutralized with a low pH glycine buffer. β-galactosidase (β-gal) production was assessed 6 h after virus entry by ONPG (ortho-nitrophenyl-β-D-galactoside) assay. Absorbance was measured at 405 nm. U251 (a) and SNB19 (b). Error bars ± 1 s.d.

Pretreatment with VPA enhances the ability of EHV-1 to spread laterally from cell to cell

To assess whether VPA pretreatment enhanced the ability of EHV-1 to spread from cell to cell, glioma cells were treated with or without VPA for 24 h and then infected with ~300 PFU of L11ΔgIΔgE for 2 h and then overlaid with 1% DMEM methylcellulose to allow for plaque development. As shown in Figure 5, pretreatment with VPA resulted in enhanced viral spread in both glioma lines. The number of plaques observed in the U251 cell line increased from 7 to 30 and the number of plaques observed in the SNB19 cell line increased from 2 to 107 with VPA pretreatment (Table 1). Plaques were defined as four or more adjacent infected cells. The number and size of plaques were both increased after VPA treatment, suggesting that the HDAC inhibitor is enabling the virus to spread more easily to neighboring cells.

DISCUSSION

Because of the refractory nature of GBM to standard cancer therapies,²⁹ oncolytic virotherapy has become an attractive therapeutic option against these aggressive tumors. An effective, clinical-grade oncolytic virus should first be safe and cause little to no adverse side effects. In addition to safety, the vector should be able to readily infect, replicate in, and ultimately lyse GBM tumor cells. Although many oncolytic viruses display some of these

Figure 5. Pretreatment with valproic acid (VPA) enhances the ability of equine herpesvirus type 1 (EHV-1) to spread laterally from cell to cell. Glioma cells were treated with or without VPA for 24 h and then infected with ~300 PFU of L11ΔgIΔgE. After 2 h, cells were overlaid with 1% methylcellulose in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and incubated. Plaque formation was visualized 96 h later by X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. Plaques were defined as four or more adjacent infected cells.

Table 1. EHV-1 spread in response to VPA pretreatment

	U251		SNB19	
	Untreated	VPA	Untreated	VPA
Single infected cells	42	87	311	231
Plaques	7	30	2	107

Abbreviations: EHV-1, equine herpesvirus type 1; VPA, valproic acid.

desired properties, most are deficient, to varying degrees, in one or more. To compensate for the deficiencies inherent in oncolytic vectors, many groups have investigated the utility of combining drug therapy with oncolytic vector administration in an attempt to enhance tumor eradication.^{12,30} One promising class of compounds that has been shown to synergize with oncolytic virotherapy is the HDAC inhibitors.^{31,32} These HDAC inhibitors have been shown to enhance oncolytic vectors in a variety of ways, including increasing the expression of virus attachment/entry receptors,²² enhancing viral replication⁸ and interfering with host innate immunity.²⁶

We previously reported that infection of human glioma cells with the animal virus EHV-1 is highly variable.¹³ The glioma cell lines A-172 and LN-18 are highly susceptible to EHV-1 infection, whereas U251 and SNB19 cells are only moderately infected.¹³ In an effort to enhance the ability of the virus to kill the moderately infected cell lines (U251 and SNB19), we investigated whether combining the HDAC inhibitor VPA with EHV-1 could augment the antitumor effects of the virus. Not only did we observe a robust increase in viral-mediated cell killing after treatment with VPA, but we also noted a distinct enhancement of the rate of virus entry. These entry data clearly showed that incubation of U251 and SNB19 cells with VPA for 24 h resulted in faster virus entry compared with untreated cells. The enhanced rate of entry was not due to increased attachment of virus to cells, as EHV-1 bound equally well to both cell lines regardless of VPA treatment (data not shown). In addition, the entry kinetics assay revealed that virus entry on both glioma lines occurred very rapidly after the temperature was shifted to 37°C, as the absorbance values at 5 min were similar to the values recorded at 60 min.

Otsuki *et al.*²⁵ demonstrated that pretreatment, but not co-treatment, with VPA enhanced the propagation of an oncolytic HSV-1 vector. The results that we obtained in this study indicate that EHV-1 infection of GBM cells is also only enhanced in cells that were pretreated with VPA. This suggests that for both alphaherpesviruses the HDAC inhibitor is sensitizing the cells to infection. In the case of EHV-1, VPA pretreatment results in a more rapid rate of virus entry into VPA-treated cells. Although a robust increase in infection and cell death was noted after VPA pretreatment, co-treatment with VPA had no effect on EHV-1 infection or viral-mediated oncolysis. These data suggest that the cellular changes induced by VPA that lead to glioma cell sensitization to infection take time to develop, and this is why no difference in infection or cytotoxicity is observed when VPA is added to cells simultaneously with EHV-1.

Interestingly, VPA has been shown to enhance major histocompatibility complex class I (MHC-I) expression in a melanoma cell line.^{23,33} The kinetics data sets obtained in this study suggest that the increased rate of virus entry observed after VPA pretreatment could be due in part to an increase in the number of MHC-I entry receptors that are available for binding by the EHV-1 glycoprotein D. Future studies will be performed to investigate this possible connection between VPA treatment and enhanced EHV-1 entry. In addition to investigating whether VPA increases the expression of EHV-1 entry receptors, we will also examine other possible cellular modifications mediated by VPA that may lead to enhanced EHV-1 infection and lysis of GBM cells. It is known that HDAC activity is required for the successful establishment of an antiviral state in cells and that treatment with HDAC inhibitors prevents transcription of interferon-stimulated genes (ISG).^{34,35} As demonstrated with HSV, inhibition of the immune response shortly after infection leads to increased viral propagation in tumor cells.³⁶ VPA in particular has been shown to counter the innate glioma immune response against oncolytic HSV by inhibiting the induction of ISG²⁵ as well as by suppressing the recruitment of immune cells to the tumor site *in vivo*.²⁶ VPA has also been shown to exhibit antiangiogenic effects in GBM models.³⁷ Taken together, the accumulated data

suggest that the ability of VPA and other HDAC inhibitors to augment virotherapy is multifactorial. The ability of VPA to downregulate the innate immune response of infected cells is of particular interest, as EHV-1 induces a robust secretion of interferon- α upon infection of equine cells.³⁸

In addition to increasing the ability of EHV-1 to enter cells, VPA treatment also enhances EHV-1 CTCS. The ability of VPA to drastically improve the lateral CTCS of EHV-1 is noteworthy and warrants further investigation. We recently showed that MHC-I expression is critically important for EHV-1 CTCS (unpublished results, AR Frampton), and thus the possible upregulation of MHC-I in GBM cells treated with VPA could contribute to both increased entry and CTCS of the virus. Similarly, the HSV-1 receptor nectin-1 has been shown to play an important role in not only HSV-1 entry but also viral CTCS.^{39,40} A common issue with current oncolytic vectors is their inability to spread fully within the tumor.^{41,42} The HDAC inhibitors VPA and trichostatin A have both been shown to enhance the spread of oncolytic viruses in cancer cells.^{20,25} Administering VPA before EHV-1 infection clearly enables the virus to spread more easily to adjacent glioma cells, an essential property for an effective oncolytic vector.

Our results clearly demonstrate that the timing of VPA administration is crucial in order to obtain a synergistic effect with the virus. This suggests an altering of the cellular environment over time in favor of the virus, and it will be necessary in future studies to further explore how the cells are modified by VPA to allow for increased viral infection, replication, CTCS and tumor cell lysis. Overall, the data presented here show that VPA pretreatment strongly augments EHV-1-mediated oncolysis in U251 and SNB19 glioma cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dennis O'Callaghan (Louisiana State University Health Sciences Center, Shreveport, LA, USA) for the RK13 cell line. We thank Michael Courchesne for his help with the preparation of this manuscript.

REFERENCES

- Lim SK, Llaguno SRA, McKay RM, Parada LF. Glioblastoma multiforme: a perspective on recent findings in human cancer and mouse models. *BMB Rep*, 2011; 44: 158–164.
- Wen PY, Kesari S. Malignant gliomas in adults. *N Engl J Med* 2008; **359**: 492–507.
- Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ *et al.* Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005; **352**: 987–996.
- Grossman SA, Ye X, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M *et al.* Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the United States. *Clin Cancer Res* 2010; **16**: 2443–2449.
- Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Caveness WK *et al.* Malignant glioma: genetics and biology of a grave matter. *Genes Dev* 2001; **15**: 1311–1333.
- Lacroix M, Abi-Said D, Fourney DR, Gokaslan ZL, Shi W, DeMonte F *et al.* A multivariate analysis of 416 patients with glioblastoma multiforme: prognosis, extent of resection, and survival. *J Neurosurg* 2001; **95**: 190–198.
- Kambara H, Okano H, Chiocca EA, Saeki Y. An oncolytic HSV-1 mutant expressing ICP34.5 under control of a nestin promoter increases survival of animals even when symptomatic from a brain tumor. *Cancer Res* 2005; **65**: 2832–2839.
- Bieler A, Mantwill K, Dravits T, Bernshausen A, Glockzin G, Kohler-Vargas N *et al.* Novel three-pronged strategy to enhance cancer cell killing in glioblastoma cell lines: histone deacetylase inhibitor, chemotherapy, and oncolytic adenovirus dl520. *Hum Gene Ther* 2006; **17**: 55–70.
- Lun X, Senger DL, Alain T, Oprea A, Parato K, Stojdl D *et al.* Effects of intravenously administered recombinant vesicular stomatitis virus (VSVΔM51) on multifocal and invasive gliomas. *J Natl Cancer Inst* 2006; **98**: 1546–1557.

- 10 Freeman AI, Zakay-Rones Z, Gomori JM, Linetsky E, Rasooly L, Greenbaum E *et al*. Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther* 2006; **13**: 221–228.
- 11 Lun X, Yang W, Alain T, Shi ZQ, Muzik H, Barrett JW *et al*. Myxoma virus is a novel oncolytic virus with significant antitumor activity against experimental human gliomas. *Cancer Res* 2005; **65**: 9982–9990.
- 12 Lun XQ, Jang J-H, Tang N, Deng H, Head R, Bell JC *et al*. Efficacy of systemically administered oncolytic vaccinia virotherapy for malignant gliomas is enhanced by combination therapy with rapamycin or cyclophosphamide. *Clin Cancer Res* 2009; **15**: 2777–2788.
- 13 Courchesne MJ, White MC, Stanfield BA, Frampton AR. Equine Herpesvirus type 1-mediated oncolysis of human glioblastoma multiforme cells. *J Virol* 2012; **86**: 2882–2886.
- 14 Davison AJ, Wilkie NM. Location and orientation of homologous sequences in the genomes of five herpesviruses. *J Gen Virol* 1983; **64**: 1927–1942.
- 15 Telford EA, Watson MS, McBride K, Davison AJ. The DNA sequence of equine herpesvirus-1. *Virology* 1992; **189**: 304–316.
- 16 Reed SM, Toribio RE. Equine herpesvirus 1 and 4. *Vet Clin North Am Equine Pract* 2004; **20**: 631–642.
- 17 Trapp S, von Einem J, Hofmann H, Köstler J, Wild J, Wagner R *et al*. Potential of equine herpesvirus 1 as a vector for immunization. *J Virol* 2005; **79**: 5445–5454.
- 18 Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr* 1996; **5**: 245–253.
- 19 Okada T, Uchibori R, Iwata-Okada M, Takahashi M, Nomoto T, Nonaka-Sarukawa M *et al*. A histone deacetylase inhibitor enhances recombinant adeno-associated virus-mediated gene expression in tumor cells. *Mol Ther* 2006; **13**: 738–746.
- 20 MacTavish H, Diallo J-S, Huang B, Stanford M, Le Boeuf F, De Silva N *et al*. Enhancement of vaccinia virus based oncolysis with histone deacetylase inhibitors. *PLoS One* 2010; **5**: e14462.
- 21 Liu T-C, Castelo-Branco P, Rabkin SD, Martuza RL, Trichostatin A, Oncolytic HSV. Combination therapy shows enhanced antitumoral and antiangiogenic effects. *Mol Ther* 2008; **16**: 1041–1047.
- 22 Segura-Pacheco B, Avalos B, Rangel E, Velazquez D, Cabrera G. HDAC inhibitor valproic acid upregulates CAR in vitro and in vivo. *Genet Vaccines Ther* 2007; **5**: 10.
- 23 Magner WJ, Kazim AL, Stewart C, Romano MA, Catalano G, Grande C *et al*. Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. *J Immunol* 2000; **165**: 7017–7024.
- 24 Kitazono M, Goldsmith ME, Aikou T, Bates S, Fojo T. Enhanced adenovirus transgene expression in malignant cells treated with the histone deacetylase inhibitor FR901228. *Cancer Res* 2001; **61**: 6328–6330.
- 25 Otsuki A, Patel A, Kasai K, Suzuki M, Kurozumi K, Chiocca EA *et al*. Histone deacetylase inhibitors augment antitumor efficacy of herpes-based oncolytic viruses. *Mol Ther* 2008; **16**: 1546–1555.
- 26 Alvarez-Breckenridge CA, Yu J, Price R, Wei M, Wang Y, Nowicki MO *et al*. The HDAC inhibitor valproic acid lessens NK cell action against oncolytic virus-infected glioblastoma cells with inhibition of STAT5/T-BET signaling and IFN γ generation. *J Virol* 2012; **86**: 4566–4577.
- 27 Vlasáková J, Nováková Z, Rossmeislová L, Kahle M, Hozák P, Hodný Z. Histone deacetylase inhibitors suppress IFN α -induced up-regulation of promyelocytic leukemia protein. *Blood* 2007; **109**: 1373–1380.
- 28 Frampton Jr AR, Smith PM, Zhang Y, Matsumura T, Osterrieder N, O'Callaghan DJ. Contribution of gene products encoded within the unique short segment of equine herpesvirus 1 to virulence in a murine model. *Virus Res* 2002; **90**: 287–301.
- 29 Niyazi M, Siefert A, Schwarz SB, Ganswindt U, Kreth F-W, Tonn J-C *et al*. Therapeutic options for recurrent malignant glioma. *Radiother Oncol* 2011; **98**: 1–14.
- 30 Fu X, Tao L, Rivera A, Zhang X. Rapamycin enhances the activity of oncolytic herpes simplex virus against tumor cells that are resistant to virus replication. *Int J Cancer* 2011; **129**: 1503–1510.
- 31 Nguyen TL-A, Wilson MG, Hiscott J. Oncolytic viruses and histone deacetylase inhibitors—a multi-pronged strategy to target tumor cells. *Cytokine Growth Factor Rev* 2010; **21**: 153–159.
- 32 Ottolino-Perry K, Diallo J-S, Lichty BD, Bell JC, Andrea McCart J. Intelligent design: combination therapy with oncolytic viruses. *Mol Ther* 2009; **18**: 251–263.
- 33 Khan A, Gregorie C, Tomasi T. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. *Cancer Immunol Immunother* 2008; **57**: 647–654.
- 34 Chang H-M, Paulson M, Holko M, Rice CM, Williams BRG, Marié I *et al*. Induction of interferon-stimulated gene expression and antiviral responses require protein deacetylase activity. *Proc Nat Acad Sci USA* 2004; **101**: 9578–9583.
- 35 Suh H-S, Choi S, Khattar P, Choi N, Lee S. Histone deacetylase inhibitors suppress the expression of inflammatory and innate immune response genes in human microglia and astrocytes. *J Neuroimmune Pharm* 2010; **5**: 521–532.
- 36 Fulci G, Breyman L, Gianni D, Kurozumi K, Rhee SS, Yu J *et al*. Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. *Proc Natl Acad Sci USA* 2006; **103**: 12873–12878.
- 37 Osuka S, Takano S, Watanabe S, Ishikawa E, Yamamoto T, Matsumura A. Valproic acid inhibits angiogenesis in vitro and glioma angiogenesis in vivo in the brain. *Neurol Med Chir* 2012; **52**: 186–193.
- 38 Wagner B, Wimer C, Freer H, Osterrieder N, Erb HN. Infection of peripheral blood mononuclear cells with neuropathogenic equine herpesvirus type-1 strain Ab4 reveals intact interferon- α induction and induces suppression of anti-inflammatory interleukin-10 responses in comparison to other viral strains. *Vet Immunol Immunopathol* 2011; **143**: 116–124.
- 39 Even DL, Henley AM, Geraghty RJ. The requirements for herpes simplex virus type 1 cell-cell spread via nectin-1 parallel those for virus entry. *Virus Res* 2006; **119**: 195–207.
- 40 Tiwari V, Oh MJ, Kovacs M, Shukla SY, Valyi-Nagy T, Shukla D. Role for nectin-1 in herpes simplex virus 1 entry and spread in human retinal pigment epithelial cells. *FEBS J* 2008; **275**: 5272–5285.
- 41 Yamamoto M, Curiel DT. Current issues and future directions of oncolytic adenoviruses. *Mol Ther* 2009; **18**: 243–250.
- 42 Kaur B, Chiocca EA, Cripe TP. Oncolytic HSV-1 virotherapy: clinical experience and opportunities for progress. *Curr Pharm Biotechnol* 2012; **13**: 1842–1851.