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Impact of a Cytomegalovirus Kinase Inhibitor on Infection and Neuronal Progenitor Cell Differentiation

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Abstract

Human cytomegalovirus (HCMV) is the leading cause of congenital infections. Symptomatic newborns present with a range of sequelae including disorders of the CNS such as visual impairment, microcephaly, mental retardation and hearing loss. HCMV congenital infection causes gross changes in brain morphology and disturbances in glial and neuronal distribution, number and migration. In these studies, we have evaluated the effectiveness of the antiviral maribavir in inhibiting HCMV infections of ES cell-derived neuronal progenitor cells (NPC). We used EZ-spheres generated from H9 ES cells which are pre-rosette NPCs that retain long-term potential to differentiate into diverse central and peripheral neural lineages following directed differentiation. Our results demonstrate that the maribavir disrupts HCMV replication and viral yield in undifferentiated EZ-sphere-derived NPCs. In addition, we observed that maribavir limits HCMV replication and reduces the percentage of infected cells during differentiation of NPCs. Finally, early steps in differentiation are maintained during infection by treating with maribavir, likely an indirect effect resulting from decreased viral spread. Future studies of NPC proliferation and differentiation during infection treated with maribavir could provide the impetus for studying maribavir as an antiviral agent for congenital HCMV disease.

Keywords

Congenital infection; human cytomegalovirus; neuronal progenitor cells; viral kinase; maribavir; differentiation

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1. Introduction

Human cytomegalovirus (HCMV) is the leading cause of congenital infections in developed countries (1) and likely around the world (2). Congenital HCMV disease is the last of the major congenital infections that remains as a major health burden. While other congenital infections have been largely controlled by immunization and prevention, there is currently no vaccination or treatment to combat congenital HCMV. Furthermore, the mechanisms of pathogenesis within the developing brain are not yet fully understood. The majority of infected infants exhibit no clinical symptoms at birth but may develop hearing loss in the first few years of life (3, 4). Symptomatic newborns present with a range of sequelae including jaundice, petechiae, hepatosplenomegaly, febrile syndrome, thrombocytopenia, anemia, and disorders of the CNS such as visual impairment, microcephaly, mental retardation and hearing loss (4). The mechanisms leading to disease are poorly understood but involve neuronal damage (5-7) and impaired placental development (8-10). Studies also suggest that the late onset of hearing loss involves persistent inflammation (10, 11).

In human tissue and animal models, congenital HCMV infection causes gross changes in brain morphology and disturbances in glial and neuronal distribution, number and migration (3, 12). Cells in the subventricular zone (SVZ) were found to be especially susceptible to HCMV infection (5-7). The SVZ is made up primarily of neural progenitor cells (NPCs) (13). NPCs derived from fetal tissue, induced pluripotent stem (iPS) cells, or embryonic stem (ES) cells are permissive to HCMV infection, as are glial and neuronal cells (14-16). On the other hand, embryonic stem cells and primitive pre-rosette neural stem cells show low susceptibility to lytic HCMV infection, though the virus can enter and maintain its genome in these cells (14, 17). HCMV infection of NPCs results in cytopathic effect, decreased proliferation, loss of multipotency markers, and cell death (15, 16, 18-20).

There are currently no approved therapies for use in congenital HCMV infection (21, 22). The anti-HCMV agents are not approved for use during pregnancy due to concerns about teratogenesis, impaired fertility and bone marrow suppression in fetuses from animal studies (22). Hyper immunoglobulin has been used with some success in small studies (22). In addition, ganciclovir and valganciclovir have been used to treat infants following congenital HCMV infection with positive results, though toxicity remains a concern (22). Identifying treatments that could be used safely in newborns and during pregnancy is a primary focus of HCMV research. The anti-HCMV compound maribavir (MBV) specifically inhibits the viral kinase, pUL97 (23, 24). Though MBV has been screened for inhibition against other kinases, off-target effects have yet to be identified (25, 26). Toxicity in humans is low and includes manageable symptoms such as taste disturbances, headache and nausea (23). MBV was not genotoxic or teratogenic in animal studies (25, 26). In addition, MBV demonstrates high bioavailability, can be taken orally, and crosses the blood-brain barrier (23).

We have evaluated the effectiveness of MBV in inhibiting HCMV infections of ES cell-derived NPC. We found that MBV inhibited viral protein expression and viral yield while maintaining expression of a subset of NPC markers in both undifferentiated and differentiated conditions. This study provides evidence that MBV may be a valuable anti-HCMV agent for treatment of congenital HCMV infection.

2. Material and Methods

2.1 Biological Reagents

Viral stocks were prepared by co-transfecting BAC DNA with the pCGN-pUL82HA expression vector into primary human foreskin fibroblasts (HFF). The wild-type TB40/E Bac4 (27) or TB40/E Bac4 containing the mCherry gene expressed from an SV40 promoter, generously provided by Dr. Eain Murphy (Cleveland Clinic), were used. The pCGN-UL82HA plasmid was generously provided by Dr. Robert Kalejta (University of Wisconsin-Madison) (28). To concentrate viral stocks, culture media and cleared cell lysate was pelleted through a sorbitol cushion (20% D-sorbitol, 50 mM Tris-HCl pH 7.2, 1 mM MgCl₂) at 55,000 × g for 1 h. Virus was reconstituted in DMEM. Titers were defined as infectious units (IU) per ml and determined by using serial dilutions of viral stocks to infect HFFs in 12-well dishes, fixing cells at 48 hours post infection (hpi), staining with an antibody against IE1, and counting IE1-positive cells per well.

Neural progenitor cells (NPCs) were derived from the H9 human embryonic stem (ES) cell line (NIH Stem Cell Registry 0062) as described by Ebert et al. (29). Briefly, ES cells were cultured in feeder free conditions on Matrigel in Nutrastem medium (Stemgent). Intact colonies were removed following dispase treatment and cultured as floating aggregates in NPC media, consisting of Stemline media (Sigma-Aldrich) supplemented with 100 ng/ml basic fibroblast growth factor (FGF-2, EMD Millipore), 100 ng/ml epidermal growth factor (EGF, EMD Millipore), and 5 µg/ml heparin (Sigma-Aldrich). The cells were cultured in ultra-low attachment flasks and were passaged weekly using a chopping technique for 11-16 passages (30, 31). To promote differentiation, twenty-four hours before infection, cells were dissociated and plated in 24 well plates at 50,000 cells/well in DMEM/F12 supplemented with 2% B27 (ThermoFisher Scientific). Cells were infected at a multiplicity of infection of 1 IU per cell in NPC media containing 1% pen/strep or in DMEM/F12 supplemented with 2% B27 containing 1% pen/strep and exposed to the viral inoculum for 2 h, unless otherwise noted. Cells were analyzed 2-4 weeks post-infection. Compounds were added to the culture media at the start of infection and reapplied after removing the inoculum. Cells were treated with maribavir (provided by Shire) ranging from 1.25 to 40 µM to identify toxicity. Based on toxicity, all other experiments were carried out using 1 µM maribavir, or DMSO as a vehicle control (Sigma-Aldrich).

Antibodies used for Western blot (WB) or immunofluorescence analysis (IF) are listed below: Mouse anti-GAPDH clone 0411 (WB) (Santa Cruz Biotechnology); mouse anti-IE1 clone 1B12 (WB, IF) and mouse anti-pUL83 clone 8F5 (WB) were generously provided by Dr. T. Shenk (Princeton University); mouse anti-pUL44 (WB) (Virusys); mouse anti-nestin (IF) (EMD Millipore); mouse anti-Tuj1 (IF) (Sigma-Aldrich); rabbit anti-GFAP (IF) (Dako); goat anti-mouse IgG HRP and donkey anti-rabbit IgG HRP (WB) (Jackson ImmunoResearch); donkey anti-mouse IgG (H+L) Alexa Fluor 488; goat anti-rabbit IgG Alexa Fluor 568, and donkey anti-mouse IgG (H+L) Alexa Fluor 568 (IF) (ThermoFisher Scientific); and mouse Zenon AF488 (IF) (ThermoFisher Scientific).

2.2 Analysis of Protein

Western blot analysis of steady-state protein levels were performed on cells lysed by sonication in lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.5% NP-40). Protein from lysates were resolved by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by semi-dry transfer to PROTRAN nitrocellulose membrane (GE Healthcare Life Sciences). Blocking was completed in 5% milk in phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-T). Membranes were incubated in primary antibody followed by a secondary antibody conjugated to HRP, both diluted in 5% milk in PBS-T. Antibodies were detected with ECL (GE Healthcare Life Sciences).

For immunofluorescence analysis, cells were plated approximately 50,000 cells per well in 24-well dishes containing glass cover slips. Immunofluorescence was initiated by fixing and permeabilizing cells in 100% methanol at -20°C for 1 hr or fixed in 4% paraformaldehyde for 15 minutes at room temperature followed by permeabilizing with 0.1% Triton-X for 15 minutes at room temperature. Blocking was performed with 3% BSA PBS-T for 30 min. Primary antibody was diluted in 3% BSA PBS-T was added for 1.5 hr. at RT then washed off three times with PBS-T. Secondary antibody conjugated to Alexa Fluor was diluted in 3% BSA PBS-T and incubated on cells for 30 min. Cover slips were put onto glass slides in ProLong Gold antifade reagent (ThermoFisher Scientific). Morphology was quantified at 200X and images were obtained at 400X on a Nikon Eclipse TS100 inverted microscope.

2.3 Cell Toxicity Assay

NPCs were treated with increasing concentrations of MBV from 1 to 40 μM . Cells were lifted from plates using trypsin, pelleted by centrifugation, and resuspended in a 1:20 dilution of crystal violet (EMD Millipore). Toxicity was determined by counting 100 cells from two biological replicates and scored as crystal violet positive (toxic) or negative. Cells were counted on a Nikon Eclipse TS100 inverted with the 10X objective lens.

2.4 Statistical Analysis

The data are representative of at least two independent experiments, and values are given as the mean of replicate experiments $\pm$ standard deviation. Viral titers were evaluated using the Students t-test ($* < 0.05$) while changes in lineage markers was evaluated using the Chi-squared analysis and Holm-Sidak test. An asterisk indicates a significant value of < 0.05 and all quantitative relationships were evaluated for significance.

3. Results

3.1 HCMV infection of EZ-sphere-derived neuronal progenitor cells

NPCs can be derived from multiple sources and methods including fetal tissue, induced pluripotent stem (iPS) cells, or embryonic stem (ES) cells. For these studies, we used EZ-spheres generated from NIH-approved H9 ES cells cultured in feeder free conditions and neuronal stem cell media (29). EZ-spheres contain pre-rosette NPCs that retain long-term potential to differentiate into diverse central and peripheral neural lineages following directed differentiation. We evaluated EZ-spheres using a subset of neuronal markers to confirm their undifferentiated state in our hands. The cells were dissociated, plated and

analyzed by immunofluorescence using antibodies against the NPC marker, Nestin, glial marker GFAP and the neuronal marker, Tuj1. We determined the number of antigen positive cells in the population relative to the total number of DAPI-positive cells in the field at 24 hr. after plating (Fig. 1A). We observed that 79% of the cells express Nestin (Fig. 1A) which is consistent with previous studies using EZ-spheres (29). We detected 3% of cells expressing GFAP and 0.4% expressing Tuj1 (Fig. 1A). Next, we determined whether the EZ-sphere-derived NPCs were susceptible to infection by HCMV. NPCs were dissociated and cultured for 24 hr. and then incubated with HCMV clinical isolate TB40/E at a multiplicity of 1 IU/cell as determined using fibroblasts. At 48 hpi, we assessed IE1 expression by immunofluorescence analysis and observed that 72% of Nestin-positive cells expressed IE1 (Fig. 1B). These results indicate that EZ-sphere-derived NPCs are predominantly undifferentiated and are susceptible to HCMV infection.

3.2 Maribavir inhibits HCMV replication in EZ-sphere derived NPCs

We were interested in determining whether the antiviral agent, maribavir (MBV) could inhibit infection of EZ-sphere NPCs. To determine the impact of MBV on cell viability, we incubated dissociated EZ-spheres with varying concentrations of MBV from 1.25 μ M to 40 μ M MBV for 24 hr. We tested for cytotoxic effects by staining for viability using Trypan-Blue and quantifying the percentage of positive cells. At 2.5 μ M MBV, we observed 0.8% Trypan-Blue-positive cells, while at 1.25 μ M MBV we did not observe Trypan-Blue-positive cells (Fig. 2A). At 5, 10, 20 and 40 μ M MBV, we detected 2.0%, 3.8%, 8.9% and 24.9%, respectively (Fig. 2A). Based upon these results, we have used 1 μ M of MBV in the remaining studies which has been shown to inhibit infection in other cell types and will have little impact on NPC viability.

To test the effect of MBV on steady-state levels of viral proteins, we infected cells with 1 IU/cell and treated with 1 μ M MBV or vehicle control. Cells were collected over a time course from 24 to 72 hpi, and lysates were analyzed by Western blot to assess steady state levels of immediate early protein IE1, early protein pUL44 and late protein pp65 (Fig. 2B). We were able to detect these proteins with similar kinetics of expression as observed in infection of other types of NPCs (15). pUL44 steady-state levels were decreased upon MBV treatment at 48 and 72 hpi and pp65 levels were reduced at 72 hpi (Fig. 2B). These data demonstrate the efficacy of MBV to disrupt steady-state levels of viral proteins in HCMV-infected NPCs. Fetally-derived NPCs produce cell-free virus to high titer as early as 3 days post infection (dpi) (15) while little to no cell free virus was detected in Hues19 ES cell-derived NPCs at 5 dpi (14). We assessed the impact of MBV on cell-free virus yield in EZ-sphere-derived undifferentiated NPCs. Cells were infected with 1 IU/cell and treated with 1 μ M MBV or vehicle control. We collected cell-free virus from infected NPCs over a 28 day time course then measured viral yields using fibroblasts by determining IE1-positive cells at 48 hpi. We observed increasing amounts of infectious cell-free virus starting at 4 dpi (Fig. 2C). In contrast, MBV treatment resulted in significant reductions in viral yields, with 1.4-log, 2.5-log and 2.5-log reductions observed at 12, 16 and 28 dpi, respectively (Fig. 2C). We also measured changes in NPC-associated virus at 13 and 28 dpi observing 1.5-log and 1.2-log reductions upon MBV treatment (Fig. 2D) and treated cells failed to exhibit HCMV-induced cytopathic affect out to 28 dpi (Fig. 2E). Our results demonstrate that the antiviral

agent MBV disrupts HCMV replication and viral yield in undifferentiated EZ-sphere-derived NPCs.

3.3 MBV partially protects against HCMV disruption of NPC differentiation

NPCs can differentiate into diverse cell types by altering the culture conditions (29). Next, we were interested in determining the impact of MBV on viral yield and NPC differentiation into glial and neuron cell populations. Using HCMV TB40/E expressing mCherry, we infected undifferentiated NPCs at 1 IU/cell. The cells were induced to differentiate by culturing in the absence of both EGF and FGF out to 28 days and were continuously treated with 1.0 μ M MBV or vehicle control. At 13 and 28 days, we quantified changes in viral yields and observed 1.2-log and 1.6-log reductions, respectively, with MBV treatment (Fig. 3A). mCherry was observed in 79% of cells at 13 days and 85% at 28 days, while the addition of MBV reduced the percentage to 12% at 13 days and 19% at 28 days (Fig. 3B). We also quantified changes in markers of differentiation (Fig. 3C-E) by immunofluorescence using antibodies against the NPC marker nestin, neuronal marker Tuj1 and glial marker GFAP (Fig. 4). Upon removal of EGF/FGF, the percentage of nestin positive cells was 4.7% at 13 days and 3.5% at 28 days (Fig. 3C) as compared to 78% in undifferentiated NPCs (Fig. 1A). HCMV infection significantly reduced the percentage to 0.6% and 0.5% at 13 and 28 days, respectively, and this change was prevented using MBV (Fig. 3C). For the neuronal marker Tuj1, removal of EGF/FGF resulted 3.3% and 11.3% positive cells at 13 and 28 days, respectively (Fig. 3D) as compared to 0.4% in undifferentiated NPC (Fig. 1A). Again, HCMV infection reduced expression to 0.6% at 13 days and 3.9% at 28 days and these changes were prevented by MBV (Fig. 3D). We also assessed the glial marker GFAP which rose from 2.9% positive NPCs (Fig. 1A) to 7.6% at 13 days and 26.4% at 28 days during differentiation (Fig. 3E). Infection reduced GFAP expression to 0.7% and 1.5% positive cells at 13 and 28 days, respectively (Fig. 3E). In contrast to nestin and Tuj1, however, MBV only partially protected against infection-induced changes in GFAP with 12.6% positive cells at 28 days (Fig. 3E). For all three markers, the percent change in lineage markers upon differentiation is consistent with previous studies using EZ-sphere-derived NPCs (29, 32, 33) and MBV did not significantly alter the percentage positive cells in uninfected cells compared to vehicle control (Fig. 3C-E). We conclude that MBV treatment inhibits virus production during NPC differentiation while providing significant protection against HCMV-mediated disruption of early lineage markers.

4. Conclusion

The majority of work studying aspects of congenital HCMV infection in cell culture has been performed using fetally-derived NPCs. ES-derived NPCs provide the advantage of using a consistent cell line and for which advanced differentiation techniques have been developed (14, 16, 29). For these studies, we used EZ sphere-derived NPCs that can generate motor neurons, dopamine neurons, striatal neurons, sensory neurons, and oligodendrocytes through several weeks of differentiation (29). We demonstrate that EZ-sphere-derived NPCs represent a suitable cell culture model of congenital HCMV infection and provide a means to evaluate how infection influences NPCs. These cells are fully permissive to HCMV infection, with infected cells demonstrating a high percentage of cells expressing HCMV

IE1 (Fig. 1B). Previous studies have shown that cell free virus yield is low in ES-NPCs at 5 dpi (14). We confirmed these results, but found that cell free viral yield increases after 5 dpi and continues out to at least 28 dpi (Fig. 2B). EZ-sphere-derived NPCs supported viral replication and release but with substantially delayed kinetics when compared to human fibroblasts. Treatment of infected NPCs with antivirals that target viral DNA synthesis partially restores NPC proliferation and differentiation (18). Those drugs which include ganciclovir and foscarnet have been shown to be teratogenic in animal studies and have significant toxicity in children and adults (34, 35). The pUL97 kinase inhibitor, maribavir (MBV) has low toxicity, is not genotoxic, and was not teratogenic in limited animal studies (23, 25). Our work, and studies from other labs have shown that inhibition of pUL97 results in decreases in viral gene expression, viral DNA synthesis and viral yield (36). The low toxicity and action of MBV at multiple stages of infection make it an attractive candidate for treatment of congenital CMV and treatment of infants following congenital CMV infection. We found that MBV treatment of infected NPCs decreased steady-state levels of viral proteins and resulted in significant decreases in cell-free and cell-associated viral yields (Fig 2B and C). Furthermore, MBV inhibited infection during NPC differentiation into neural and glial populations while maintaining lineage-specific markers. Our data suggests that MBV may be acting to decrease viral spread during differentiation. This is evidenced by an observed reduction in the percentage of cells expressing mCherry (Fig. 3B), decreased cell free viral yield in differentiating cells (Fig. 3A), and the known role of pUL97 in viral nuclear egress (37). MBV may be acting to decrease viral spread to uninfected cells, allowing those cells to differentiate. Alternatively, MBV may promote an abortive infection or limit initiation of replication in newly infected cells. Future studies are needed to identify the effect of MBV on survival and normal differentiation of the HCMV infected NPCs. Whatever the mechanism, it has been shown that infection of NPCs with HCMV limits their multipotency (18, 19). The impact of MBV on decreasing the percentage of infected NPCs could be profoundly beneficial in a developing brain during congenital CMV infection (15, 20). In the absence of infection, MBV exhibited little toxicity to NPCs (Fig. 2A) and did not alter expression of lineage markers during differentiation (Fig 3B-D). These data suggest that MBV could be an effective antiviral agent for inhibiting HCMV in NPCs. Future studies of NPC proliferation and differentiation during infection treated with MBV could provide the impetus for studying MBV as an antiviral agent for congenital HCMV disease.

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Highlights

- EZ-sphere-derived neuronal progenitor cells are fully susceptible to HCMV infection.
- The HCMV kinase inhibitor maribavir disrupts infection in undifferentiated NPCs.
- Maribavir protects NPCs while maintaining early steps in differentiation.

Figure 1. MBV disruption of HCMV infection of ESC-derived neuronal progenitor cells
 (A) Neural progenitor cells (NPCs) were derived from the H9 human embryonic stem (ES) cell line. Progenitor cells were evaluated by immunofluorescence staining for the NPC marker, Nestin, the glial cell marker, GFAP and the neuronal marker, Tuj1. Antigen positive cells were determined from five independent fields and presented as percentage of positive cells per DAPI-stained nuclei. Error bars represent standard deviation from the five fields.
 (B) NPCs were infected with HCMV strain TB40/E at 1 infectious unit per cell. Cells were fixed at 48 hpi and stained with antibodies against HCMV IE1 and Nestin. Scale 20 μ m. Antigen positive cells were quantified as IE1 positive cells per Nestin positive cells, averaged from three biological replicates. Errors bars represent standard deviation from the three biological replicates.

Figure 2. MBV disrupts HCMV infection in undifferentiated NPCs

(A) NPCs were treated with the indicated concentrations from 1.25 - 40 μ M of MBV for 24 hr. Cell viability was determined using Trypan Blue staining and the data are presented as the percentage of stain-positive cells per total cells. (B) NPCs were infected at 1 IU/cell using TB40/E and treated with 1 μ M MBV or equivalent volume of DMSO control. Levels of HCMV protein expression were analyzed by Western blot at multiple times post infection. (C) Viral titers were determined from cell-free culture supernatants of HCMV-infected NPCs using human fibroblasts. Error bars represent standard deviation of two or three (28 dpi) biological replicates and significance determined by Students t-test (* <0.05). (D) Cell-associated virus was isolated from infected NPCs and viral titers were determined as described in (C) from three biological replicates. (E) Representative brightfield images show the absence of cytopathic affect in MBV-treated HCMV-infected NPCs. Scale 20 μ m.

Figure 3. The impact of HCMV infection and MBV on NPC differentiation

(A and B) NPCs were infected at 1 IU/cell using HCMV TB40/E expressing mCherry. NPCs were then induced to differentiate by culturing cells in the absence of EGF and FGF with 1.0 μ M MBV or DMSO control. Viral titers were determined from culture supernatants of HCMV-infected NPCs using human fibroblasts (A) and percentage of mCherry positive cells per DAPI were determined from approximately 250 cells from three biological replicates (B). Experiments were performed in biological triplicate and error bars represent standard deviation from the mean. (C) Cells were evaluated by immunofluorescence staining for the NPC marker Nestin at 13 and 28 days. The data were collected from counting approximately 250 cells from five independent fields and are presented as the percentage of Nestin-positive cells per DAPI-positive cells. Significance was determined using the Chi-squared analysis and Holm-Sidak test (* <0.05). Cells were evaluated for Tuj1 (D) and GFAP (E) expression as described in above. Error bars represent the standard deviation from the mean of the five independent fields.

Figure 4. Immunofluorescence analysis of NPC markers after differentiation

Undifferentiated NPC were infected using TCMV TB40/E expressing mCherry and induced to differentiate by removal of EGF and FGF from the culture media. Both uninfected and infected NPCs were treated using 1.0 μ M MBV or DMSO. After 28 dpi, cells were evaluated using antibodies against Nestin, Tuj1 and GFAP.