Neuroblastomas vary widely in their sensitivities to herpes simplex virotherapy unrelated to virus receptors and susceptibility

P-Y Wang1, HM Swain1, AL Kunkler1, C-Y Chen1, BJ Hutzen1, MA Arnold2, KA Streby3, MH Collins4, B Dipasquale4, JR Stanek3, J Conner5, TH van Kuppevelt6, JC Glorioso7, P Grandi7 and TP Cripe1,3

Although most high-risk neuroblastomas are responsive to chemotherapy, relapse is common and long-term survival is < 40%, underscoring the need for more effective treatments. We evaluated the responsiveness of 12 neuroblastoma cell lines to the Δγ134.5 attenuated oncolytic herpes simplex virus (oHSV), Seprehvir (HSV1716), which is currently used in pediatric phase I trials. We found that entry of Seprehvir in neuroblastoma cells is independent of the expression of nectin-1 and the sum of all four known major HSV entry receptors. We observed varying levels of sensitivity and permissivity to Seprehvir, suggesting that the cellular anti-viral response, not virus entry, is the key determinant of efficacy with this virus. In vivo, we found significant anti-tumor efficacy following Seprehvir treatment, which ranged from 6/10 complete responses in the CHP-134 model to a mild prolonged median survival in the SK-N-AS model. Taken together, these data suggest that anti-tumor efficacy cannot be solely predicted based on in vitro response. Whether or not this discordance holds true for other viruses or tumor types is unknown. Our results also suggest that profiling the expression of known viral entry receptors on neuroblastoma cells may not be entirely predictive of their susceptibility to Seprehvir therapy.

INTRODUCTION

Neuroblastoma is the most common extra-cranial solid tumor of childhood. These tumors arise from the embryonic sympathoadrenal lineage of the neural crest and display extreme genetic and phenotypic heterogeneity. Neuroblastoma is a major cause of death for pediatric patients between ages 1 and 5, and is responsible for ~ 15% of childhood cancer-related deaths in the United States.1 Although most high-risk cases of neuroblastoma are responsive to chemotherapy and complete responses can be achieved, relapses are also fairly common and only 40% of patients survive long term. More effective treatments for this disease are clearly needed.

Oncolytic herpes virotherapy is an emerging form of therapy that uses attenuated herpes viruses to selectively infect and destroy cancerous tissue. Over the past decade, numerous preclinical models and clinical trials have demonstrated the efficacy and safety of this approach,2,3 including a successful phase III trial with the oncolytic herpes simplex virus (oHSV) Talimogene laherparepvec.4 Current oHSV vector designs mutate or selectively express the virulence factor γ134.5 as a means of restricting toxicity to tumor cells. Most oHSV vectors also retain their thymidine kinase gene (tk), making them susceptible to anti-herpetic agents in the unlikely event of an off-target infection.5

HSV has a wide range of cellular tropism, partly due to its ability to interact with multiple entry receptors. These include the cellular adhesion molecules, nectin-1 and nectin-2 (for certain mutant strains), various moieties of heparin sulfate (HS), and tumor necrosis factor-receptor superfamily member 14, otherwise known as herpes virus entry mediator (HVEM).6 The entry process begins when an enveloped virus attaches to HS on the host cell surface via envelope glycoproteins B and C (gB and gC). This interaction allows the major receptor-binding protein, glycoprotein D, to engage one of its receptors, triggering fusion of the viral envelope with the host cell membrane and the subsequent release of the virion into the cytosol. The virion can then traffic toward the cell nucleus, allowing transcription of the viral genome and the initiation of virus replication. Our laboratory and others have previously shown that HSV entry receptor expression on various cell types correlates with or determines their susceptibility to HSV infection.7–10 More recently, Jackson et al.11 reported that increasing nectin-1 expression in cell lines derived from malignant peripheral nerve sheath tumors enhanced the replication of wild-type HSV, but had no effect on the replication of Δγ134.5 viruses. Their study implies that cellular sensitivity to killing by such oHSV mutants is less dependent on the expression of virus entry receptors (susceptibility to infection) than it is on the host cell’s ability to mount an anti-viral response (permissivity to infection).

Several studies have investigated the use of oHSV therapy in treating preclinical models of neuroblastoma and have shown significant anti-tumor efficacy with both γ134.5-intact and Δγ134.5 viruses.12–14 Whether the expression of viral entry receptors on neuroblastoma cells contributes to the susceptibility (virus entry), sensitivity (virus-mediated cytototoxicity) and/or permissivity (productive virus infection) has not been thoroughly examined. In the present study, we evaluated the response of a panel of...
human neuroblastoma cell lines and preclinical neuroblastoma models to Seprehvir (a Δγ134.5 virus) treatment and examined the relationship between HSV entry receptor expression and anti-tumor efficacy. We show that human neuroblastoma cells express varying amounts of the known major HSV entry receptors with nectin-1 and 3-OS HS found in the greatest abundance. Conversely, levels of HVEM were virtually undetectable. The relative expression of any given HSV receptor by each cell line did not correlate with its sensitivity and susceptibility to Seprehvir in vitro. Our *in vivo* studies suggest that anti-tumor efficacy only partially correlates with virus production in the tumor. Furthermore, factors other than direct oncolysis, such as the induction of an innate immune cell response, may also contribute to the anti-tumor effect.

**RESULTS**

**oHSV entry, sensitivity and susceptibility in neuroblastoma cells**

We evaluated 12 human neuroblastoma cell lines for oHSV therapeutic potential in this study (see Supplementary Figure S1 for demographic details). We first examined the ability of oHSV to enter each neuroblastoma cell line (classically defined as susceptibility). We infected each cell line with the clinical-grade oHSV Seprehvir (HSV1716) at a multiplicity of infection (MOI) of 20 infectious virus particles per cell. After 30 min of infection, the cells were washed with phosphate-buffered saline (PBS) and an acidic buffer solution to remove any bound, but non-internalized virus. We then isolated genomic DNA from each sample and determined the amount of HSV that had entered the cells via quantitative PCR (qPCR), normalizing copies of the HSV-tk to the house-keeping gene *gapdh*. Our panel of neuroblastoma cell lines showed a wide range of susceptibility to Seprehvir infection, with the most susceptible cell lines (CHP-134 and CHLA-20) showing a five-fold increase in virus entry over the least susceptible cell lines (SH-SY5Y and NB-EBc1; Figure 1a). These findings correlated well with virus entry studies using K26GFP, a wild-type KOS-strain oHSV that shares an identical gD protein (based on amino acid sequence) with Seprehvir (Supplementary Figures S2 and S3).

Next, we evaluated the cytotoxicity and viral replication of Seprehvir within the 12 neuroblastoma cell lines. We quantified in vitro cytotoxicity with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell survival assays conducted 6 days post-virus infection. Representative cytotoxicity data from four of these cell lines (CHLA-20, CHP-134, SK-N-AS and SK-NBE2) are shown in Figure 1b, and illustrate a range of varying sensitivities to Seprehvir treatment. For comparative purposes, the IC50 values for the remaining neuroblastoma cell lines were graded and summarized in Figure 1c. To measure cell permissivity to virus, we evaluated Seprehvir replication in the same four cell lines at multiple timepoints following infection. In general, each cell line showed a steady increase in virus production over time, and the

---

**Figure 1.** Neuroblastoma cell lines display differential responses to oHSV treatment *in vitro*. (a) *In vitro* virus entry assay. Cells were infected with Seprehvir (MOI = 20) for 30 min before genomic DNA isolation and qPCR analysis. Data are presented as HSV-tk gene copies per *gapdh*. Error bars represent s.e.m. (n = 6). (b) *In vitro* cytotoxicity of oHSV in neuroblastoma cells. Cells were infected with Seprehvir at MOIs of 0.001, 0.01, 0.1 and 1.0 and MTS viability assays were performed 6 days post-virus infection. Cell survival is presented as the percent of viable cells compared with uninfected controls. Data are presented as a mean plot to compare cell survival among MOI and cell group with 95% confidence limits (s.e., n = 6). (c) Day 6 IC50 of Seprehvir for the 12 neuroblastoma cell lines. +++ indicates IC50 falls between MOI 0.001–0.01; ++ means IC50 falls between MOI 0.1–1; + indicates IC50 falls between MOI 0.01–0.1; − represents IC50 > MOI = 1. (d) *In vitro* virus replication assay. Neuroblastoma cells were treated with Seprehvir at MOI = 0.01 and virus yields were determined by plaque assays performed 2, 24, 48 and 72 h post-virus infection. Data are presented as a mean plot for virus titer over time with 95% confidence limit (s.e., n = 9). Linear Mixed Model with Tukey-adjusted P-values is used for multiple comparisons. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
extents of this replication correlated well with each cell line’s observed cytotoxicity; CHLA-20, the least sensitive of the neuroblastoma cell lines, showed only a <2log increase in Seprehvir replication compared with the ~3.5log increase seen in the more susceptible CHP-134 line (Figure 1d). Based on our observations, human neuroblastoma cells appear to respond differently to Seprehvir treatment in vitro, with virus replication being more closely correlated to cytotoxicity than virus entry.

Evaluation of HSV-1 entry receptor expression in human neuroblastoma cell lines

As HSV entry mainly relies on the binding of its surface glycoproteins to their cognate cellular receptors, we reasoned that it would be important to evaluate the expression of HSV-1 entry receptors in primary human neuroblastoma cases and further study their relationship with the overall response to oHSV therapy. To this end, we evaluated the expression of nectin-1 in a neuroblastoma tissue microarray obtained from Children’s Oncology Group (COG). This tissue microarray contained primary neuroblastoma tumor specimens from 56 individual patients with different stages of disease. Figure 2 shows representative nectin-1 immunohistochemical staining for several neuroblastoma variants, including both differentiated and undifferentiated forms of disease, a comparatively less aggressive ganglioneuroblastoma, and a benign ganglioneuroma. We assigned grades of nectin-1 expression for each specimen, ranging from negative to 3+. These data are summarized in Table 1. In total, 97% of the tested neuroblastoma specimens were found to express nectin-1 with the expression grade predominantly >2+, a finding consistent with previously reported data obtained from a smaller number of clinical specimens.14 Nectin-1 expression levels in these neuroblastoma specimens did not appear to be associated with the disease stage, N-myc gene amplification status, age or sex (data not shown). We also profiled HSV entry receptor expression levels in our panel of neuroblastoma cell lines. The relative abundance of nectin-1, nectin-2, HVEM and 3-OS HS was determined by quantitative reverse transcription PCR (Figure 3a) and flow cytometric analysis (Figure 3b). A heat map of these data, representing cell surface protein expression as quantified by mean fluorescence intensity values, is shown in Figure 3c. Each cell line expressed relatively high levels of 3-OS HS and moderate to high levels of nectin-1. Levels of nectin-2 and HVEM were considerably lower or absent altogether, suggesting that these receptors do not have significant roles in mediating HSV entry in neuroblastoma. In general, receptor RNA expression correlated well with surface protein expression, however we did observe some slight discordance with nectin-1 levels among the intermediate expressing cell lines. Likewise, the cell surface expression of nectin-2 by SK-N-SH, which we expected to be high based on quantitative reverse transcription PCR, was instead only moderate. Despite the high level of 3-OS HS in most neuroblastoma lines in the panel, we only detected relatively low levels of heparan sulfate 3-O-sulfotransferase 3 (HS3ST3), which previous studies used as an surrogate marker for 3-OS HS,15 indicating that other heparan sulfate 3-O-sulfotransferase isoforms16 might be responsible for this receptor expression on neuroblastoma cells. We also mined the Pediatric Preclinical Testing Program (PPTP) gene expression database and found that most neuroblastoma xenograft tumors in that panel express relatively high levels of nectin-1 (Supplementary Figure S4, yellow columns, mRNA Z-score >0) at the RNA level compared with other pediatric cancer models in the panel. Consistent with our findings, the PPTP database also showed lower than average expression of HVEM in neuroblastoma cells (Supplementary Figure S4, green columns, mRNA Z-score <0). The mRNA levels of HS3ST3A1 and HS3ST3B1 were also low in this panel (Supplementary Figure S4, blue and purple columns). Given the fact that nectin-2 is selectively used by HSV-2 and only a subset of HSV-1 GD mutant strains19,20 as well as the lack of HVEM expression in neuroblastoma cells, these data suggest that oHSV entry in neuroblastoma cells is mainly through nectin-1 and/or 3-OS HS. Nevertheless, the expression profile of nectin-1 or the sum of all four receptors did not correlate with virus entry or virus-mediated cytotoxicity as shown in Figure 3d. This discordance suggests that virus entry in neuroblastoma cells might involve additional unknown mediators and/or mechanisms.

Anti-tumor efficacy of oHSV in xenograft neuroblastoma models

To determine the susceptibility of neuroblastoma tumors to oHSV therapy in vivo, four neuroblastoma lines were selected for survival studies based on their distinct HSV receptor profiles (shown in Figure 3b), N-myc gene amplification status and the duration of successful tumor engraftment in athymic nude mice (Supplementary Figure S1, highlighted). We treated mice with subcutaneously implanted tumors with intratumoral injection of 1e7 plaque-forming units (pfu) of Seprehvir for a total of 3 doses at days 0, 2 and 4 when their tumor volumes reached 150–300 mm3. Kaplan–Meier survival curves for the CHLA-20, CHP-134, SK-N-AS and SK-N-BE(2) models are shown in Figures 4a–d, respectively, with the corresponding tumor growth plots for each animal included in Figures 4e–h. Consistent with the in vitro killing assay, Seprehvir-treated CHP-134-bearing mice showed the greatest anti-tumor efficacy (Figure 4b) with complete responses achieved in six out of ten mice (Figure 4f). Seprehvir treatment in SK-N-AS tumors, the second most responsive line among the 12 in vitro, only slowed tumor growth, prolonging median survival from 14 to 19 days (Figures 4c and g). Interestingly, tumors derived from CHLA-20, the least sensitive in vitro, had a significant anti-tumor response to Seprehvir treatment, leading to complete responses in three out of seven mice (Figures 4a and e). The remaining neuroblastoma model, SK-N-BE(2), initially showed some evidence of efficacy following Seprehvir treatment, but six out of seven treated animals eventually succumbed to excessive tumor growth (Figures 4d and h).

Separate sets of tumor-bearing animals were used to assess in vivo Seprehvir replication. Unlike the animals in the previous efficacy studies, these mice received only a single 1e7 pfu dose of Seprehvir and were sacrificed at 3, 24, 72 and 144 h thereafter. We then excised and processed their tumors to quantify virus production via HSV plaque assays (Figure 5). Similar to our in vitro observations (Figure 1d), Seprehvir replication was greatest in CHP-134 tumors, suggesting that the efficacy we observed in this model may be more dependent on direct oncolysis. Conversely, Seprehvir replication in the highly aggressive SK-N-AS model declined nearly 10-fold between 24 and 72 h post infection, indicating that virus spread or replication in these tumors may be inadequate to slow tumor growth.

Because type I interferons are known to inhibit virus replication, we also sought to determine if differences in cellular responsiveness to exogenous type I interferons (IFN) might explain differential virus production. We performed analyses of the IFN response in these four cell lines by adding exogenous human IFN (mouse type I IFNs do not cross-react with human cells21) and evaluating the induction of IFN-stimulated genes (ISGs). On this basis, we found that CHP-134 appears to be the least sensitive to IFN-beta treatment, which may partially explain why it is the most sensitive line both in vitro and in vivo (Supplementary Figure S5). However, our results do not adequately explain the disconnection between in vitro and in vivo efficacy observed in SK-N-AS, which displayed substantial induction of all five tested ISGs upon IFN-beta treatment. Therefore, we think the IFN response may only partially explain the differences of how cells react to virus treatment in our study, and other factors, such as infiltrating immune cells and the concomitant cytokine response may also have a role.
DISCUSSION
The cure of high-risk neuroblastoma patients remains an ongoing challenge. Despite significant advancements in our understanding of this disease and its management, overall survival rates remain unacceptably low and provide an impetus to develop novel and more effective therapies. Attenuated oncolytic herpes viruses such as Seprehvir are promising therapeutic agents not only because they can safely and selectively destroy cancerous cells and release progeny virus to do likewise (the central paradigm of oncolytic virotherapy), but also because of their ability to stimulate an antitumor immune response. Two ongoing clinical trials are investigating the use of Seprehvir in young patients with either CNS

Figure 2. Nectin-1 expression by different primary neuroblastoma specimens. Representative H&E and nectin-1 immunohistochemical staining for (a and e) differentiated neuroblastoma, (b and f) undifferentiated neuroblastoma, (c and g) ganglioneuroblastoma and (d and h) ganglioneuroma.
In this study we evaluated a panel of 12 human neuroblastoma cell lines for their expression of HSV entry mediators and assessed their responses to Seprehvir treatment. Most cells showed high expression of nectin-1 and 3-OS HS, two HSV entry receptors that have been shown to be important determinants of HSV susceptibility in various cell types. Unexpectedly, we found that HSV receptor expression did not necessarily correlate with virus entry. For example, the neuroblastoma cell line NB-1643, which had the highest overall expression of nectin-1 in addition to high levels of 3-OS HS, fell into the relatively low virus uptake group (Figures 1a and 3c). Likewise, IMR-32, one of the highest 3-OS HS-expressing cells, also showed lower virus uptake (Figures 1a and 3c). Although the PCR-based assay we used for virus entry study only measures an average value over the entire population of cells, we consistently observe a Poisson distribution of gene expression when using flow cytometry and GFP-expressing oHSVs (not shown). Thus, some cells are more susceptible than others and our assay does not allow us to correlate virus entry with permissivity on a cell-by-cell basis.

Table 1. Nectin-1 expression on NB tissue microarray

| Clinical Information | Number of cases |
|----------------------|-----------------|
| Histology            | Stage           | Cell type | 0 | 1+ | 2+ | 3+ |
| Neuroblastoma        | I               | Neuroblast | 5 | 4  |    |    |
|                      | II              | Neuroblast | 1 | 4  | 1  | 5  |
|                      | III             | Neuroblast | 2 | 5  |    |    |
|                      | IV              | Neuroblast | 1 | 3  | 6  | 16 |
|                      |                | Neuropil   | 1 | 4  | 6  | 15 |
| Ganglioneuroma       | NA              | Ganglion   | 2 |    |    |    |
| Ganglioneuroblastoma |                | Neuroblast | 3 |    |    |    |
| Tonsil               | NA              | NA         | 2 |    |    | 3  |
| Total NB cases       |                 |            | 2 | 4  | 17 | 33 |
| Percentage           |                 |            | 3 | 7  | 30 | 59 |

Abbreviations: NA, not applicable; NB, neuroblastoma.

Figure 3. HSV entry receptor expression in the neuroblastoma cell line panel. (a) Quantitative reverse transcription PCR analysis for mRNA expression of nectin-1, nectin-2, HVEM and HS (glucosamine) 3-O-sulfotransferase 3 (HS3ST3, surrogate marker for 3-OS HS). Data are presented relative to Gapdh. Error bars represent s.d. (n = 3). Linear mixed model with Tukey-adjusted P-values is used for multiple comparisons. The expression levels of nectin-1, nectin-2 or HS3STS in the indicated cell lines are significant higher than the rest. ****P < 0.0001. (b) Representative flow cytometric analysis of the HSV entry receptors in four select neuroblastoma cell lines. (c) A heat map summarizing the surface HSV-1 receptor expression profile of the 12 neuroblastoma cell lines. Data are presented as average mean fluorescence intensities (MFI) of nectin-1, nectin-2, HVEM and 3-OS HS relative to isotype or unstained controls from three independent results as determined by flow cytometric analysis. (d) Correlation of nectin-1 or the sum of HSV entry receptors expression with Seprehvir entry and cytotoxicity in neuroblastoma cell panel. Pearson’s correlation coefficients were calculated between nectin-1/the sum of all four HSV entry receptors expression and virus entry (Figure 1a) or cell survival following Seprehvir treatment (Figure 1c).
Nevertheless, our finding of a discordance of receptor expression with entry in cells on average could indicate that (i) other unknown entry receptors may be expressed and used in neuroblastoma cells or, (ii) nectin-1 or other receptor-mediated viral entry pathways do not function properly in some of these neuroblastoma lines. Interestingly, although we did not see a correlation between Seprehvir entry and cytotoxicity, we did find a correlation between entry of the wild-type (γ134.5-intact) K26GFP virus and viral gene transfer (GFP) (Supplementary Figures S2 and S3b). This finding suggests that something other than susceptibility (entry) is responsible for sensitivity to Seprehvir killing, such as cell-autonomous anti-viral responses, which would be in agreement with a recent report from Jackson and colleagues. Whatever the case, profiling neuroblastoma tumor cells for currently known HSV entry receptors does not appear predictive for determining the degree of their susceptibility to oHSV.

We observed similar variability in vivo, where peak levels of Seprehvir replication in SK-N-AS tumors were comparable to those achieved in the better responding CHLA-20 and SK-N-BE(2) tumor models. We postulate that the failure of Seprehvir to induce a complete response in the SK-N-AS model may be due to the high intrinsic growth rate of these tumors relative to our other models (Figure 4, PBS-treated tumors), and that tumor cell proliferation may simply outpace virus replication in that model. Conversely, the relative resistance of CHLA-20 to Seprehvir in vitro may mainly due to its autonomous anti-viral responses as its in vitro growth rate is similar to SK-NBE(2) and relative slower than CHP-134 (Supplementary Figure S6).

Another possibility is that discrete differences amongst the tumor microenvironments of the neuroblastoma models may be influencing the therapeutic response. Preliminary cell recruitment studies performed in these four neuroblastoma models show that Seprehvir treatment induces the infiltration of differential leukocyte subsets, including many key components of the innate immune response (unpublished data). Further studies will need to be conducted to understand the contribution of each of these components in the anti-tumor activity of Seprehvir in neuroblastoma, but are beyond the scope of this present work.

In support of this concept, it is well established that wild-type HSV infection induces both innate and adaptive immune responses within the host, including the recruitment of immune effector cells and the production of inflammatory cytokines. Our lab and others have demonstrated that this response also occurs within the context of oncolytic herpes virotherapy. We previously showed that oHSV treatment of the A673 Ewing sarcoma model led to a concomitant influx of CD11b+ myeloid cells (monocytes/macrophages, granulocytes, natural killer cells and so on) into the tumor. The net effect of this cellular infiltration...
was a dampening of oHSV therapeutic efficacy, as tumor growth was further delayed when these CD11b+ cell populations were depleted.26 More recently, we demonstrated that tumor shrinkage in syngeneic rhabdomyosarcoma models following oHSV treatment was not mediated by direct oncolysis, but rather by the induction of anti-tumor T cells.21 Similar studies by Workenhe et al.28 suggest that the immunogenic cell death caused by HSV can lead to further activation of anti-tumor immunity (mainly via CD8+ T cells), which may be more important to a positive treatment outcome than the persistence of virus replication. The relative roles of virolysis versus immunologic response in tumor shrinkage has been a major issue for discussion in the field, which continues to pose a conundrum for preclinical testing; while human tumors are typically the most susceptible to human virus infection, animals bearing human tumor xenografts lack an adaptive immune response. Conversely, mouse tumors in immunocompetent animals are typically not very susceptible and/or permissive to human viruses. Certainly this difference exists for human HSVs. Thus studies in human xenografts typically only measure lytic or innate immunologic effects, whereas studies in mouse tumors primarily measure immunologic effects. To complicate matters further, there are species-specificities with respect to molecular interactions, particularly for the immune system. In light of these observations, we are presently conducting studies to better understand how the tumor microenvironment affects oHSV therapy in both xenograft and syngeneic in various pediatric tumor models.

In summary, our study demonstrates that Seprehvir is differentially effective amongst neuroblastoma cell lines and in vitro cytotoxicity is not completely predictive of in vivo therapeutic response. In vitro sensitivity appears to be less related to the host cell’s expression of putative HSV entry receptors than it is to permissiveness of virus replication. Although virus replication appears to be important for the in vivo neuroblastoma therapeutic response, it is also not the sole determinant of efficacy, and other factors such as the tumor microenvironment are likely modulating the anti-tumor response.

Figure 5. Seprehvir replication in neuroblastoma xenograft tumors. Subcutaneously implanted CHLA-20, CHP-134, SK-N-AS and SK-NBE (2) tumors were given a single intratumoral dose of 10^7 plaque-forming unit (pfu) Seprehvir and harvested at 3, 24, 72 and 144 h post infection for plaque assay. Data are presented as mean pfu per tumor from triplicate plaque assays of four tumors at each time point. Error bars represent s.d. (n = 12). Bonferroni corrected pairwise comparisons were made to look for differences in virus yields (PFU) amongst the four groups at 24 h. **p < 0.01. BDL, below detectable level.

RNA extraction, reverse transcription PCR and qPCR Total RNA was isolated from 1 × 10^5 cells using the RNeasy Plus Mini Kit (Qiagen) per manufacturer’s instructions. The concentration and purity of the recovered RNA was determined measuring the optical density at 260 and 280 nm. cDNA was generated using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific Inc.) per the manufacturer’s instructions. For qPCR, 5 μl of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific Inc.), 5 μl of 1:25 diluted cDNA and 500 nm of each primer of interest was used in a 10 μl reaction. The reaction was performed using a 7900 real-time PCR System (Thermo Fisher Scientific Inc.). The samples were run at 50°C for 2 min, 95°C for 10 min, 40 cycles of 94°C for 15 s, 58°C for 35 s, and 72°C for 20 s. The amplified product was detected using a melting curve analysis with a temperature range of 50°C to 95°C. The comparative quantitation method was used for data analysis. The results were presented as expression fold relative to GAPDH: 2−ΔΔCt. Primers used in the study were described previously.10 Results shown are representative of three independent experiments.

Flow cytometry
Single-cell suspensions of approximately 1 × 10^6 elements were treated with 10% FcR blocking reagent (130-059-901, Miltenyi Biotec Inc. Auburn, CA) for 10 min at 4°C and then stained with anti-human nectin-1 (R1.302.12) (sc-69718, Santa Cruz Biotechnology, Inc. Dallas, TX), nectin-2 (R2.525) (sc-32804, Santa Cruz Biotechnology, Inc.) or HVEM (CW10) (sc-21718, Santa Cruz Biotechnology, Inc.). After applying the unconjugated primary Abs or their isotype controls, cells were stained with a secondary fluorescein isothiocyanate-conjugated goat anti-mouse IgG (554001, BD Biosciences, San Jose, CA, USA). For 3-O-Sulfated Heparan Sulfate (3-O HS) analysis, cells were first fixed in 1% paraformaldehyde (PFA) for 10 min at 4°C followed by incubation with antibody HSAC4 (ref. 10) for 1 h at 4°C. Next, cells were stained with unconjugated mouse anti-IVS (PSD4; V5507, Sigma-Aldrich Corp., St. Louis, MO, USA) for 30 min at 4°C.
Finally, cells were stained with fluorescein isothiocyanate goat anti-mouse Ig (S54001, BD Biosciences) for 20 min at 4 °C. Analyses were performed on the BD LSRII flow cytometer (BD Biosciences) using FlowJo v.9.8.2 analyzing software (FlowJo, LLC, Ashland, OR, USA). The analysis has been performed on the neuroblastoma panel for three independent times. Average mean fluorescence intensities of nectin-1, nectin-2, HVEM and 3-OS HS relative to isotype (for nectin-1, nectin-2 and HVEM) or unstained controls (for 3-OS HS) were calculated and presented as a heatmap in Figure 3c.

Tissue microarray and immunohistochemistry

Neuroblastoma tissue microarray chips were obtained from Children’s Oncology Group (Nationwide Children’s Hospital, Columbus, OH, USA) that contained neuroblastoma specimens from 56 individual patients. For immunohistochemistry staining, the reagents described below were all from Ventana Medical Systems, Inc. Tucson, AZ, USA. After deparaffinization, slides were pre-treated with Cell Conditioning 1 (CC1, #950-124) for 90 min for antigen retrieval, incubated with the nectin-1 antibody (R1.302.12, Santa Cruz Biotech) at 1:10 dilution for 2 h at 42 °C, followed by blocking the slides for 8 min with blocking reagent (#760-1028). The slides were then subjected for detection using UltraView detection kit (#760-500) per manufacturer’s protocol, followed by staining with hematoxylin (#760-2021) for 4 min, and Bluing Reagent (#760-2037) for 4 min. The degree of nectin-1 staining intensity of tumor samples was graded negative as 0, weak as 1+, moderate as 2+ and strong as 3+, by board-certified pathologist, M.H.C.

Cell survival/MTS assay

Cells were plated in 96-well dishes at 4000 cells per well, incubated at 37 °C for overnight and then infected with Seprehvir at MOI 0.001, 0.01, 0.1 and 1.0 in hexaplicate. The assays were performed using Cell Titer96 AQueous Non-Radioactive Cell Proliferation Assay (G5421, Promega, Madison, WI, USA) per manufacturer’s instructions on days 2, 4 and 6. Results were presented as percent cell survival compared with uninfected controls. Results shown are representative of three independent experiments.

In vitro virus production

Neuroblastoma cell lines were plated in 12-well dishes at 5 × 10^4 cells per well in triplicate, incubated at 37 °C for over-night, and infected with Seprehvir at MOI 0.01. Plates were gently shaken for 2 h at 42 °C, 2 h at 24, 48, and 72 h post infection, both cells and supernatants were harvested, freeze-thawed three times, diluted and titered by standard plaque assay on Vero cells.

Animal studies

Animal studies were approved by the Nationwide Children’s Hospital Institutional Animal Care and Use Committee (IACUC), protocol number AR12-00045. To establish tumors, 5 × 10^7 of CHLA-20, CHP-134, SK-N-AS and SK-N-BE(2) cells were injected subcutaneously with 33% Matrigel (356234, BD Biosciences) into the flanks of 6–8-week-old female athymic nude mice (Harlan Laboratories, Inc. Indianapolis, IN, USA). When tumors reached 150–300 mm³, animals were pooled and randomly assigned into two groups. One group of mice was treated intratumoral with Seprehvir (10⁵ pfu in 100 μl) every other day for a total of three injections. Control mice received intratumoral PBS following the same regimen. Tumor volume was determined by the formula V = (L × W²)/2, where L is the length of the tumor and W is the width. Animals were monitored for tumor volumes two times per week not blinded for 80 days after initial treatment, until tumor volume exceeded 2500 mm³, or until tumor diameter reached 2 cm. Initial sample size was estimated based on previous study for 10 mice per group. Animals that did not have successful tumor engraftment within the estimated period (see Supplementary Figure S1 for the time length of tumor growth) were excluded from the study.

In vivo virus production

Subcutaneous xenografted human neuroblastoma tumors were established in 5–6-week-old athymic nude mice as described above. Tumors were treated with one dose of Seprehvir (10⁵ pfu in 100 μl) intratumoral when reached 350–500 mm³. Tumors were then harvested at 3, 24, 72 and 144 h post-virus infection, four tumors per time point, followed by homogenizing, freeze-thawed three times and the lysates were titered by standard plaque assay (triplicates) on Vero cells.

Statistical analysis

All statistical analyses were performed using Statistical Analysis System software 9.3 (SAS Institute, Cary, NC, USA). Statistics for Figures 1b and d were analyzed using linear mixed models adjusted for unequal variances to compare cell groups. For Figure 1b, survival for the four cell groups was compared over different MOIs. For Figure 1d, viral titer (measured as log PFU) was compared over time for each of the four cell groups. Figures 1b and d have 95% confidence limits to aid in showing where statistically significant differences exist between the cell groups. For Figures 4a–d Kaplan–Meier survival curves were analyzed by log-rank test to compare the survival of Seprehvir and PBS. In Figure 5, Bonferroni corrected pairwise comparisons were made to look for differences in virus yields (PFU) amongst the four groups at 24 h (** for P-value < 0.01).

CONFLICT OF INTEREST

JC is an employee of Virttu Biologics Ltd. The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Brian Geier for analysis of the Pediatric Preclinical Testing Program gene expression database, and Peter Houghton (Greehey Children’s Cancer Research Institute) for cell lines. This work was supported in part by Alex’s Lemonade Stand Foundation for Childhood Cancer (PWF), the Research Institute at Nationwide Children’s Hospital and NIH grant R21-CA133663-01A1 (TPC). Oncolytic HSV Seprehvir used in the study was from Virttu Biologics, Ltd (Glasgow, UK).

REFERENCES

1 Park JR, Eger A, Caron H. Neuroblastoma: biology, prognosis, and treatment. Pedi atr Clin North Am 2008; 55: 97–120; x.
2 Patel MR, Kratzke RA. Oncolytic virus therapy for cancer: the first wave of translational clinical trials. Transl Res 2013; 162: 355–364.
3 Pol J, Bloy N, Obrit F, Eggemorter A, Galon J, Cremer I et al. Trial watch: oncolytic viruses for cancer therapy. Oncoimmunology 2014; 3: e28694.
4 Andtbacka RH, Kaufman HL, Collicchio F, Amatruda T, Senzer N, Chesney J et al. Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. J Clin Oncol 2015; 33: 2780–2788.
5 Balfour Jr HH. Antiviral drugs. N Engl J Med 1999; 340: 1255–1268.
6 Spear PG. Herpes simplex virus: receptors and ligands for cell entry. Cell Microbiol 2004; 6: 401–410.
7 Huang YY, Yu Z, Lin SF, Li S, Fong Y, Wong RJ. Nectin-1 is a marker of thyroid cancer sensitivity to herpes oncolytic therapy. J Clin Endocrinol Metab 2007; 92: 1965–1970.
8 Yu Z, Adusumulli PS, Eisenberg DP, Darr E, Ghoasein RA, Li S et al. Nectin-1 expression by squamous cell carcinoma is a predictor of herpes oncolytic sensitivity. Mol Ther 2007; 15: 103–113.
9 Friedman GK, Langford CP, Coleman JM, Cassidy KA, Parker JN, Markert JM et al. Engineered herpes simplex viruses efficiently infect and kill CD133+ human glioma xenografts that express CD111. Journal of Neuro-Oncology 2009; 95: 199–209.
10 Wang PY, Currier MA, Hansford L, Kaplan D, Chiocca EA, Uchida H et al. Expression of HSV-1 receptors in EBV-associated lymphoproliferative disease determines susceptibility to oncolytic HSV. Gene Ther 2013; 20: 761–769.
11 Jackson JD, McMorrism AM, Roth JC, Coleman JM, Whitley RJ, Gillespie GY et al. Assessment of oncolytic HSV efficacy following increased entry-receptor expression in malignant peripheral nerve sheath tumor cell lines. Gene Ther 2014; 21: 984–990.
12 Parikh N, Currier MA, Adams LC, Mahller YY, DiPasquale B, Collins MH et al. Oncolytic herpes simplex virus mutants are more efficacious than wild-type adenovirus for the treatment of high-risk neuroblastomas in preclinical models. Pediatr Blood Cancer 2005; 44: 469–478.
13 Mahller YY, Williams JP, Baird WH, Mitton B, Grossheim J, Saeki Y et al. Neuroblastoma cell lines contain pluripotent tumor initiating cells that are susceptible to a targeted oncolytic virus. PLoS One 2009; 4: e4235.
14 Gillory LA, Megison ML, Stewart JE, Mroczek-Muselman E, Nabers HC, Waters AM et al. Preclinical evaluation of engineered oncolytic herpes simplex virus for the treatment of neuroblastoma. PLoS One 2013; 8: e77753.
15 Desai P, Person S. Incorporation of the green fluorescent protein into the herpes simplex virus type 1 capsid. J Virol 1998; 72: 7563–7568.
16 Tiwari V, Clement C, Xu D, Valyi-Nagy T, Yue BY, Liu J et al. Role for 3-O-sulfated heparan sulfate as the receptor for herpes simplex virus type 1 entry into primary human corneal fibroblasts. J Virol 2006; 80: 8970–8980.
17 Choudhary S, Marquez M, Alencastro F, Spors F, Zhao Y, Tiwari V. Herpes simplex virus type-1 (HSV-1) entry into human mesenchymal stem cells is heavily dependent on heparan sulfate. J Biomed Biotechnol 2011; 2011: 264350.

18 Tiwari V, Tarbutton MS, Shukla D. Diversity of heparan sulfate and HSV entry: basic understanding and treatment strategies. Molecules 2015; 20: 2707–2727.

19 Warner MS, Geraghty RJ, Martinez WM, Montgomery RI, Whitbeck JC, Xu R et al. A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. Virology 1998; 246: 179–189.

20 Lopez M, Cocchi F, Menotti L, Avitabile E, Dubreuil P, Campadelli-Fiume G. Nectin2alpha (PRR2alpha or HveB) and nectin2delta are low-ef- ficiency mediators for entry of herpes simplex virus mutants carrying the Leu25Pro substitution in glycoprotein D. J Virol 2000; 74: 1267–1274.

21 Leddon JL, Chen C-Y, Currier MA, Wang P-Y, Jung FA, Denton NL, Cripke KM et al. Oncolytic HSV virotherapy in murine sarcomas differentially triggers an antitumor T-cell response in the absence of virus permissivity. Mol Ther Oncolytics 2015; 1: 14010.

22 Uchida H, Shah WA, Ozuer A, Frampton Jr AR, Goins WF, Grandi P et al. Generation of herpesvirus entry mediator (HVEM)-restricted herpes simplex virus type 1 mutant viruses: resistance of HVEM-expressing cells and identification of mutations that rescue nectin-1 recognition. J Virol 2009; 83: 2951–2961.

23 Chew T, Taylor KE, Mossman KL. Innate and adaptive immune responses to herpes simplex virus. Viruses 2009; 1: 979–1002.

24 Miller CG, Fraser NW. Role of the immune response during neuro-attenuated herpes simplex virus-mediated tumor destruction in a murine intracranial melanoma model. Cancer Res 2000; 60: 5714–5722.

25 Alvarez-Breckenridge CA, Yu J, Price R, Wojton J, Pradarelli J, Mao H et al. NK cells impede glioblastoma virotherapy through NKp30 and NKp46 natural cytotoxicity receptors. Nat Med 2012; 18: 1827–1834.

26 Currier MA, Eshun FK, Sholl A, Chernoguz A, Crawford K, Divanovic S et al. VEGF blockade enables oncolytic cancer virotherapy in part by modulating intratumoral myeloid cells. Mol Ther 2013; 21: 1014–1023.

27 Thorne AH, Meisen WH, Russell L, Yoo JY, Bolyard CM, Lathia JD et al. Role of cysteine-rich 61 protein (CCN1) in macrophage-mediated oncolytic herpes simplex virus clearance. Mol Ther 2014; 22: 1678–1687.

28 Workenhe ST, Simmons G, Pol JG, Lichy BD, Halford WP, Mossman KL. Immunogenic HSV-mediated oncolysis shapes the antitumor immune response and contributes to therapeutic efficacy. Mol Ther 2014; 22: 123–131.

29 MacLean AR, ul-Fareed M, Robertson L, Harland J, Brown SM. Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the 'a' sequence. J Gen Virol 1991; 72: 631–639.

30 Ten Dam GB, Kupup S, van de Westerlo EM, Versteeg EM, Lindahl U, Spillmann D et al. 3-O-sulfated oligosaccharide structures are recognized by anti-heparan sulfate antibody HS4C3. J Biol Chem. 2006; 281: 4654–4662.

Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt).