Diazadispiroalkane Derivatives Are New Viral Entry Inhibitors

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ABSTRACT Herpesviruses are widespread and can cause serious illness. Many currently available antiviral drugs have limited effects, result in rapid development of resistance, and often exhibit dose-dependent toxicity. Especially for human cytomegalovirus (HCMV), new well-tolerated compounds with novel mechanisms of action are urgently needed. In this study, we characterized the antiviral activity of two new diazadispiroalkane derivatives, 11826091 and 11826236. These two small molecules exhibited strong activity against low-passage-number HCMV. Pretreatment of cell-free virus with these compounds greatly reduced infection. Time-of-addition assays where 11826091 or 11826236 was added to cells before infection, before and during infection, or during or after infection demonstrated an inhibitory effect on early steps of infection. Interestingly, 11826236 had an effect by addition to cells after infection. Results from entry assays showed the major effect to be on attachment. Only 11826236 had a minimal effect on penetration comparable to heparin. Further, no effect on virus infection was found for cell lines with a defect in heparan sulfate expression or lacking all surface glycosaminoglycans, indicating that these small molecules bind to heparan sulfate on the cell surface. To test this further, we extended our analyses to pseudorabies virus (PrV), a member of the *Alphaherpesvirinae*, which is known to use cell surface heparan sulfate for initial attachment via nonessential glycoprotein C (gC). While infection with PrV wild type was strongly impaired by 11826091 or 11826236, as with heparin, a mutant lacking gC was unaffected by either treatment, demonstrating that primary attachment to heparan sulfate via gC is targeted by these small molecules.

KEYWORDS antiviral activity, entry inhibitors, heparan sulfate binding, human cytomegalovirus, new mode of action, pseudorabies virus, virus attachment

Human cytomegalovirus (HCMV) can cause life-threatening diseases. It persists lifelong in the infected host, while reactivation during immunosuppression leads to recurrent episodes of disease (1). In neonates as well as immunocompromised adults, HCMV can cause fatal organ damage (2). HCMV has a global seroprevalence of up to 100% in adults. Different antiviral compounds alone or in combination have been used for treatment of HCMV diseases. Nearly all currently available drugs are targeted to the viral DNA polymerase. In clinical use, mutations in the viral DNA polymerase confer resistance to ganciclovir, foscarnet, and cidofovir (3, 4). Moreover, mutations in the terminase subunits pUL56 and, less commonly, pUL89 or pUL51 convey resistance to letermovir, used as an HCMV prophylaxis (5). The major drawbacks of ganciclovir and cidofovir are low bioavailability, significant side effects, dose-dependent toxicity, and a low barrier to resistance. Compounds with a novel mechanism of action are urgently

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FIG 1 Structures of the diazadispiroalkane derivatives DSTP-27, 11826091, and 11826236.

needed. Consequently, to broaden therapies for HCMV infections, an inhibitor targeting an early process of infection would be of great value.

One unique mechanism of action concerning viral invasion is prevention of virus adsorption by means of specific blockage of heparan sulfate (HS) receptors. A variety of viruses have been analyzed, including herpes simplex viruses (HSV-1/2) (6, 7), human papillomavirus (HPV) (8, 9), HCMV (10), human immunodeficiency virus (HIV) (11), respiratory syncytial virus (RSV) (12, 13), human hepatitis B and C viruses (HBV and HCV) (14), and several enteroviruses (15). One class of compounds, diazadispiroalkanes, binds to heparan sulfate glycoconjugates, thus leading to impairment of virus binding to host cells and a block of viral replication. DSTP-27, a member of this group, has been studied more extensively (16–18). It targets two sulfate groups located on adjacent sugar residues of the glycoconjugates. The mode of action is an electrostatic interaction between negative charges on the sulfated groups and positive charges of nitrogen atoms of the compounds. Similar interactions can occur with a carboxyl group of octasaccharides, a key region for binding of heparan sulfate glycoconjugates (10, 16–18). However, DSTP-27 has a low metabolic stability due to its furoxane rings (19), and releases nitric oxide *in vivo*. Therefore, DSTP-27 is unsuitable for further development. In this study, we designed and synthesized new promising small molecules that possess anti-herpesvirus activity. The mode of action of these novel compounds was characterized by analyzing virus-host cell interactions.

RESULTS

Synthesis of the compounds. In order to design novel compounds we investigated the role of the cycle size on the antiviral activity. In addition, we studied the introduction of different heterocycles (such as triazines). We started the synthesis with formylpiperazine leading to dispiro systems with cycles of different sizes (Fig. S1 in the supplemental material). At the end, we obtained the diazadispiroalkane derivatives 11826236 and 11826091. The structures of the compounds are shown in Fig. 1.

Antiviral activity. The antiviral activity was analyzed by plaque reduction assays. HELF Fi301 cells were infected with HCMV TB40/E expressing green fluorescent protein (GFP) fused to pp150 (TB40/E-pp150GFP) or ganciclovir (GCV)-resistant isolates (multiplicity of infection [MOI] 0.01) in the presence of various concentrations of 11826236, 11826091, or DSTP-27. The mean 50% effective concentrations (EC_{50s}) against HCMV were $2.74 \pm 0.95 \mu\text{M}$ for 11826236 and $8.95 \pm 2.92 \mu\text{M}$ for 11826091, compared to $2.40 \pm 1.22 \mu\text{M}$ for DSTP-27 (Fig. 2) (10). The EC_{50s} against GCV-resistant HCMV were $1.38 \pm 0.04 \mu\text{M}$ for 11826236 and $2.73 \pm 0.42 \mu\text{M}$ for 11826091 (Table 1). To exclude that unspecific effects could affect the antiviral activity, the cytotoxicity of the compounds was investigated. The mean 50% cytotoxic concentrations (CC_{50s}) were $416.67 \pm 0.09 \mu\text{M}$ for 11826091 and $321.00 \pm 0.08 \mu\text{M}$ for 11826236 (Table 1). This

FIG 2 Antiviral activity of the compounds. HELF Fi301 cells were infected with HCMV TB40/E-p150GFP (MOI 0.1) in the presence of different concentrations of 11826091 or 11826236 under plaque assay conditions. Plaques were counted at seven dpi. Plaque reduction was calculated as percentage inhibition of PFU obtained in the absence of compounds. The mean 50% (EC_{50}) and 90% (EC_{90}) effective concentrations ranged from 8.95 to 21.67 μ M for 11826091 and 2.75 to 9.12 μ M for 11826236. Results were obtained from three independent experiments. Error bars represent the standard deviations (SD).

result demonstrated that all diazadispiroalkane derivatives were active against HCMV. Derivatives 11826091 and 11826236 were not cytotoxic below 300 μ M.

To further investigate the antiviral effect, viral yield assays were performed. HELF Fi301 cells pretreated with the EC_{90} of the compounds or heparin (10 μ g/ml) or left untreated were infected with TB40/E-p150GFP (MOI 1). Viral titers in the supernatant were determined by plaque assay at the indicated time points. The virus yield was reduced about 1.5 logs at 96 h postinfection (hpi) and about 2.8 logs for both compounds at 120 hpi (Fig. 3). This analysis confirmed the high antiviral activity of the new compounds.

Effects on viral replication. To compare the antiviral effect of 11826091 and 11826236 with DSTP-27, time-of-addition analyses were performed. HELF Fi301 cells were infected with HCMV TB40/E-p150GFP (MOI 1) and treated with the substances at EC_{90} (11826091, 21.67 μ M; 11826236, 9.12 μ M; DSTP-27, 10.95 μ M) or heparin (10 μ g/ml) at different times of infection or left untreated (Fig. 4A). The effect on viral replication was quantified by plaque reduction assays. If the compounds were added before infection (before inf.) and removed after 30 min prior to infection, viral replication was inhibited by $\geq 80\%$, whereas addition of heparin led to a reduction of approximately 20% (Fig. 4B). Addition of the inhibitors 30 min before and for 90 min during infection (before + during inf.) resulted in plaque reduction by 60 to 70%. Similar observations were made by addition during infection (during inf., Fig. 4B). Addition of 11826091 and heparin after infection (after inf.) had no effect on HCMV replication (Fig. 4B). However, addition of 11826236 led to a reduction of $\leq 70\%$ and DSTP-27 led to a 50% reduction (Fig. 4B). These experiments suggest the compounds primarily act on early steps of infection.

TABLE 1 Antiviral activities of new diazadispiroalkane derivatives against HCMV

Virus	Compound	Mean EC_{50} (μ M) ^{a,b} plaque reduction	Mean EC_{90} (μ M) ^{a,b} plaque reduction	SI (μ M) ^c
TB40/E-p150GFP	11826091	8.95 $\pm$ 2.92	21.67 $\pm$ 2.38	46.48
	11826236	2.75 $\pm$ 0.95	9.12 $\pm$ 2.24	116.73
Ganciclovir-resistant	11826091	2.73 $\pm$ 0.42	6.15 $\pm$ 0.21	152.62
	11826236	1.38 $\pm$ 0.04	2.80 $\pm$ 0.14	232.61

^aThe EC_{50}/EC_{90} was defined as the concentration of compound that resulted in a 50%/90% plaque reduction compared to the untreated control. Values represent means $\pm$ standard deviation from four independent experiments.

^b CC_{50} : 11826091 = 416.67 $\pm$ 0.09 μ M; 11826236 = 321.00 $\pm$ 0.08 μ M.

^cSI, selectivity index, CC_{50}/EC_{50} .

FIG 3 Growth kinetics in the presence of 11826091 or 11826236. HELF Fi301 cells were infected with HCMV TB40/E-p150GFP (MOI 1) in the absence (w/o) or presence of 21.67 μ M 11826091, 9.12 μ M 11826236, or 10 μ g/ml heparin. At each time point, supernatants were harvested and titers determined on HELF Fi301 cells. Given are mean values and corresponding SD of three independent experiments.

Influence on viral attachment and penetration. To determine the effect of 11826091 and 11826236 on viral attachment (Fig. 5A), prechilled HELF Fi301 cells were treated with 21.67 μ M, 11826091, 9.12 μ M 11826236, and 10 μ M DSTP-27 or left untreated on ice for 30 min at 4°C. After removal of the compounds, cells were infected with prechilled HCMV TB40/E-p150GFP (MOI 1) for 2 h at 4°C. Free virus was removed by three washing steps with phosphate-buffered saline (PBS) and the cells were incubated under plaque assay conditions (Fig. 5A). Both inhibitors led to a plaque reduction of about 90%, while DSTP-27 reduced plaque formation to only 62 to 70% (Fig. 5A). Heparin showed no block of attachment.

For penetration assays (Fig. 5B), prechilled HELF Fi301 cells were infected with HCMV TB40/E-p150GFP (MOI 1) for 2 h at 4°C. Thereafter cells were treated with 10 μ M or 50 μ M of both compounds or left untreated. Penetration was allowed at 37°C for 10 min and was stopped by low pH treatment (pH 3.0). Heparin (10 μ g/ml) was used as a control. Regardless of the concentration, all inhibitors had only marginal effects on virus penetration (Fig. 5B). In contrast to attachment, 11826236 reduced this process only up to 30 to 38% (Fig. 5B). Heparin treatment led to a reduction of 30% to 45% (Fig. 5B). These results imply that 11826236 and 11826091 prevent attachment of HCMV TB40/E-p150GFP, whereas 11826236 had some additional effect on penetration.

Impact on fusion activity. To investigate the ability of the compounds to block activity of the herpesvirus core fusion glycoprotein B (gB), human HEK293T cells were transfected with nonfusogenic gB (gB-EGFP) or a fusion-active gB-VSV-G chimera fused with a C-terminal enhanced green fluorescent protein (EGFP) (Fig. 6A). Quantification of syncytia formed in transfected HEK293T cells that were treated with either 25 μ g/ml gB-specific antibody or 10 μ M of the respective inhibitor is shown in Fig. 6B. Higher magnification showed the strong inhibitory effect of the gB-specific antibody on syncytium formation, as recently reported (Fig. 6C) (20). Interestingly, no inhibitors reduced, but all slightly enhanced syncytium formation (Fig. 6D). These results indicate that fusion activity of gB is not affected by any of the compounds.

Effect of heparan sulfate glycosaminoglycan and chondroitin sulfotransferase. To analyze whether the inhibitors bind to heparan sulfate glycosaminoglycans (HS) and/or chondroitin 4-O-sulfotransferase-1 (CS), a murine fibroblast cell line unable to express HS on the cell surface (gro2C) and a cell line that expresses neither HS nor CS (sog9) were treated with the compounds prior to infection with TB40/E-p150GFP. After 4 days, the supernatants were transferred to HELF Fi301 cells and analyzed after 7 days by plaque assay. All inhibitors showed a significant decrease in the mouse

FIG 4 Time-of-addition analysis. (A) Diagram of the experimental settings. (B) HELF Fi301 cells treated with 21.67 μ M 11826091, 9.12 μ M 11826236, 10 μ M DSTP-27, or 10 μ g/ml heparin before infection, before and during infection, during and after infection, or without treatment (w/o) were subjected to plaque reduction assays. Shown are mean values and SD of three independent experiments.

parental L cells (Fig. 7). However, the compounds as well as heparin were not able to reduce the infection further in the absence of HS (gro2C) and in the absence of HS and CS (sog9) (Fig. 7). These experiments demonstrated that (i) HCMV infection is facilitated by the presence of glycosaminoglycans and (ii) the compounds target HS on the cell surface.

Effect of the small molecules against PrV. For alphaherpesviruses, it is well known that attachment is mediated by binding of gC to heparan sulfate moieties on the cell surface (reviewed in reference 21). To study the effect of the compounds against pseudorabies virus (PrV) infection, RK13 cells were infected with PrV-Ka in the presence of various concentrations of 11826091 and 11826236 (Fig. 8A). The mean 50% effective concentrations (EC_{50} s) against PrV were 1.32 μ M for 11826091 and 0.55 μ M for 11826236. These results demonstrated that all compounds were also active against PrV. None of the compounds was cytotoxic lower than 1 mM. In order to examine further the antiviral effect, time-of-addition analyses were performed. RK13 cells were infected PrV-Ka (MOI 0.001) and treated with 20 μ M of each compound or heparin (10 μ g/ml) as control at the indicated time points. The effect on infection was quantified by counting plaques after 2 days (Fig. 8B). Similar to heparin treatment, both compounds showed a reduction of about 75 to 80% by addition before infection and by

FIG 5 Effects on viral attachment and penetration. (A) HELF Fi301 cells were treated with 21.67 μ M 11826091, 9.12 μ M 11826236, 10 μ M DSTP-27, 10 μ g/ml heparin or left untreated (w/o) for 30 min at 4°C. After removing the inhibitors, cells were infected with prechilled HCMV TB40/E-p150GFP (MOI 1) for 2 h at 4°C. After three washing steps and aspiration of unattached virus, cells were overlaid with methylcellulose and incubated for 7 days. (B) Prechilled HELF Fi301 cells were infected with TB40/E-p150GFP (MOI 1) for 2 h at 4°C. Cells were treated with 10 μ M or 50 μ M 11826091 or 11826236, 10 μ g heparin, or left untreated for 10 min at 37°C. After washing steps with Tris-HCl (pH 3.0), cells were overlaid with methylcellulose and incubated for 7 days. Error bars on the histogram are SD from three independent experiments.

addition before and during infection, as well as during infection. Treatment after infection had no effect on the number of plaques (Fig. 8B). These experiments indicate that, as seen with HCMV, the compounds block attachment. To investigate this, a mutant lacking the attachment protein gC (PrV- Δ gC) was used (20), which is the only viral glycoprotein productively interacting with cell surface proteoglycans (20). As shown in Fig. 8C, while treatment of cells before and during infection reduced the number of plaques for PrV-Ka to approximately 20%, no reduction was found for PrV- Δ gC with either compounds or with heparin.

DISCUSSION

Human cytomegalovirus (HCMV) is a life threatening, opportunistic pathogen (21, 22). To date no prophylactic vaccination is available, and nearly all anti-HCMV drugs are inhibitors of the viral DNA polymerase. The major drawbacks of ganciclovir and cidofovir are low bioavailability, significant side effects, dose-dependent toxicity, and a low barrier to resistance. (23, 24). New non-nucleosidic inhibitors with different modes of action are urgently needed. Consequently, an inhibitor targeting an early process within infection would give an alternative to available antivirals. One unique mechanism of action is the prevention of virus adsorption by specific blockage of heparan sulfate (HS) receptors. Previously, we identified the N-N-(bis-5-nitropyrimidyl) dispirotriperazine derivative (DSTP-27), a new class of low molecular weight inhibitors, as an antiviral agent against HSV-1 and HCMV (10, 15). However, further development of DSTP-27 was discontinued due to the metabolic instability associated with nitric oxide release *in vivo*.

In this study, we describe the synthesis and antiviral activity of two new diazadispiroalkane derivatives, 11826091 and 11826236. Plaque reduction assays revealed EC_{50} s

FIG 6 Influence on fusion activity of gB. (A) HEK293T cells were transfected with nonfusogenic gB (gB-EGFP) or the fusion-active gB/VSV-G chimera (gB/VSV-G EGFP). (B) Quantification of syncytia formed in transfected HEK293T cells that were treated either with 25 μ g/ml gB-specific antibodies (1G2, C23, or 27-287) or with 10 μ M of the respective compounds as indicated. The numbers in the left corner of each image represent the syncytium counts of each well as calculated by the software ImmunoSpot (version 6.0.0.2). This program marked counted spots that exceeded the size of 0.02 mm² by blue edging and spots that did not exceed this size by white edging. (C) Magnification showing the strong inhibitory effect of the gB specific antibody 1G2 on syncytium formation. (D) Quantification of 620 syncytium formation. Syncytium counts from (B) were calculated relative to the level of the mock control gB/VSV-G without antibody (w/o), which was set to 100%.

against HCMV TB40/E-pp150GFP to be at low micromolar concentrations, indicating potent antiviral activity. All compounds were well tolerated at concentrations lower than 300 μ M (Table 1). Together with the reduction of viral yield over nearly three logs, both compounds are promising new antivirals. Further, time-of-addition analysis with HCMV revealed that 11826236 is not only active before infection, it also leads to a decrease of about 70% when added after infection. One reason might be that the compound binds to the cell surface throughout the infection, as has been reported for papillomavirus (25). Therefore, this compound could be useful not only for prophylactic treatment but also as a therapy for infected patients.

Our results showed the compounds prevent virus attachment. It has been demonstrated that HCMV glycoprotein M (gM) and B (gB) are involved in this step (26). In addition, by using gB-null mutants, it was demonstrated that gB is required for entry

FIG 7 Effects on cells lacking either heparan sulfate glycosaminoglycans (HS; gro2C) or HS and chondroitin 4-O-sulfotransferase-1 (CS; sog9). Murine fibroblasts (L-parental), gro2C, or sog9 cells were treated with 21.67 μ M 11826091, 9.12 μ M 11826236, 10 μ M DSTP-27, or 10 μ g/ml heparin, or else left untreated (w/o), prior to infection with HCMV TB40/E-p150GFP (MOI 3). After an incubation of 4 days, the supernatants were harvested, used for infection of HELF Fi301 cells, and titers were determined after 7 days. Shown are mean values of three independent assays with SD.

(27). In order to get further insight into the effect of the compounds, we investigated their ability to inhibit the fusion activity of gB. For this purpose, two constructs were used, one without fusion activity (gB-EGFP) and one chimera of gB fused with VSV-G that had fusion activity (gB/VSV-G-EGFP). However, our fusion studies demonstrated that 1182601 or 11826236 do not affect fusion activity. In line with this, studies performed with HSV-1 (15) and papillomaviruses (25) revealed that DSTP-27 prevents virus infection by binding to cell surface heparan sulfate glycosaminoglycans (HS) moieties. DSTP-27 blocks infection by cell-bound papillomaviruses, leading to a noninfectious entry pathway (28). We performed a set of experiments to address the question of whether 11826091 and 11826236 bind to HS. For this approach, a murine fibroblast cell line was used that does not express HS on the cell surface (gro2C) (29) and another cell line that expresses neither HS nor chondroitin 4-O-sulfotransferase-1 (CS; sog9) (30). On the other hand, glycoprotein C (gC) is important for attachment in PrV via binding of heparan sulfate proteoglycans (31, 32). Our analysis with PrV- Δ gC demonstrated that one target of both compounds is binding of PrV gC to heparan sulfate. These experiments confirmed that HCMV infections depend on glycosaminoglycans but, as shown for alphaherpesviruses, this binding is not essential for infection.

Overall, the present study shows that the two new small molecules (i) have a high potency as viral entry inhibitors and (ii) are promising candidates for development of a novel antiviral therapy. Due to their mode of action, these compounds will be useful for prophylaxis as well as in therapies. *In vivo* studies will show if these molecules will have a potential for preclinical studies.

MATERIALS AND METHODS

Cells and virus. Human embryonic lung fibroblasts Fi301 (HELF Fi301, authenticated by the German Collection of Microorganisms and Cell Culture; DSMZ, Germany) and all mouse L-cell lines were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and gentamicin (60 μ g/ml). HELF Fi301 at passages 10 to 15 were used for infection, and experiments were carried out with confluent cell monolayers. Infection of HELF Fi301 with HCMV TB40/E-p150GFP (kindly provided by C. Sinzger, Ulm University Medical Center, Institute of Virology (33)) or a GCV-resistant clinical isolate (kindly provided by K. Hamprecht, Institute of Medical Virology, Tübingen) was carried out as described before (34). The HCMV clinical isolate used is resistant against ganciclovir because it carries the substitution M460I and M406V in HCMV UL97. Rabbit kidney epithelial cells (RK13; CCLV-RIE-0109) were cultivated in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS) and infection with PrV wild-type strain Kaplan (PrV-Ka) (29) or PrV- Δ gC was done as described (30).

Mouse cell lines. The parental L cell line (clone 1D) is a derivative of LMtk-murine fibroblasts. Mouse L-cells, mouse L-gro2C cells (35), and mouse L-sog9 cells (36) were obtained from the Collection of Cell Lines in Veterinary Medicine (CCLV), FLI, Insel Riems. While the cell line L-gro2C is defective in

FIG 8 Effects on PrV. (A) RK13 cells were infected with PrV wild-type strain Ka (PrV-Ka) (approximately 100 PFU/well) in the absence or presence of different concentrations of 11826091 or 11826236 for 1 h. The inoculum was removed and cells were overlaid with methylcellulose. Plaques were counted after 2 to 3 days and calculated as percentage of plaques obtained in the absence of compounds (w/o). The EC_{50} ranges were from 1.32 μ M for 11826091 and 0.55 μ M for 11826236. (B) Time-of-addition assay. RK13 cells were treated with 20 μ M 11826091, 20 μ M 11826236, or 10 μ g/ml heparin before infection, before and during infection, during and after infection, or left untreated (w/o). Mean values of four independent assays with corresponding SD are given. (C) Effect on PrV lacking the attachment glycoprotein C. RK13 cells were infected with PrV-Ka or PrV- Δ gC in the presence of 20 μ M 11826091, 20 μ M 11826236, or 10 μ g/ml heparin. Number of plaques was counted after 2 to 3 days and calculated in relation to the number of plaques in the control wells (w/o), set as 100%. Mean values of three independent experiments with corresponding SD are given.

biosynthesis of heparan sulfate but still expresses chondroitin sulfate (CS) (37, 38), the cell line L-sog9 is defective in glycosaminoglycans, including CS biosynthesis (35).

Compounds. The diazadispiroalkane derivatives 11826091 and 11826236 or DSTP-27 were synthesized by V. Makarov. The synthesis of 11826091 and 11826236 is described in Fig. S1. Heparin was used as a reference compound (Sigma-Aldrich, Deisenhofen, Germany). All compounds were dissolved in Aqua bidest. Stock solutions of 10 mg/ml were stored at -20°C before use.

Plaque reduction assay. HELF Fi301 cells were seeded in 24-well plates (5×10^4 cells per well) and treated in triplicate with various concentrations (1.0, 3.0, 5.0, 7.0, 9.0, 11.0, 15.0, 20.0, 25.0, 50.0, 55.0, and 70.0 μM) 11826091 or 11826236 for 30 min, or left untreated. The inoculum was discarded and the cells were washed three times with PBS prior to infection with TB40/E-pp150GFP (MOI 0.1). After 1 hpi, the inoculum was discarded and the cells were washed three times with PBS and overlaid with 2 ml methylcellulose (Methocel MC; Fluka) containing DMEM with 7.5% FBS. After incubation for 7 days p.i. (dpi), the cells were fixed with ethanol-acetone composition (95:5) prior to staining with crystal violet (2% wt/vol) for 15 min at room temperature. Plaques were counted by using a microscope and compound effects were calculated by comparing compound-treated cells versus untreated cells.

RK13 cells seeded in 24-wells (approximately 2×10^5 cells/well) were infected the next day with 100 PFU/well of PrV-Ka or PrV- ΔgC in the absence (w/o) or in the presence of different concentrations of 11826091 and 11826236 (1, 2, 5, 10, and 20 μM) for 1 h. The inoculum was removed and cells were overlaid with methylcellulose. After 2 to 3 days, the cells were fixed with 5% formaldehyde and stained with crystal violet. Plaques were counted and calculated in relation to the number of plaques in the control wells (w/o). Assays were repeated at least 4 times.

Cytotoxicity determination. HELF Fi301 cells (5×10^4) were seeded in 96-well plates. Subconfluent cells (70%) were incubated with several concentrations of 11826091 or of 11826236 in a final volume of 100 μl for 7 days at 37°C . Cytotoxicity (50% cytotoxic concentration, CC_{50}) profiling of the diazadispiroalkane derivatives was determined by the use of Cell Proliferation kit II (XTT, Roche), according to the manufacturer's recommendations. Briefly, after the incubation time, 50 μl of the labeling solution (XTT labeling reagent and electron coupling reagent, 50:1) was added to the cells, followed by incubation for 4 h at 37°C . The absorbance at 492 nm with a reference wavelength of 650 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader. The assay is based on the cleavage of the yellow tetrazolium salt XTT and the formation of the orange formazan dye by metabolically active cells. An increase in the number of living cells directly correlates to the amount of orange formazan formed, as monitored by the absorbance.

In addition, cytotoxicity produced in stationary HELF Fi301 cells was determined by microscopic inspection of cells not affected by the virus used in plaque assays, similar to Turk et al. (39).

RK13 cells were seeded in 96-well plates at 70 to 80% confluence and treated the next day with different concentrations of 11826091 and 11826236 (10, 50, 100, 250, 500, 750, and 1,000 μM) for 24 h. Then 10 μl of Presto blue (Thermo Fisher Scientific) was added and plates were further incubated at 37°C for 30 min. Fluorescence was measured in a microplate reader (Tecan). Toxicity experiments were repeated three times with triplicates of each condition.

Time-of-addition experiments. HELF Fi301 cells (5×10^4 cells per well) were seeded in 24-well plates and grown until confluence. The derivatives 11826091 (21.67 μM), 11826236 (9.12 μM), or DSTP-27 (10 μM) or heparin (10 $\mu\text{g}/\text{ml}$) were added at the following times of addition: (i) 30 min before infection and the compound was removed by three washing steps with PBS before infection (before inf.); (ii) during infection (during inf.); or (iii) after infection in the methylcellulose overlay (after infect.). Infection was performed with TB40/E-pp150GFP (MOI 0.01) for 60 min at 37°C . After this, the inoculum containing nonadsorbed virus was removed by three washing steps with PBS. Afterward, 2 ml of methylcellulose (Methocel MC; Fluka) containing DMEM with 7.5% FBS was added and the plaque assay was performed as described above.

For experiments with PrV, RK13 cells were seeded in 24-well plates (2×10^5 cells per well) and treated with 11826091 (20 μM), 11826236 (20 μM), or heparin (10 $\mu\text{g}/\text{ml}$) either 30 min before infection, 30 min before and during infection (1 h), during infection, or after infection for 1 h with approximately 100 PFU/well of either PrV-Ka or PrV- ΔgC . Thereafter the inoculum was removed and cells were overlaid with 2 ml methylcellulose. Cells were fixed after 2 days with 5% formaldehyde, stained with crystal violet, and the number of plaques was calculated as the percentage of the control without inhibitor. Experiments were repeated four times.

Viral yield assay. HELF Fi301 cells (5×10^4 cells per well) were seeded in 24-well plates. Confluent cells were cultured with DMEM containing 2% FBS for 24 h prior to a 30-min treatment with 11826091 (21.67 μM), 11826236 (9.12 μM), or heparin (10 $\mu\text{g}/\text{ml}$). Compounds were removed by three washing steps with PBS before infection with TB40/E-pp150GFP (MOI of 1). At 24, 48, 72, 96, and 120 hpi, the supernatants were harvested and frozen at -80°C . After collection of all time points, the supernatants were thawed and transferred to a 12-well plate and, after 7 days, titers were determined by plaque assay. Fixation and staining were as described for the plaque reduction assay.

Attachment assay. HELF Fi301 cells were seeded in 12-well plates (1×10^5). Prechilled confluent cells were treated with 21.67 μM 11826091, 9.12 μM 11826236, 10 μM DSTP-27, 10 $\mu\text{g}/\text{ml}$ of heparin, or left untreated for 30 min at 4°C . The compounds were removed and the cells were washed three times with prechilled PBS prior to infection with prechilled HCMV TB40/E-pp150GFP (MOI 1) for 2 h at 4°C . Thereafter, unattached virus was removed from the cells and the cells were subjected to plaque reduction assays at 37°C for 8 days.

Penetration assay. Confluent HELF Fi301 cells (4×10^6) were cooled to 4°C and infected with HCMV TB40/E-pp150GFP (MOI 1) for 2 h at 4°C . After removing inoculums by three washing steps with

precooled PBS, cells were treated with 10 μ M or 50 μ M 11826091 and/or 11826236, 10 μ g/ml heparin, or control medium for 10 min at 37°C. The compound dilutions were aspirated and the cells were rinsed with Tris/HCl pH 3.0 in order to inactivate any nonpenetrant virus. The cells were subjected to plaque reduction assays and analyzed after 7 days.

Fusion activity assay of gB. In order to investigate the ability of the compounds to block the fusion activity of gB, HEK293T cells were transfected with nonfusogenic gB or the hyperfusogenic chimera gB/VSV-G, exactly as described by Reuter et al. (40). Briefly, cells were transfected by calcium phosphate precipitation. At 4 h after transfection, the cell culture medium was replaced with medium containing 25 μ g/ml gB-specific monoclonal antibody (MAbs-1G2, C23, or 27–287) or 10 μ M of the respective compound (11826091, 11826236, or DSTP-27). After 48 h, cells were fixed with 3% paraformaldehyde and plates were imaged with a CTL Immunospot S6 analyzer (Cellular Technology Limited, Bonn, Germany). Then, syncytium formation was quantified using ImmunoSpot, version 6.0.0.2, software (Cellular Technology Limited, Bonn, Germany). Syncytia were defined as EGFP signals that were larger than the fluorescent spots detected for nonfusogenic gB-EGFP.

Analysis of cell surface proteins as targets. Possible host cell surface targets could be heparan sulfate glycosaminoglycans (HS) and/or chondroitin 4-O-sulfotransferase-1 (CS). We used a murine fibroblast cell line that does not express HS on the cell surface (gro2C) and another cell line that expresses neither HS nor CS (sog9). Murine fibroblast L cells, gro2C cells, or sog9 cells were treated with 21.67 μ M 11826091, 9.12 μ M 11826236, 10 μ M DSTP-27, 10 μ g/ml heparin, or left untreated prior to infection with HCMV TB40/E-pp150GFP (MOI 1). After 4 dpi, supernatants were harvested and used for infection of HELF F1301 cells. The cells were overlaid with 2 ml methylcellulose (Methocel MC) containing DMEM with 7.5% FBS and the plaque reduction was analyzed after 7 days.

Statistical analysis. All experiments were performed in duplicate or triplicate at minimum three times. Data were expressed as mean $\pm$ standard deviation (SD).

Data availability. All data and materials are available on request for HCMV to E.B., for PrV to B.G.K. or T.C.M., for gB fusion to T.C.M., and for compounds to V.M.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

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R.A. performed most of the experiments, J.S. performed analysis of the mouse cells, B. K. performed the gB fusion assays, N.M. synthesized and validated the compounds, and J.E. H. performed the assays with PrV. R.A. and E.B. managed the research. R.A., M.T., B.G.K., V.M., and E.B. conceptualized the experiments, validated the results, and wrote the original draft. R.A., M.T., B.G.K., T.C.M., V.M., and E.B. reviewed and edited the manuscript.

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