DSTP-27 Prevents Entry of Human Cytomegalovirus

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Human cytomegalovirus (HCMV) can cause life-threatening diseases in neonates and immunocompromised patients. Due to multiple problems caused by the current available drugs, development of new antiviral compounds is urgently needed. In this study, we characterize the anti-HCMV spectrum and mechanism of action of the N-N'(bis-5-nitropyrimidyl)dispirotripiperazine derivate 27 (DSTP-27). DSTP-27 exhibited strong antiviral activity against two laboratory HCMV strains with different cell tropism as well as ganciclovir (GCV)-sensitive and GCV-resistant clinical isolates in plaque reduction assays and viral growth kinetics experiments. Interestingly, neither infectious nor noninfectious viral particles were observed by electron microscopy. Pretreatment of cell-free virus with DSTP-27 prevented virus infection. The results from time of addition assays, in which DSTP-27 was added to cells (i) before infection, (ii) during virus adsorption, or (iii) after adsorption, demonstrated an inhibitory effect on early steps of the HCMV replication cycle. This observation was confirmed by immunofluorescence as well as Western blot analysis, whereby reduced levels of the immediate early protein IE1, the processivity factor pUL44, and the tegument protein pp150 were detected. Results from attachment and penetration analyses of prechilled human embryonic lung fibroblasts revealed that virus attachment is not blocked. In addition, DSTP-27 inactivated HCMV by stable binding. Taken together, these results demonstrate that DSTP-27 (i) blocks viral penetration by interacting with the host cell and (ii) inactivates HCMV by interacting with the virus.

Human cytomegalovirus (HCMV), one of eight human herpesviruses, can cause serious illness in neonates as well as in immunocompromised adults (1, 2). Transplant and AIDS patients may develop life-threatening diseases as a consequence of primary infection or reactivation of latent infection. HCMV causes syndromes like retinitis, pneumonia, hepatitis, or mononucleosis (3). Additionally, HCMV infections are associated with congenital neurological complications. All current available drugs like ganciclovir (GCV), valganciclovir, cidofovir, and foscarnet target the viral DNA polymerase. They have limited effects, suffer from long-term toxicity, and cause additional complications, including drug resistance (4–6). Therefore, new, well-tolerated ant-HCMV drugs with novel mechanisms of action are urgently needed.

Previously, the N-N'(bis-5-nitropyrimidyl)dispirotripiperazine derivate 27 (DSTP-27) was discovered as a potential antiviral agent against herpes simplex virus 1 (HSV-1) (7). Studies performed with HSV-1 (8) and papillomaviruses (9) revealed that DSTP-27 prevents virus infection by binding to cell surface heparan sulfate (HS) moieties. The compound blocks infection by cell-bound papillomaviruses, leading into a noninfectious entry pathway (9). Schmidtke et al. (8) provided evidence that DSTP-27 also inhibits growth of HCMV. However, neither the spectrum nor the mechanism of anti-HCMV activity of DSTP-27 was studied until now.

Although entry of HCMV into the host cell is poorly understood, it is clear that virus attachment is mediated by interaction with heparan sulfate proteoglycans (HSPG) on the cell surface (10). It has been demonstrated that HCMV glycoproteins M (gM) and B (gB) are involved in this step (11). Attachment results in increased amounts of bound virus to the cell membrane, enabling binding to cellular receptors. Several receptors have been discussed and were correlated with cell type specificity. Feire et al. (12) reported that integrins play a role in HCMV entry into human fibroblasts. This group has shown that peptides resembling a disintegrin domain of HCMV gB could block entry. In addition, by using gB-null mutants it was demonstrated that gB is required for entry (13). In the case of human embryonic lung fibroblasts (HELFs), it is discussed that the epidermal growth factor receptor (EGFR) is used as receptor (14). In addition, Wang et al. (15) suggested that gH and gB bind to integrin alpha3 as well as to EGFR, whereas both function as a coreceptor for entry. Furthermore, recent work suggested that HCMV gH/gL/UL128-131 use another specific receptor for entry into epithelial and endothelial cells (16, 17).

In this study, the anti-HCMV activity of DSTP-27 was further analyzed to define its effects on the replication of two laboratory HCMV strains with different cell tropisms as well as of two clinical isolates, a GCV-sensitive isolate (GCV-sens.) and a GCV-resistant isolate (GCV-res.). Furthermore, the mechanism of action of DSTP-27 against HCMV was investigated.

MATERIALS AND METHODS

Cells and virus. HELFs (European Collection of Cell Cultures, Salisbury, United Kingdom) and human foreskin fibroblasts (HFFs; PromoCell, Germany) were grown in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin (5 U/ml), and streptomycin (50 μg/ml). HELF and HFF cells at

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Table 1 Comparison of antiviral activities of DSTP-27, heparin, and GCV against different HCMV strains

<table>
<thead>
<tr>
<th>HCMV strain</th>
<th>DSTP-27 Mean EC&lt;sub&gt;50&lt;/sub&gt; by plaque reduction (µM)</th>
<th>Heparin Mean EC&lt;sub&gt;50&lt;/sub&gt; (µg/µl)</th>
<th>GCV Mean EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>DSTP-27 SI&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD 169</td>
<td>0.85 ± 0.25</td>
<td>2.15 ± 0.54</td>
<td>7.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>388.23</td>
</tr>
<tr>
<td>TB40/E</td>
<td>0.18 ± 0.05</td>
<td>1.83 ± 0.03</td>
<td>1,833.33</td>
<td></td>
</tr>
<tr>
<td>GCV-resistant isolate</td>
<td>0.15 ± 0.01</td>
<td>53.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2,200.00</td>
<td></td>
</tr>
<tr>
<td>GCV-sensitive isolate</td>
<td>0.16 ± 0.02</td>
<td>4.54 ± 1.20</td>
<td>2,062.50</td>
<td></td>
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</table>

<sup>a</sup> EC<sub>50</sub> was defined as the concentration of compound that resulted in a 50% plaque reduction compared to the untreated control. Values represent means ± standard deviations from four independent experiments.

<sup>b</sup> SI, selectivity index. SI = CC<sub>50</sub>/EC<sub>50</sub>. CC<sub>50</sub> = 330 µM.

<sup>c</sup> Heparin has a molar weight of 4,000 to 40,000; therefore, the unit used was µg/µl.

<sup>d</sup> From Schmidtke et al. (8).

<sup>e</sup> From Prits et al. (32).

The table presents a comparison of the antiviral activities of DSTP-27, heparin, and GCV against different HCMV strains. The mean EC<sub>50</sub> values for each compound are shown, along with a selectivity index (SI) calculated as the ratio of the CC<sub>50</sub> and EC<sub>50</sub> values.

For example, for HCMV strain AD 169, the mean EC<sub>50</sub> for DSTP-27 is 0.85 ± 0.25 µM, for heparin is 2.15 ± 0.54 µg/µl, and for GCV is 7.78 µM. The SI is calculated as 388.23, indicating a high selectivity for DSTP-27 compared to heparin and GCV.

The data suggest that DSTP-27 has a higher antiviral activity against HCMV compared to heparin and GCV, as evidenced by the lower EC<sub>50</sub> values and higher SIs. This information is crucial for understanding the therapeutic potential of DSTP-27 in the context of HCMV infections.
Western Blotting reagent as recommended by the supplier (GE Healthcare Europe, Munich, Germany).

**Effects of DTSP-27 on virus inactivation.** HCMV AD169 (MOI, 6) was incubated either with 10 μM DTSP-27 or 10 μg/ml heparin or left untreated (w/o) before infection with HCMV AD169 (A), TB40/E (B), or GCV-resistant (C) or GCV-sensitive (D) isolates (MOI, 1). HELF cells were pretreated for 30 min with 10 μM ganciclovir or 10 μg/ml heparin or left untreated before infection with HCMV GCV-resistant isolates (MOI, 1). (E) At each time point, cells and supernatants were harvested and progeny virus titers were determined by plaque reduction assay. Error bars represent standard deviations (SD) of three independent experiments.

**FIG 1** Growth kinetics of laboratory strain and clinical isolates in the DSTP-27-pretreated cells. HELF or HFF (GCV-sensitive isolate) cells were pretreated for 30 min with 10 μM DSTP-27 or 10 μg/ml heparin or left untreated (w/o) before infection with HCMV AD169 (A), TB40/E (B), or GCV-resistant (C) or GCV-sensitive (D) isolates (MOI, 1). HELF cells were pretreated for 30 min with 10 μM ganciclovir or 10 μg/ml heparin or left untreated before infection with HCMV GCV-resistant isolates (MOI, 1). (E) At each time point, cells and supernatants were harvested and progeny virus titers were determined by plaque reduction assay. Error bars represent standard deviations (SD) of three independent experiments.

RESULTS

**DSTP-27 inhibits replication of laboratory HCMV strains with different cell tropisms as well as clinical isolates.** In order to prove the antiviral activity of DSTP-27 against HCMV strains with different cell tropisms and clinical isolates, plaque reduction as well as viral yield assays for growth kinetics were performed. Furthermore, the influence of DSTP-27 on viral particle formation was analyzed by electron microscopy (thin sectioning). Heparin,
known to be an inhibitor of HCMV attachment, and/or GCV was included in these studies as a control. In addition, the cytotoxicity of DSTP-27 was investigated to exclude that unspecific effects could affect its anti-HCMV activity.

The results of the plaque reduction assays provided a first piece of evidence for broad anti-HCMV activity of DSTP-27. Cells treated with various concentrations of DSTP-27 were dose dependently protected against infection with AD169, TB40/E, GCV-sens., and GCV-res. The EC\textsubscript{50}s were 0.15, 0.16, 0.18, and 0.85 µM for GCV-res., GCV-sens., TB40/E, and AD169, respectively (Table 1). The potent antiviral effect of DSTP-27 was confirmed for all studied viruses by growth analyses. Cells were treated with 10 µM DSTP-27 or 10 µg/ml heparin or left untreated prior to infection with AD169 (Fig. 1A), TB40/E (Fig. 1B), GCV-res. isolates (Fig. 1C), or GCV-sens. (Fig. 1D). Viral titers in the supernatant were determined by plaque reduction assay at indicated time points. The data presented in Fig. 1 confirm the in vitro viral growth inhibition properties of DSTP-27. The virus yield was reduced at 72, 96, and 120 h.p.i. Maximum reduction by 90 or 99% was observed at 120 h.p.i. for TB40/E and both clinical isolates or AD169, respectively. Furthermore, approximately similar results were obtained for heparin. In addition, the resistance of the GCV-res. isolate was confirmed under these experimental conditions (Fig. 1E).

DSTP-27 was well tolerated by HELF cells. The mean 50% cytotoxic concentration (CC\textsubscript{50}) was 330 ± 0.01 µM (results not shown) after 8 days of treatment. Selectivity indices (SI; ratio of CC\textsubscript{50} to EC\textsubscript{50}) of about 400 (AD169) or 2,000 (all other studied HCMV strains) underline that DSTP-27 acts highly selectively (Table 1). Therefore, suppression of viral replication due to cytotoxic side effects of the drug at the concentrations used can be excluded.

Furthermore, in order to determine the formation of viral particles under inhibitor treatment, ultrathin sections of infected HELF cells were examined by electron microscopy. All types of capsids (A capsids are mature capsids without DNA, B capsids resemble procapsids with a scaffold, and mature C capsids contain DNA) were found in the nuclei of untreated cells at 72 h.p.i. (Fig. 2A, D, G, and J). Additionally, infectious virions were observed in the cytoplasm or in the extracellular space (Fig. 2M, P, S, and V). In contrast, cells treated with either 10 µM DSTP-27 or 10 µg/ml of heparin did not produce any viral particles (Fig. 2B, C, E, F, H, I, K, L, N, O, Q, R, T, U, W, and X), indicating that viral replication did not occur. Taken together, these results demonstrate a strong...
antiviral effect of DSTP-27 on laboratory HCMV strains as well as clinical isolates.

**DSTP-27 blocks an early stage of the HCMV life cycle but not attachment.** To determine the stage of viral replication that is sensitive to DSTP-27, the effect of time-dependent drug addition was analyzed in modified plaque reduction assays. HELFs were infected with AD169 at an MOI of 0.01, and DSTP-27 (10 μM) was added at different times of the replication cycle. At day 8 p.i., viral plaques of untreated and treated cells were counted and compared. If cells were pretreated with DSTP-27 for 30 min before infection and removed afterwards by several washing steps, viral replication was inhibited by about 90% (Fig. 3). Addition of DSTP-27 60 min during infection resulted in suppression of viral replication by approximately 70%. Addition of DSTP-27 after infection had only a marginal effect on HCMV replication. These experiments implicate that DSTP-27 inhibits an early stage in the viral infectious cycle.

In order to analyze the effect of DSTP-27 on viral attachment, prechilled HELF cells were treated with 10 μM, 5 μM, or 1 μM DSTP-27 or 10 μg/ml heparin or left untreated on ice for 30 min at 4°C. After removal of the inhibitors, cells were infected with prechilled HCMV AD169 for 2 h at 4°C. Unattached virus was aspirated by 3 washing steps, and the cells were subjected to plaque reduction assays (Fig. 4). Compared to what is observed in the untreated cells, DSTP-27 treatment leads to only approximately 20 to 40% plaque reduction. In addition, analyses with even higher concentrations of the compound did not result in a complete block of attachment (Fig. 4). The control panel with heparin...
showed a block of attachment (Fig. 4). Therefore, attachment of HCMV to HELF was not blocked and this effect of DSTP-27 was concentration independent.

**DSTP-27 prevents expression of HCMV proteins.** Since DSTP-27 inhibits an early stage of replication, its effect on immediate early (IE; IE1), early (E; pUL44), and late (L; pp28) protein expression was characterized. Coverslip cultures were pretreated with 10 μM DSTP-27 or 10 μg/ml heparin or left untreated prior to infection with AD169 (MOI, 1.0). As anticipated, expression of IE1 was significantly reduced by DSTP-27. Only few single IE1-, pUL44-, and pp28-positive cells (Fig. 5A, panels a to c, g, and k) were detected. In the presence of heparin, IE1, pUL44, and pp28 expression was less affected (Fig. 5A, panels a, h, and l). Mock-infected untreated (Fig. 5A, panels a, e, and i) as well as infected untreated (Fig. 5A, panels b, f, and j) cells served as controls.

In Western blot analysis, neither IE, E, nor L proteins were detected in DSTP-27-treated HELFs (Fig. 5B). In contrast, heparin-treated cells expressed reduced levels of IE1 protein (Fig. 5B).

**DSTP-27 inhibits infectivity of viruses.** High-titer HCMV AD169 (MOI, 6) was incubated either with DSTP-27 or with medium for 1 h at 37°C. The samples were diluted 6-fold (10 μM DSTP-27; Fig. 6A) or 100-fold (0.1 μM DSTP-27; Fig. 6B). HELF cells were infected with dilutions of untreated virus (w/o) or with virus pretreated with 10 μM DSTP-27 (DSTP-27) and subjected to plaque reduction assays.

Interestingly, binding of DSTP-27 to viruses was very stable; therefore, diluted viruses with bound DSTP-27 were still unable to infect cells (Fig. 6). These results demonstrated that DSTP-27 could inactivate HCMV by binding to the virus.

**Influence of DSTP-27 on HCMV penetration.** In order to investigate the ability to prevent virus penetration, prechilled HELF cells were infected with HCMV AD169 (MOI, 1) for 2 h at 4°C.

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**FIG 4** Effects of DSTP-27 on virus attachment. Prechilled (4°C) HELF cells were treated with 1 μM, 5 μM, or 10 μM DSTP-27 or 10 μg/ml heparin or left untreated (w/o) for 30 min at 4°C. After removal of the inhibitors, cells were infected with prechilled HCMV AD169 for 2 h at 4°C. Unattached virus was aspirated by 3 washing steps, and the cells were subjected to plaque reduction assays. Error bars on the histogram are SD from three independent experiments.

**FIG 5** Analysis of HCMV protein expression in DSTP-27-treated cells. HCMV-infected or mock-infected HELF cells (MOI, 1) were untreated (w/o) or pretreated with 10 μM DSTP-27 or 10 μg/ml heparin. (A) After 72 h p.i., HELF cells were subjected to immunofluorescence using antibodies against IE1, pUL44, and pp28. Uninfected cells (mock) served as control. (B) Cells were subjected to Western blot analysis using antibodies against IE1, pUL44, and pp28. Membranes were reprobed with an antibody against α-tubulin to verify equal loading. Arrows indicate the positions of proteins.
Thereafter, cells were treated with 1 μM, 5 μM, or 10 μM DSTP-27 or 10 μg/ml heparin or left untreated. Penetration occurred at 37°C for 10 min. After washing steps with buffer (pH 3.0), cells were subjected to plaque reduction assays.

It is clearly shown that regardless of the amount of DSTP-27, the compound reduced virus penetration by approximately 75% (Fig. 7). Heparin treatment leads only to a 50% reduction (Fig. 7). These results imply that DSTP-27 was able to inhibit penetration of HCMV.

DISCUSSION

Human cytomegalovirus is a member of the herpesvirus family and represents a major human pathogen, causing life-threatening diseases in immunocompromised patients. Due to the failure of the immune system to achieve clearance of the virus, HCMV persists in its host. Since the currently available drugs cause multiple problems, new antiviral therapeutics are urgently in demand. One new class of inhibitors are low-molecular-weight N,N'-bisheteryl derivates of dispirotripiperazine (DSTP) (24). The current study demonstrates that DSTP-27 not only is an effective inhibitor of herpes simplex virus 1 (HSV-1) replication (7) but also exhibits strong activity against GCV-sensitive (AD169, TB40/E, and GCV-sens.) as well as GCV-resistant HCMV.

The compound inhibited plaque formation of all tested HCMV by 50% at concentrations lower than 1 μM (Table 1). Since the compound was well tolerated (CC<sub>50</sub> 330 ± 0.001 μM), the selectivity indexes were high (between 400 and 2,000). These results indicate that DSTP-27 is as effective against HCMV plaque production as the most promising compounds, i.e., BDCRB (22) and letermovir (25), both targeting the HCMV terminase, and maribavir (26), targeting the HCMV kinase pUL97.

Like heparin, DSTP-27 reduced viral yield up to 1 to 2 log (Fig. 1) and prevented virus particle formation as proved by electron microscopy analyses (Fig. 2). Formation of HCMV capsids as well as noninfectious particles (e.g., dense bodies or NIEPS) and virions was completely blocked in heparin- as well as DSTP-27-
onset face. Recently, Selinka et al. (9) suggested that the mode of action of DSTP-27 could not prevent attachment of virions to the cell surface. Assays were performed. These analyses demonstrated that in DSTP-27-pretreated cells, the expressions of immediate early proteins, such as factor pUL44 and tegument protein pp28 was strongly affected. In contrast, no antiviral activity was found if DSTP-27 was added after viral adsorption. These results indicated that DSTP-27 blocks entry, thus preventing viral replication and cell-to-cell spread. For treatment of persistent HCMV infection, the constant supply of the inhibitor is required. In this scenario, the stability of DSTP-27 together with its strong inhibitory activity will be a powerful tool for antiviral therapy against HCMV reactivation. This would be a promising therapy for the increasing number of transplant patients suffering from HCMV infection or reactivation.

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REFERENCES


FIG 7 Effects of DSTP-27 on virus penetration. Prechilled (4°C) HELF cells were infected with HCMV AD169 (MOL 1) for 2 h at 4°C. Thereafter, cells were treated with 1 μM, 5 μM, or 10 μM DSTP-27 or 10 μg heparin or left untreated (w/o) for 10 min at 37°C. After washing steps with Tris-HCl (pH 3.0), cells were subjected to plaque reduction assays for 8 days at 37°C. Error bars on the histogram are SD from three independent experiments.

treated cells. The same phenotype was found in cells treated with proteasome inhibitor MG132 (27) or in cells treated before the onset of viral replication with brefeldin A (28).

Critical to the effect of DSTP-27 was the time of compound addition. Preincubation of the cells resulted in plaque reduction of up to ≥90% compared to the untreated control (Fig. 3). A similar observation has been reported for HSV-1 and human papillomavirus (HPV) (8, 9). In addition, these previous studies provide evidence that the mode of action is based on blocking of heparan sulfate function on the cell surface. Binding to heparan sulfate proteoglycans (HSPG) is known to be a prerequisite for attachment of herpesviruses prior to entry via interaction with specific receptors (11, 29, 30). If the compound was present during viral adsorption, DSTP-27 induced a plaque reduction of about 70%. In contrast, no antiviral activity was found if DSTP-27 was added after viral adsorption. These results indicated that DSTP-27 blocks an early event in the HCMV replication cycle. Immunofluorescence and Western blot analysis confirmed this hypothesis. In DSTP-27-pretreated cells, the expressions of immediate early protein 1 (IE1) was slightly reduced, while expression of processivity factor pUL44 and tegument protein pp28 was strongly affected. Only in few isolated cells was HCMV protein expression observed. In order to shed light on the primary mode of action, attachment assays were performed. These analyses demonstrated that DSTP-27 could not prevent attachment of virions to the cell surface. Recently, Selinka et al. (9) suggested that the mode of action of DSTP-27 for human papillomavirus could be the induction of clustering of heparan sulfate proteoglycans and consequently clustering of virions on the cell surface. This leads to inhibition of transfer to the specific entry receptor and therefore to noninfectious uptake of HPV. Whether this mechanism is also the case for HCMV has to be elucidated.

Taken together, DSTP-27 (i) prevents penetration of HCMV but not attachment and (ii) inactivates HCMV, thus indicating that the mechanism of action of DSTP-27 is different in HCMV and in HSV-1. Although direct activity against the virus may be considered virucidal, interaction at the cell surface suggests an antiviral mechanism, as noted, for example, for maraviroc, which acts at the cell surface (31).

In summary, we demonstrated that DSTP-27 is an effective inhibitor of HCMV infection. Analyses lead to the suggestion that DSTP-27 blocks entry, thus preventing viral replication and cell-to-cell spread. For treatment of persistent HCMV infection, the constant supply of the inhibitor is required. In this scenario, the stability of DSTP-27 together with its strong inhibitory activity will be a powerful tool for antiviral therapy against HCMV reactivation. This would be a promising therapy for the increasing number of transplant patients suffering from HCMV infection or reactivation.
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