Novel non-nucleoside inhibitors of cytomegaloviruses (BAY 38-4766): in vitro and in vivo antiviral activity and mechanism of action

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For two decades it has been impossible to develop drugs with novel mechanisms of action against herpesviruses, and treatment has been confined largely to the use of inhibitors of viral DNA polymerase. As a representative of a novel inhibitory approach, the non-nucleosidic BAY 38-4766 was identified as a highly selective inhibitor of human cytomegalovirus (HCMV). The compound selectively inhibits not only HCMV strains, including ganciclovir-resistant, ganciclovir/foscarnet and ganciclovir/cidofovir double-resistant clinical isolates, but also a number of monkey and rodent cytomegaloviruses. In a murine cytomegalovirus (MCMV) pathogenicity model in mice, antiviral efficacy and excellent tolerability were demonstrated. BAY 38-4766-resistant HCMV and MCMV strains are not cross-resistant to the nucleoside analogues ganciclovir and cidofovir or the pyrophosphate analogue foscarnet, indicating a different mode of action. Mechanistic studies demonstrated that the high selectivity of this drug class is most likely due to the inhibition of a late stage of the viral replication cycle. Sequence analyses of resistant HCMV and MCMV strains revealed mutations in UL89 and UL104, proteins known to be involved in viral DNA cleavage and packaging. Consequently, the drug is highly specific for the viral as opposed to cellular functions, since UL89 is related to a bacteriophage terminase and no human equivalent exists. In addition, because some of the genes of the viral DNA cleavage and packaging complex are highly conserved among herpesviruses, development of broad-spectrum agents covering additional human herpesviruses might be possible using this approach.

Introduction

In the past, antiviral chemotherapy has been confined largely to nucleoside analogues that inhibit viral DNA polymerases or reverse transcriptases (RTs) as substrate analogues. The most prominent examples are aciclovir [9-(2-hydroxyethoxymethyl)guanine] for the treatment of herpes simplex virus (HSV) and varicella-zoster virus infections and azidothymidine (3′-azido-2′,3′-dideoxythymidine), the first drug for the treatment of human immunodeficiency virus (HIV) infections. New anti-HIV agents, such as non-nucleosidic RT inhibitors (NNRTIs), which bind to a hydrophobic pocket at the active site, and protease inhibitors were successfully introduced into clinical practice. The search for protease inhibitors of herpesviruses has so far failed despite the fact that several herpes protease structures have been elucidated. As several herpesviruses, in particular the human cytomegalovirus (HCMV), cause severe, often life-threatening diseases in immunocompromised hosts such as transplant recipients,
AIDS patients and newborns, a significant medical need still exists for novel antiviral agents/approaches.

HCMV is widespread in the human population. In immunocompetent individuals, infection is inapparent or associated with mild symptoms. However, HCMV infection is the leading cause of neurological disease and hearing loss in congenitally infected newborns affecting some 8000 newborns per year in the USA. Furthermore, following the first 100 days after transplantation, HCMV-induced pneumonia develops in about 50% of heterologous bone marrow transplants with an 80% mortality rate if untreated. Approximately 15–70% of patients with kidney, liver, bone marrow and heart/lung transplant recipients are affected by HCMV hepatitis and pneumonia resulting in decreased graft and patient survival. HCMV-positive graft donors and HCMV-negative graft recipients are the major risk factors in solid organ transplantation. HCMV was found in 90% of AIDS patients at autopsy. Before the advent of highly active antiretroviral therapy (HAART), HCMV retinitis occurred in about 10–45% of patients with late-stage AIDS. In addition, some 7–10% of AIDS patients may have HCMV diseases of the gastrointestinal tract and the nervous system presenting with colitis, oesophageitis and wasting syndrome. While the use of HAART has diminished the impact of HCMV disease significantly, cessation of treatment in patients with virological and immunological failure under potent antiretroviral therapy led to recurrence of HCMV retinitis. Antiviral resistance emerges in 14–37% of AIDS patients with HCMV retinitis treated for 9 months with ganciclovir, cidofovir or foscarnet.

Currently, only inhibitors of herpesviral DNA polymerases are licensed for the prophylaxis and treatment of HCMV infections, but these anti-HCMV therapies do not eliminate virus or eradicate infection in any individual. Despite their antiviral potential all of these medications are associated with multiple side effects, such as dose-limiting bone marrow and kidney toxicity, as well as the emergence of single and double drug resistance. Even the recently developed phosphorothioate antisense oligonucleotide fomivirsen (ISIS 2922) for the treatment of HCMV retinitis in AIDS patients can only be applied intraocularly and is associated with increased intraocular pressure and ocular inflammation in 25% of treated patients.

We report on BAY 38-4766 [3-hydroxy-2,2-dimethyl-N-[4-[[5-(dimethylamino)-1-naphthyl]sulfonyl]amino]-phenyl]-N-propanamide (Figure 1), a novel oral non-nucleosidic inhibitor of HCMV that targets virus-specific proteins known to be required for the cleavage and packaging of viral DNA by processing high molecular weight viral DNA concatemers to monomeric genome length. In particular, as this viral DNA processing machinery has no counterpart in human cells but is highly conserved among herpesviruses, and therefore very specific for these viruses, this approach facilitates the generation of novel drugs with excellent tolerability and also has the potential for broad-spectrum action against a number of human herpesviruses.

Figure 1. Structure, antiviral activity and selectivity of BAY 38-4766 (CC50 = 50% cytostatic concentration; EC50 = 50% antivirally effective concentration; HELF = human embryonic lung fibroblasts; HCMV = human cytomegalovirus strain Davis; MCMV = murine cytomegalovirus strain Smith; MEF = murine embryonic fibroblasts; SI = selectivity index).

Materials and methods

Viruses, cells and drugs

The laboratory strains HCMV-Davis [ATCC VR 807 of the American Type Culture Collection (ATCC), Rockville, MD, USA], HCMV-AD169 (ATCC VR 538), HCMV-Towne (ATCC VR 977), as well as the clinical isolates HCMV-He (kindly provided by Dr A. Eis, University of Bonn, Institute of Medical Microbiology, Germany), HCMV-UlmB, HCMV-B.K./19684, HCMV-R.R./17727, HCMV-MF./16445 and HCMV-isolate 2 (obtained from Professor J. Mertens, University of Ulm, Germany), were propagated in human embryonic lung fibroblast (HELF) or normal human dermal fibroblast (NHDF; CellSystems, St Katharinen, Germany) cells at passages 10–40 or 6–20, respectively. HELF and NHDF cells were cultivated in Eagle’s minimal essential medium with Earle’s salts (EMEM) supplemented with 10% (v/v) fetal calf serum (FCS), hereafter referred to as EMEM/10. Murine cytomegalovirus (MCMV) strain Smith (ATCC VR 194) was propagated in murine embryonic lung fibroblast (HELF) or normal human dermal fibroblast (NHDF; CellSystems, St Katharinen, Germany) cells at passages 10–40 or 6–20, respectively. HELF and NHDF cells were cultivated in Eagle’s minimal essential medium with Earle’s salts (EMEM) supplemented with 10% (v/v) fetal calf serum (FCS), hereafter referred to as EMEM/10. Murine cytomegalovirus (MCMV) strain Smith (ATCC VR 194) was propagated in murine embryonic fibroblast (MEF) or NIH 3T3 cells (ATCC CRL 1658). MEF cells were prepared from embryos of late-stage pregnant BALB/c mice by trypsin–EDTA disintegration and stored in liquid nitrogen at early passages, and were grown in Dulbecco’s modified Eagle’s medium (DMEEM) supplemented with 10% (v/v) FCS. NIH 3T3 cells were propagated in EMEM/10. All cell culture media and supplements were from Gibco-BRL, manufactured by Life Technologies Ltd (Paisley, UK). The

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following reference drugs were used: iv formulations of Cymevene (ganciclovir sodium; Syntex/Roche, Grenzach Wyhlen, Germany), Foscavir (foscarnet sodium; Astra Pharmaceuticals, Wedel, Germany) and Vistide (cidofovir; Pharmacia & Upjohn SA, Luxembourg, Luxembourg) as 50 mM solutions in 0.9% saline. BAY 38-4766 was used as a 50 mM solution in dimethylsulphoxide (DMSO).

Preparation of virus stocks

Confluent HELF cell cultures were infected with cell-associated or cell-free virus at a multiplicity of infection (moi) of 0.001–0.005. After removal of the viral inoculum, the cells were incubated at 37°C/5% CO₂ for a further 2 days after reaching 100% cytopathic effect (CPE). Culture supernatant containing cell-free virus was cleared by low speed centrifugation and stored at −140°C or in liquid nitrogen. Virus-infected cells were collected by trypsinisation and distributed to confluent HELF tissue culture bottles. After reaching complete CPE, infected cells were collected in freezing medium (EMEM plus 20% FCS and 10% DMSO). Aliquots were carefully frozen and stored at −140°C or in liquid nitrogen. Confluent monolayers of MEF or NIH 3T3 cells were infected with MCMV strain Smith as described above. Reaching a complete CPE, infected cell cultures were harvested by three freezing and thawing cycles and subsequent sonification for 30 s. After low speed centrifugation, aliquots of cell-free virus were stored at −140°C.

Anti-HCMV and anti-MCMV cytopathogenicity assays

Test compounds were used as 50 mM DMSO stock solutions. Ganciclovir, foscarnet and cidofovir served as reference drugs. Addition of 50, 5, 0.5 and 0.05 mM DMSO-stock solutions to culture medium in duplicates was followed by serial two-fold dilutions in 96-well microtitre plates. Each well was supplemented with 1 × 10⁴ to 3 × 10⁴ cells of a suspension of infected and uninfected HELF or NHDF cells (moi = 0.001–0.002). Wells without either drug and virus or wells without drug served as cell and virus controls, respectively. Final drug concentrations were between 250 and 0.0005 μM. Plates were incubated for 6 days at 37°C/5% CO₂ until infected virus controls reached 100% CPE. A neutral red dye solution (3.33 g/L) was added to the wells (final concentration 56 μg/mL) and plates were stored at 37°C for 1 h followed by a 2 h staining in the dark at room temperature (RT). Neutral red-stained monolayers were fixed with 4% formaldehyde by incubation for 30 min at RT. After three washing cycles with deionized water, plates were dried at 56°C followed by visual evaluation using an overhead microscope (Plaque multiplier; Technomara, Zurich, Switzerland). The following data were drawn from the assay plates: EC₅₀ (HCMV) = concentration of drug that inhibits the CPE by 50% compared with an untreated virus-infected control; CC₅₀ (HELF) = highest concentration of drug with no visible cytopathic effects on cells compared with the untreated cell control; SI = selectivity index = CC₅₀ (HELF)/EC₅₀ (HCMV).

For anti-MCMV assays the same procedure was used, with some exceptions. A concentrated suspension of MEF or NIH 3T3 cells was mixed with a cell-free virus suspension (moi 0.05–0.1) and incubated for 15 min before being diluted to 1.3 × 10⁵ cells/mL and added to the drug dilutions in 96-well plates. Incubation for 5 days was followed by fixation with 4% formaldehyde/Giemsa solution for 30 min. Plates were washed three times, dried and evaluated as described above.

HCMV and MCMV plaque assays

HEL and NHDF cells (1 × 10⁴ to 2 × 10⁴/well) or MEF and NIH 3T3 cells (1 × 10⁴/well) were seeded in 24-well tissue culture plates. Confluent cell monolayers were infected with 40–60 plaque-forming units (pfu) per well. After a 1 h adsorption period, a 0.5% methylcellulose (MC)–EMEM/10 overlay medium and the appropriate drug solutions were added. HCMV-infected cell cultures were stained after 7–12 days with medium changes every 3–4 days and MCMV plaque assays after 5 days without overlay exchange, using neutral red dye or Giemsa’s solution, respectively. Plaques were counted visually with the aid of an overhead microscope (Plaque viewer). The number of plaques in the treated wells was expressed as a percentage of untreated virus control and plotted against the logarithm of drug concentration. Drug concentrations producing 50% reduction in plaque formation (EC₅₀) were determined graphically from the dose–response curves. Assays were carried out two to four times in duplicate. For titration of crude virus stocks, confluent monolayers of cells were infected with a serial log dilution (10⁻¹–10⁻⁸) of stock virus and processed accordingly.

Cytotoxicity and antiviral fluorescence assays

In order to evaluate drug toxicity, 96-well microtitre plates were prepared with 100 μL of EMEM/10 per well. After addition of 2 μL of 50 mM compound stock solutions in duplicate into 198 μL in row 2, serial two-fold dilutions were made with 100 μL up to row 12 and 100 μL of a HELF, NHDF or 3T3 cell suspension (5 × 10⁵ cells/mL) were added per well. Row 1 served as an untreated cell control. After incubation for 6 days at 37°C and 5% CO₂, the cells were washed once with phosphate-buffered saline (PBS), and 200 μL of a 10 μg/mL fluorescent dye solution in PBS, pH 7.2 (fluorescein diacetate) were dispensed per well. After 45 min, the fluorescence signal was measured
with a Fluorskan Ascent fluorimeter (Labsystems, Finland) (excitation filter 485 ± 11 nm, emission filter 530 ± 15 nm). The relative fluorescence units (RFUs) of treated cells were expressed as percentages of untreated cell controls and 

$CC_{50}$ values were determined graphically. The anti-HCMV and anti-MCMV cytopathogenicity assays followed the same procedure as described above, with the exception that the moi values were 0.03 and 0.2 and the incubation times 15 days and 7 days, respectively. Thereafter, medium was removed and the wells were washed with 200 μL of PBS, and 45 min after addition of 200 μL of the fluorescence dye solution, signals were measured as described. The RFUs of treated wells were expressed as percentages of the difference between RFUs of the untreated cell and virus controls and 

$CC_{50}$ and 

$EC_{50}$ values were estimated graphically.

Selection of HCMV- and MCMV-resistant strains

To select drug-resistant viral mutants, HCMV strain AD169 was serially passaged in HELF cells in the presence of increasing compound concentrations (two-fold steps). The resistant mutants of HCMV strain AD169, as well as MCMV strain Smith, were growing at 50-fold and 500-fold above the 

$EC_{50}$, respectively. Resistant progeny virus was plaque-purified by limiting dilution in the presence of the respective compounds. Stability of resistance was tested by serially passaging (10–12 times) plaque-purified viruses without selective drug pressure.

Preparation of viral DNA for sequencing and Southern blot analysis

Confluent monolayers of HELF cells ($5 \times 10^5$) were infected with resistant HCMV AD169 at an moi of 0.25–0.5 and treated with various drug concentrations until untreated, infected control cells showed 100% CPE. Supernatant was harvested, cell debris removed by low-speed centrifugation and extracellular virions were collected by ultracentrifugation for 1 h, at 45 000g and 4°C. Pellets were resuspended using a Potter–Elvehjem homogenizer and further purified by a centrifugation step through a 15%/40% sucrose cushion for 1 h and 150 000g. The Qiagen Blood & Cell Culture DNA kit (Qiagen, Hilden, Germany) was applied as instructed by the manufacturer. Construction of an overlapping cosmid library and sequence analyses were carried out by Qiagen. Resistant MCMV Smith was propagated using NIH 3T3 cells (moi = 0.005) and harvested as described above. Sequences homologous to mutant HCMV AD169 were amplified by PCR using a 

$pfu$ polymerase and analysed by double-stranded sequencing. For Southern blot analyses, viral DNA was prepared using the Qiagen Blood & Cell Culture DNA kit as instructed by the manufacturer after disruption of collected cells by repeated freezing and thawing cycles. To quantify viral DNA, dot-blot hybridization was carried out using a DIG-labelled, randomly primed 300 bp PCR fragment (HCMV genome position 703–1524). DNA (2.5 μg per lane) was digested overnight with 20 U KpnI, size-fractionated on a 0.7% agarose gel by electrophoresis and subjected to capillary transfer on positively charged nylon membranes. After UV-crosslinking, the blot was incubated four times for 3 min each time with Soak I (0.5 M NaOH; 1 M NaCl), twice with Soak II (3 M NaCl; 0.5 M Tris–HCl, pH 7.4), baked at 120°C for 30 min, prehybridized in a standard hybridization buffer (5 × SSC, formamide, 50%; N-lauroylsarcosine, 0.1% w/v; SDS, 0.02%; blocking reagent, 2%; 20 mL/100 cm$^2$ filter) for 2 h at 42°C and hybridized overnight in the presence of randomly DIG-labelled DNA probe (20 ng/mL). Detection was carried out by luminescence as instructed by the manufacturer (Roche, Germany).

MCMV pathogenesis model: in vivo passage of MCMV

Salivary glands of BALB/c mice were harvested 2–3 weeks post-infection and homogenized in EMEM/10 using an Ultra Turrax (IKA Labortechnik, Staufen, Germany). After freezing (–80°C) and thawing, the debris was removed by centrifugation at 2000g for 15 min at 4°C. Virus was concentrated by ultracentrifugation through a 15% sucrose cushion using a SW28 rotor at 100 000g for 90 min and titre was determined by plaque assay on MEF cells.

Infection and treatment of mice

Five- to 6-week-old male homozygous NOD/LtSz-SCID/j mice (Jackson Laboratory, Bar Harbor, ME, USA) were infected ip using 5 × 10$^5$ pfu in 0.2 mL. Approximately 20 h after infection, test compounds were administered as a suspension in 0.5% tylose to the animals twice daily for 8 consecutive days po using a gavage. Daily dosages of 3, 10, 30 and 100 mg/kg body weight (bw) per application in a total application volume of 10 mL/kg bw were given. Animals were killed 16 h after the last application and organs were removed for further analyses.

Isolation of nucleic acids and dot-blot analysis

The nucleic acids were extracted using the QIAGEN Tissue Kit (Qiagen) as instructed by the manufacturer. After quantification of genomic DNA, dot-blot hybridization was carried out with a DIG-labelled, randomly primed 1300 bp PCR-amplified fragment from the HindIII J fragment of
MCMV strain Smith. DNA (10 μg) was blotted on to a nylon membrane soaked four times for 3 min each time with 0.5 M NaOH/1 M NaCl, twice with 3 M NaCl/0.5 M Tris–HCl, pH 7.4, baked at 120°C for 30 min, prehybridized in a standard hybridization buffer (5 x SSC, formamide, 50%; N-lauroylsarcosine, 0.1% w/v; SDS, 0.02%; blocking reagent, 2%; 20 mL/100 cm²) for 2 h at 42°C and hybridized overnight in the presence of the probe (25 ng/mL). After washing, immunological detection was carried out using the CDP-Star system (Roche) as instructed by the manufacturer. Intensity of chemiluminescence signals was quantified using the LumiImager system (Roche). The LumiImager signals were analysed by descriptive statistics and compared by Variance analysis with post hoc comparison of means (Statistica; StatSoft, Tulsa, OK, USA).

Results

In vitro antiviral activity and selectivity

The anti-HCMV activity and selectivity of BAY 38-4766 (Figure 1) and the reference drug ganciclovir were evaluated against different laboratory and human field strains in a plaque inhibition and an HCMV cytopathogenicity assay (6 day; neutral red dye staining) as well as an HCMV cytopathogenicity assay (15 day; fluorescence dye staining) using HELF or NHDF cells (Table 1; Figures 1 and 2). Results of plaque assays showed that all strains were sensitive to BAY 38-4766 with an averaged EC₅₀ of 1.03 ± 0.57 μM. Most notably, the drug was also active against the ganciclovir-resistant clinical isolates, as well as the ganciclovir/foscarnet and ganciclovir/cidofovir double-resistant clinical isolates. Ganciclovir was about four times less active with an averaged EC₅₀ of 4.32 ± 1.82 μM for the sensitive strains. Using two different HCMV pathogenicity assays antiviral activity data were confirmed for both drugs, although somewhat lower drug concentrations were needed to achieve 50% inhibition of cytopathogenicity of the laboratory strain HCMV Davis (EC₅₀ = 0.3 and 1.9 μM for BAY 38-4766 and ganciclovir, respectively). No activity was found against HSV type 1 and type 2, varicella-zoster virus or human herpesvirus 6, nor against HIV, hepatitis B virus, adenovirus type 5 or measles virus. In contrast, BAY 38-4766 was found to be active against various monkey CMV strains (EC₅₀ < 1 μM), but most pronounced inhibitory effects were found for various rodent CMV strains (data not shown) including the MCMV (see Figure 1), offering the possibility of assessing the impact of the drug in a MCMV pathogenicity model in mice (see below). Compared with the HCMV Davis strain the impact of BAY 38-4766 on the replication of the MCMV strain Smith was nearly 10-fold stronger in both the plaque and cytopathogenicity assays (EC₅₀ = 0.05 and 0.04 μM, respectively). Cytostatic activities of BAY 38-4766 in human NHDF and HELF cells as well as in murine 3T3 cells observed visually after staining from the respective HCMV or MCMV screening assays (Figure 1) compared favourably with quantitative data of HCMV and MCMV screening assay procedures using a fluorescence dye signal evaluation (Figure 2) as well as with data from 6 day cytotoxicity fluorescence assays (data not shown) using uninfected NHDF and 3T3 cells (CC₅₀ = 93 and 63 μM, respectively). Based on these data, selectivity indices (SI = CC₅₀/EC₅₀) of nearly 300 (HCMV) and 1600 (MCMV) were calculated.

Mechanism of action

The laboratory strain HCMV AD169 was made resistant to increasing concentrations of a BAY 38-4766-related compound over multiple in vitro passages (time > 6 months) starting with the EC₅₀. The resistant strain showed cross-resistance to BAY 38-4766 (Table 1) and to various other BAY 38-4766-related structures (data not shown). As indicated by the resistance index (RI), no cross-resistance was shown for the nucleoside reference drugs, or the pyrophosphate analogue foscarnet, indicating a different mode of action. The same results were obtained with the resistant MCMV strain (Table 2b) generated with increasing con-
centrations of BAY 38-4766 (within nearly 2 months). Interestingly, the MCMV-resistant strain showed much higher resistance compared with the resistant HCMV AD169 strain. The marketed reference drugs did not discriminate between the wild-type and resistant strains, again indicating a different target of drug interference.

Sequence analyses of the resistant HCMV AD169 strain revealed seven mutations, deletions and inversions, which led to amino acid exchanges in the corresponding viral gene products [affected open reading frames (ORFs): UL78, UL81/82, UL89, UL104]. Four mutations mapped to UL89 and UL104 (see Table 3), which are known to be essential.

Figure 2. Antiviral and anticellular activity of BAY 38-4766 and ganciclovir. Mixtures of HCMV Davis-infected and uninfected NHDF cells (m.o.i. = 0.03) and of 3T3 cells with MCMV strain Smith (m.o.i. = 0.2) were incubated in 96-well plates in the presence of various concentrations of BAY 38-4766 (250–0.0005 μM) or ganciclovir (250–0.5 μM) at 37°C, 5% CO₂ for 15 or 7 days, respectively. After this incubation period medium was removed, the wells were washed with PBS, and 45 min after addition of a fluorescence dye solution, fluorescence signals produced by activity of esterases in living cells was measured as described in Materials and methods. Relative fluorescence units = percentage of untreated, uninfected cell control. [○, NHDF/HCMV (BAY 38-4766); △, NHDF/HCMV (ganciclovir); ●, 3T3/MCMV (BAY 38-4766)].

**Table 2a.** Anti-HCMV AD169 activity of BAY 38-4766 and reference drugs (plaque inhibition assay data)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>EC₅₀&lt;sup&gt;a&lt;/sup&gt; (μM)</th>
<th>HCMV&lt;sup&gt;w&lt;/sup&gt; (wild type)</th>
<th>HCMV&lt;sup&gt;r&lt;/sup&gt; (resistant)</th>
<th>RI&lt;sup&gt;b&lt;/sup&gt; = EC₅₀ (HCMV&lt;sup&gt;r&lt;/sup&gt;) / EC₅₀ (HCMV&lt;sup&gt;w&lt;/sup&gt;)</th>
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<tr>
<td>BAY 38-4766</td>
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<td>Cidofovir</td>
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<td>0.17</td>
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<td>Ganciclovir</td>
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<td>1.6</td>
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<td>Foscarnet</td>
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<sup>a</sup>EC₅₀ = 50% effective concentration.

<sup>b</sup>RI = resistance index.

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<tr>
<th>Drugs</th>
<th>EC₅₀&lt;sup&gt;a&lt;/sup&gt; (μM)</th>
<th>MCMV&lt;sup&gt;w&lt;/sup&gt; (wild type)</th>
<th>MCMV&lt;sup&gt;r&lt;/sup&gt; (resistant)</th>
<th>RI&lt;sup&gt;b&lt;/sup&gt; = EC₅₀ (MCMV&lt;sup&gt;r&lt;/sup&gt;) / EC₅₀ (MCMV&lt;sup&gt;w&lt;/sup&gt;)</th>
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<tr>
<td>BAY 38-4766</td>
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<td>Foscarnet</td>
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<td>280.0</td>
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**Table 2b.** Anti-MCMV strain Smith activity of BAY 38-4766 and reference drugs (plaque inhibition assay data)
Novel non-nucleoside inhibitors of cytomegaloviruses

Apparantly, mutations of MCMV strain Smith resistant to BAY 38-4766 also map to the corresponding genes within the murine homologues of UL89 and UL104. These observations indicate that the gene products of UL89 and UL104 are potential molecular targets of the drug.

Northern blot analyses of late viral RNA transcripts confirmed that viral DNA replication is not affected in the presence of this new inhibitor class (data not shown). Therefore, inhibition of functional viral DNA cleavage activity was examined by Southern blot analysis in vitro. Viral DNA was isolated from inhibitor-treated and untreated HCMV-infected cells and digested with the restriction enzyme KpnI. A terminal digoxigenin-labelled DNA probe was employed to determine processing of concatemeric DNA (see Figure 3). Analysis of DNA from infected cells demonstrated that de novo synthesis of viral DNA was abolished by ganciclovir (Figure 3; left lane), but continued in the presence of BAY 38-4766. However, specific cleavage of viral DNA (maturation, indicated by the 4 kbp fragment) was inhibited in the presence of the drug in a dose-dependent manner with concentrations >0.5 μM being completely inhibitory.

In vivo antiviral activity: lethal infection of SCID mice with MCMV

Due to its inhibitory potential against rodent CMV strains, BAY 38-4766 was tested in a murine pathogenicity model, in which immunodeficient mice were inoculated with MCMV. This viral infection is lethal. Spread of the virus into various organs occurs in this model as is also observed in human CMV infections under conditions of immunodeficiency. Survival of the mice after po treatment with BAY 38-4766 was compared with that of ganciclovir. Uninfected mice treated similarly with the test compounds did not develop weight loss. In acute toxicity studies the lethal dose, LD50, in mice and rats was >2000 mg/kg bw after oral administration of BAY 38-4766 (data not shown). Treatment of MCMV-infected animals for 8 days with increasing doses of ganciclovir and BAY 38-4766, respectively, showed a comparable effect of both drugs on survival, which was dose dependent (Figure 4). To clarify the in vivo drug activity in more detail, weight reduction and reduction of MCMV DNA in different target organs was determined for ganciclovir and BAY 38-4766. In this murine model, BAY 38-4766 decreased viral load in target organs comparably to ganciclovir. The clinical parameter (weight loss as a consequence of viral infection) showed a similar efficacy as well (see Tables 4a and b).

Discussion

In spite of many efforts, including rational drug design of inhibitors against the proteases of herpesviruses,9 no new

Table 3. Resistance mutations are found in components of the DNA cleavage and packaging complex

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon position</th>
<th>Mutation type</th>
<th>Sequence change</th>
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<tr>
<td>UL89</td>
<td>exon I</td>
<td>substitution</td>
<td>c-&gt;t</td>
</tr>
<tr>
<td></td>
<td>exon II</td>
<td>substitution</td>
<td>c-&gt;t</td>
</tr>
<tr>
<td>UL104</td>
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<td>inversion</td>
<td>gaga-&gt;cttc</td>
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<tr>
<td></td>
<td>exon II</td>
<td>deletion</td>
<td>cagc-&gt;gagg</td>
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In the viral DNA concatemer cleavage process. Apparently, mutations of MCMV strain Smith resistant to BAY 38-4766 also map to the corresponding genes within the murine homologues of UL89 and UL104. These observations indicate that the gene products of UL89 and UL104 are potential molecular targets of the drug.
Figure 3. Southern blot analysis of viral DNA in the absence or presence of BAY 38-4766. HELF cells were infected with HCMV AD169 at an moi of 1 and maintained at the inhibitor concentrations indicated. Isolated viral DNA was digested with KpnI and size-fractionated by gel electrophoresis. After blotting, hybridization was carried out with a terminal DNA probe (genome position 703–1524; position indicated by grey rectangle). Cleaved and uncleaved genomic DNA can be distinguished by the size of the fragment (4 kbp: cleaved product).

Table 4a. Reduction of MCMV DNA in salivary gland, liver and kidney of MCMV-infected NOD-SCID mice after treatment with either BAY 38-4766 or ganciclovir

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>ganciclovir</th>
<th>BAY 38-4766</th>
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<tbody>
<tr>
<td></td>
<td>salivary gland</td>
<td>liver</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*The compounds (in 0.5% tylose) were administered po twice daily for 8 consecutive days at dosages of 0 (placebo), 3, 10, 30 and 100 mg/kg bw in a total volume of 10 mL/kg bw (n = 6 animals/group). The placebo group was set as 100% MCMV DNA.

Table 4b. Weight reduction and MCMV DNA content in target organs after treatment of MCMV-infected mice with ganciclovir and BAY 38-4766

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ganciclovir*</th>
<th>BAY 38-4766</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight reduction (%)</td>
<td>100 ± 12.5</td>
<td>33 ± 12.5</td>
<td>31.2 ± 10.4</td>
</tr>
<tr>
<td>MCMV DNA (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td>100</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>spleen</td>
<td>100</td>
<td>1.0</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Starting 20 h after infection the compounds were administered po at a dose of 25 mg/kg bd for 8 days with n = 10 animals per group. Animals were killed 16 h after the last application and organs were removed for further analysis. The placebo group was set as 100% MCMV DNA or 100% total weight reduction. Uninfected mice treated similarly with the test compounds did not produce weight loss. In acute toxicity studies the LD50 in mice and rats was >2000 mg/kg bw after oral administration of BAY 38-4766 (data not shown).
Novel non-nucleoside inhibitors of cytomegaloviruses

The only new anti-herpesviral approach introduced recently for the treatment of CMV retinitis has been based on anti-sense oligonucleotides, which unfortunately have been limited to intravitreal administration. In clinical practice, HCMV diseases are managed by the following drugs. Several formulations of ganciclovir [GCV; 9-(1,3-dihydroxy-2-propoxymethyl)guanine] are used for the treatment of HCMV diseases in AIDS patients and for the prophylaxis and treatment of HCMV diseases developing after bone marrow and solid organ transplantation. Additionally, foscarnet (PFA; trisodiumphosphonoformate) and cidofovir [S-HPMPC, GS 504; S-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine] are used for the iv treatment of HCMV retinitis in AIDS patients.

With the exception of PFA, all of these drugs are nucleoside analogues and most of them act by the inhibition of HCMV-specific DNA polymerase (reviewed by Field). BDCRB seems to be an inhibitor of an HCMV-specific maturation event (HCMV UL89 terminase) and benzimidavir may have an effect on Epstein–Barr virus (EBV) DNA processivity factor (HCMV UL97 protein kinase). However, for various reasons, development of all these new drugs was discontinued.

BAY 38-4766 is a representative of our newly discovered non-nucleoside class of inhibitors of the human β-herpesvirus, HCMV (Figure 1). A large panel of laboratory HCMV strains and clinical isolates were shown to be several times more sensitive to BAY 38-4766 than to ganciclovir. Ganciclovir-resistant as well as ganciclovir/foscarnet and ganciclovir/cidofovir double-resistant clinical isolates were as susceptible to BAY 38-4766 as wild-type strains. These latter results indicate that BAY 38-4766 may act by a mode of action distinct from all these different DNA polymerase inhibitors. Calculated selectivity of anti-CMV activity in HELF or NHDF cells (reflecting three and four cell or virus generations) was nearly 300 in case of HCMV and nearly 1600 for MCMV in 3T3 cells (Figures 1 and 2). In contrast to the lack of activity against the human α-herpesviruses (HSV type 1, type 2 and varicella-zoster virus), against a human γ-herpesvirus, EBV, as well as the EBV-related mouse γ-herpesvirus (MHV-68) and even against the human β-herpesvirus 6 (HHV-6), BAY 38-4766 was found to be inhibitory to various monkey CMV strains but most particularly to rodent CMV strains.

Sequence analyses of the genomes of two drug-resistant CMVs revealed several amino acid exchanges in UL89, encoding part of the putative viral terminase and UL104, a minor structural component of virions and capsids. Both proteins are essentially involved in the process of viral DNA concatemer cleavage and packaging of genomes into procapsids, as has been shown for respective homologues of the HSV or bacteriophage T4 maturation and packaging complex. These data together with our DNA cleavage analysis indicate that both UL89 and UL104, alone or by interaction, most likely represent the molecular antiviral drug target. Therefore, maturation and spread of viral particles to uninfected host cells are inhibited by a new unique mechanism of action. Although it was proposed that inhibition of HCMV DNA maturation by the benzimidazole ribonucleoside BDCRB is mediated through the UL89 gene product and resistance to TCRB maps to the two ORFs UL89 and UL56, we did not find cross-resistance of our HCMV AD169 sulphonamide-resistant strain to BDCRB. Unfortunately, because BDCRB is not active against MCMV, we could not confirm this result using our MCMV Smith BAY 38-4766-resistant strain. The fact that only two identified mutations of the highly resistant MCMV Smith map to UL89 exon II and UL104 indicates that in the case of HCMV, development of resistance is more complex compared with the murine virus.
virus, which was also reflected by a markedly prolonged generation time for drug-resistant HCMV. Thus, it can be expected that the requirement to accumulate multiple mutations to generate a resistant phenotype may translate into a relatively slow development of clinical HCMV resistance. However, future mutational analyses have to elucidate which amino acid exchanges confer resistance. In addition, the mechanism, which is distinct from those of the marketed drugs, will offer the possibility of treating patients who have acquired resistance to these agents.

Apart from offering a new highly specific approach to the inhibition of herpesviruses, this new mechanism of action could potentially also have beneficial immunological consequences. During treatment with BAY 38-4766, viral protein synthesis continues, but due to the lack of monomeric genomic length DNA, only empty particles (dense bodies) can be formed. It is conceivable that these non-infectious viral particles could aid the establishment of an antiviral immune response, leading to better control of the virus by the host. This mechanism appears possible in all cases where an immuno-incompetent host (re)gains immune competence (newborns, transplant recipients). However, proof of this theoretical benefit will have to await clinical studies.

In summary, by studying the mechanism of action of this novel drug class, which in vitro and in vivo selectively inhibits cytomegaloviruses, we have discovered an antiviral approach that may be of consequence for other members of the herpesvirus group as well, potentially allowing for the generation of broad-spectrum drugs. In addition, since a similar DNA maturation process does not occur in higher cells, this principle offers the potential for high selectivity, in contrast to many of the viral DNA polymerase inhibitors, which also interact with cellular enzymes and hence can have severe side effects. Furthermore, favourable pharmacokinetic data of BAY 38-4766 and its main active metabolite in mice, rats and dogs as well as excellent safety, tolerability and pharmacokinetic data after single oral doses in healthy male subjects with expected therapeutic tolerability and pharmacokinetic data after single oral doses in healthy male subjects with expected therapeutic

Acknowledgements

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References

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