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Inhibition of murine cytomegalovirus and human cytomegalovirus by a novel non-nucleosidic compound in vivo

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Abstract

Novel non-nucleosidic compounds have recently been identified as potent inhibitors of the human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV) in vitro. We have now investigated the antiviral activity of these compounds in MCMV-infected NOD/LtSz-*scid*/*j* mice that lack functional T, B and, in contrast to C.B-17/Icr *scid*/*scid* mice, natural killer cells, and represent a novel model for cytomegalovirus infection in immunocompromised hosts. BAY 38-4766 (3-hydroxy-2,2-dimethyl-*N*-[4({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)-phenyl]propanamide) was identified as the most potent representative of this class of antiviral compounds. Per os administration of BAY 38-4766 at dosages ≥ 10 mg/kg body weight led to antiviral effects that were comparable to ganciclovir 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (*Cymevene*®) as measured by survival and levels of viral DNA in organs of infected mice. In order to assess the anti-HCMV activity of BAY 38-4766 in vivo, we used a model, in which HCMV-infected human cells were entrapped in hollow fibers and subsequently transplanted into immunodeficient mice. Using this model, we demonstrated antiviral activity of BAY 38-4766 similar to that of ganciclovir. We conclude that BAY 38-4766 shows potential as an anti-HCMV drug. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Antiviral activity; Animal models; Human cytomegalovirus; Murine cytomegalovirus

1. Introduction

Human cytomegalovirus (HCMV) is an ubiquitous human virus. Most infections occur during

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childhood without clinically apparent disease. However, in immunocompromised individuals, including patients with organ transplants (Rubin, 1990; de Jong et al., 1998; Falagas et al., 1998), late-stage cancer patients and AIDS patients (Tyms et al., 1989; Jabs, 1995), HCMV may induce life-threatening diseases. Depending on the time of infection, HCMV may also cause severe clinical manifestations in the fetus and neonate. Clinical conditions arising from congenital infection include jaundice, respiratory distress and convulsive seizures that may result in mental retardation, deafness, neurologic disability or death (Stagno et al., 1982; Arvin and Alford, 1990).

Four drugs, 9-(1,3-dihydroxy-2-propoxymethyl)-guanine (ganciclovir, *Cymevene*®), trisodium phosphonoformate (foscarnet, *Foscavir*®), (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (cidofovir, *Vistide*®) and an antisense oligonucleotide (fomvirsen, *Vitravene*®) are currently available for the treatment of HCMV infections. Although of clinical benefit, these compounds may lead to the emergence of drug resistant viruses and/or have significant side effects such as neutropenia or kidney failure (Knox et al., 1991; Sarasini et al., 1995; Harada et al., 1997; Jabs et al., 1998). In addition, fomvirsen is limited to intravitreal administration. Therefore, our major goal is to identify and develop new drugs that have better tolerability and that solve the clinical problems resulting from drug resistance against marketed drugs.

BAY 38-4766 (3-hydroxy-2,2-dimethyl-*N*-[4({[5-(dimethylamino)-1-naphthyl]sulfonyl}amino)-phenyl]propanamide) is a member of a new class of non-nucleosidic anti-HCMV agents, which inhibits processing of viral DNA-concatemers (Hallenberger et al., 1999). In this study, we assessed the antiviral activity of this novel class of compounds in vivo. Evaluations of antiviral compounds have been performed with mice (Kern et al., 1984; Neyts et al., 1992; Smee et al., 1992, 1994, 1995; Yang et al., 1996), rats (Neyts et al., 1993) or guinea pigs (Lucia et al., 1984) with cytomegaloviruses natural to those species. Among these models, the MCMV-infected mouse represents the best characterized system. Recent National Institute of Health recommendations (Laughlin et al., 1991) indicate the need to evaluate treatments in immunocompromised animals in order to correlate the results to appropriate treatments in humans. We used a model of MCMV infection in NOD/LtSz-*scid*/*j* (NOD-SCID) mice that lack functional T and B lymphocytes and, unlike C.B-17/Icr *scid*/*scid* (SCID) mice (Shultz et al., 1995), also exhibit a functionally less mature macrophage population (Prochazka et al., 1992; Christianson et al., 1993). In addition, these mice have a markedly reduced activity of their natural killer (NK) cells, a population of immune cells that has been reported to be important in cytomegalovirus clearance (Welsh et al., 1991; Polic et al., 1996).

In order to investigate the anti-HCMV activity of BAY 38-4766 in vivo, we used a model in which HCMV-infected human cells were entrapped in hollow fibers and subsequently transplanted into immunodeficient mice (Hollingshead et al., 1995a,b).

2. Material and methods

2.1. Virus and cell culture

The laboratory strain HCMV-Davis (American Type Culture Collection, Manassas, VA; ATCC No. VR 807) was propagated in human embryonic lung fibroblasts (HELF) cells passages 10–40. The HELF cell line derived from normal lung tissue of a male embryo that was isolated 1980 according to the state regulations. HELF cells were cultivated in Eagle's minimal essential medium with Earl's salts (EMEM) without glutamine supplemented with 1% (v/v) L-glutamine (200 mM), 1% (v/v) penicillin/streptomycin (10 000 U penicillin and 10 000 μg/ml streptomycin in physiological saline; GIBCO) and 10% (v/v) fetal calf serum (FCS; GIBCO), hereafter referred as EMEM/10.

MCMV strain Smith (ATCC No. VR-194) was propagated in NIH 3T3 cells (ATCC No. CRL 1658). NIH 3T3 cells were propagated as already described.

2.2. Virus preparation and plaque assay

Confluent HELF cell cultures in 150 ml plastic cell culture bottles (ca. $(0.5–1) \times 10^7$ cells) were infected with cell-free virus or HCMV-infected HELF cells with a multiplicity of infection (MOI) $\approx 0.001–0.005$ for 60–90 min. After removal of the viral inoculum, cells were incubated for several days at 37°C/5% CO_2 until the monolayer became infected indicated by a typical rounding of the fibroblasts. Two days after maximal cytopathic effect (CPE), virus stock was prepared. Culture supernatant containing cell-free virus was collected and spun at $800 \times g$ for 10 min at 4°C. Infected HELF cells containing cell-associated virus were collected by trypsinization followed by centrifugation at $800 \times g$ for 10 min at 4°C and resuspended in freezing medium (EMEM/20% FCS and 10% dimethylsulfoxide) (Sigma, Deisenhofen, Germany). After titration, 0.5 ml aliquots of virus stocks were frozen (1°C/min) in 1 ml plastic ampoules and stored at −80°C until usage.

Confluent monolayers of NIH 3T3 cells were infected with MCMV Smith as already described. Reaching a nearly complete CPE, the infected cell cultures were harvested, followed by repeatedly freezing and thawing (three times) and subsequent sonification for 30 s. After centrifugation at $800 \times g$ for 10 min at 4°C, 0.5 ml aliquots of cell-free virus were stored in ampoules at −80°C until usage. Titers were estimated in a plaque assay. HELF cells ($(1–2) \times 10^5$/well) or MEF and NIH3T3 cells (1×10^5/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/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 change, 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 were expressed as percentages of untreated virus controls and plotted against the logarithm of drug concentrations. Drug concentrations producing 50% reduction of plaque formation (EC_{50}) were determined graphically from the dose–response curves. Assays were performed 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} to 10^{-6}) of stock virus and processed accordingly.

2.3. MCMV infection in immunodeficient mice

2.3.1. Animals

Male, 5- to 6-week-old homozygous NOD/LtSz-*scid*/*j* mice were purchased from a commercial supplier (The Jackson Laboratory, Bar Harbor, ME). In one experiment, female 6- to 8-week-old C.B-17/Icr *scid*/*scid* mice were used (Bomholtgard Breeding and Research Centre, Ry, Denmark). The mice were held under barrier conditions in a minimal disease facility with sterile bedding, food, water, and cages with filter tops.

2.3.2. Virus preparation

MCMV was passaged in BALB/c mice and purified by ultracentrifugation. Salivary glands of mice were harvested 2–3 weeks post infection and homogenized in EMEM/10 medium using an ultra turrax. After freezing (−80°C) and thawing the material, the debris were removed by centrifugation at $2000 \times g$ for 15 min at 4°C. Virus was enriched by ultracentrifugation through a 15% sucrose cushion using a SW28 rotor $100\,000 \times g$ for 90 min. The titer was estimated as described.

2.4. Infection and treatment of mice

Mice were infected intraperitoneally using 5×10^5 PFU in a total volume of 0.2 ml. Twenty hours after infection, the compounds were administered to the animals twice daily for eight consecutive days per os using a gavage. In order to assess the in vivo antiviral activity of the compounds, they were formulated as a suspension in 0.5% Tylose (Hoechst, Frankfurt Germany) and administered twice daily at a dosage of 25 mg/kg body weight (b.w.) per application in a total application volume of 10 ml/kg b.w. The 0.5% Tylose served as a negative placebo control, the

drug ganciclovir (*Cymeven*® i.v.; Syntex/Roche) as a positive treatment control. Animals were sacrificed 16 h after the last application and organs were removed for further analyses. The protocols for other pharmacodynamic studies are described in results.

2.5. Isolation of nucleic acids and Dot–Blot analysis

The nucleic acids were extracted using the QIAamp Tissue Kit (Qiagen, Hilden Germany) as instructed by the manufacturer. DNA concentrations were estimated by the formula $OD_{260} \times 50 = mg/ml$ by photometrical measurement (Power wave 200, Bio-Tek instruments). Dot–Blot hybridisation was carried out with a DIG-labelled, randomly primed 1300 bp polymerase chain reaction-amplified fragment from the HindIII J fragment of MCMV strain Smith. The DNA probe was prepared using the DIG-labelling and detection kit (Boehringer Mannheim, Germany) as instructed by the manufacturer. Blotting was carried out using a standard dot-blot apparatus (Dunn, Germany). Ten micrograms of DNA was spotted onto a nylon membrane (Boehringer Mannheim, Germany) soaked four times for 3 min each time with Soak I (0.5 N 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; sodium dodecyl sulfide (SDS), 0.02%; Blocking reagent, 2%; 20 ml/100 cm^2) without a probe for 2 h at 42°C and hybridized overnight in the presence of a probe (25 ng/ml). The filter was subsequently washed twice for 5 min with washing solution I (3 × SET; 1% SDS) buffer at room temperature (RT) and twice with washing solution II (1 × SET; 1% SDS) at 65°C. The immunological detection was carried out using the CDP-Star™ system (Boehringer Mannheim, Germany) as instructed by the manufacturer. Intensity of chemiluminescence signals were quantified using the LumiImager™ system (Boehringer Mannheim, Germany).

2.6. Statistics

The LumiImager signals were analyzed by descriptive statistics and compared by Variance analysis with post-hoc comparison of means (LSD test) (Statistica; StatSoft, Tulsa, Oklahoma). Survival analysis was carried out using the log-rank analysis procedure.

2.7. Hollow fiber model

2.7.1. Preparation for hollow fibers filled with infected cells

Biocompatible polypropylene tubes (Enka Membranes, Akzo Nobel, Wuppertal, Germany Accurel® ppS6/20) were cut into pieces of 10 cm, flushed with 70% ethanol and incubated in 70% ethanol at RT for a minimum of 96 h. Following three washes with deionized water, the fibers were placed into deionized water for sterilization. All subsequent steps were carried out under sterile conditions. The fibers were cut into pieces of ≈ 2.5 cm, heat-sealed at one end by clamping hot tweezers across the ends, and transferred into medium containing 20% FCS and stored at 4°C for a minimum of 12 h. HELF cells were infected with cell-free HCMV-Davis at MOI = 0.1. After 2 h, cells were collected by trypsinization followed by centrifugation at RT for 5 min at $800 \times g$. Cells were resuspended in EMEM/10 and counted. The cell suspension was drawn into a 1 cm^3 syringe and the conditioned fibers were filled with 45 μl cell suspension (0.5×10^6 cells) via a 20-gauge needle using a micromanipulator at RT and closed by heat sealing. Subsequently, the fibers were transferred to culture dishes containing EMEM/10 followed by transplantation into mice.

2.7.2. Transplantation procedure and drug administration

NOD/LtSz-*scid*/*j* mice, 20–30 g body weight, were anesthesized with 0.015–0.017 ml/g body weight Avertin 2.5% (Avertin 100% consists of 10 g tribromoethyl alcohol (Aldrich) in 10 ml tertiary amyl alcohol (Merck, Darmstadt Germany)). After shaving and cleaning the belly aseptically, the abdomens were opened and the fibers inserted

intra-abdominally. The abdomens were closed with two suture layers (5/0 Softcat, Braun Melsungen, Melsungen, Germany; and 5/0 Miralene, Braun Melsungen). Only asymptomatic animals were included in the study. Starting 1 day after transplantation, the mice were treated with the compounds at indicated dosages twice daily for four consecutive days per os. In preliminary experiments, viral peak titers were observed on day 5 under these conditions. Therefore, we concluded that this therapeutic scheme was feasible.

2.7.3. Harvesting of fibers and plaque assay

Mice were sacrificed, the abdomens were opened and the fibers were removed aseptically, followed by transfer into culture dishes containing EMEM/10. The fibers were opened at both ends and transferred into sterile Eppendorf tubes containing complete medium. The cell suspension was sonicated in a water bath for 15 s and flushed one time. Plaque assays were performed on 24-well plates (Costar, Cambridge, MA) on HS 68 (ATCC No. CRL 1635) monolayers in duplicates. The virus preparation was diluted in a series of tenfold dilutions starting with a 1:10 dilution of the first 1 ml cell suspension. The plates were incubated at 37°C, 5% CO_2 for 8 days with changing the overlay medium at day 4. At day 8, the plates were processed as already described.

3. Results

3.1. Antiviral activity of BAY 38-4766 in MCMV-infected NOD-SCID mice

In preliminary experiments, the inhibitory concentration 50 (IC_{50}) of ganciclovir against HCMV was estimated as 0.74 μM, against HELF (CC_{50}) with 788 μM. The IC_{50} against MCMV was estimated to be 23.4 μM and against NIH3T3 cells 250 μM. The IC_{50} of BAY 38-4766 against HCMV in vitro was estimated as 0.34 μM and the IC_{50} (CC_{50}) against HELF with 85 μM., the IC_{50} against MCMV in vitro was estimated as 0.039 μM and the IC_{50} (CC_{50}) against NIH 3T3 with 62.5 μM.

In a first set of experiments in MCMV-infected immunodeficient mice, we assessed the antiviral activity of a number of different compounds with excellent anti-HCMV activity in vitro at one dosage treatment (twice daily at a dosage of 25 mg/kg body weight (b.w.) per administration in a total volume of 10 ml/kg b.w.) and compared with the positive treatment control ganciclovir and the negative treatment control placebo. The compounds were formulated as a Tylose suspension and administered as described in Section 2. Compound BAY 38-4766 (Fig. 1A) was identified as having excellent antiviral activity in vivo. The experiment was repeated using different dosages in order to compare BAY 38-4766 and ganciclovir in more detail. The results are depicted in Table 1. BAY 38-4766 reduced the amount of viral DNA in liver, kidney and salivary gland comparable with ganciclovir. The differences between both drugs were not statistically significant in all dosages (variance analysis with post-hoc comparison). We also demonstrated that treatment with either ganciclovir or BAY 38-4766 had a signifi-

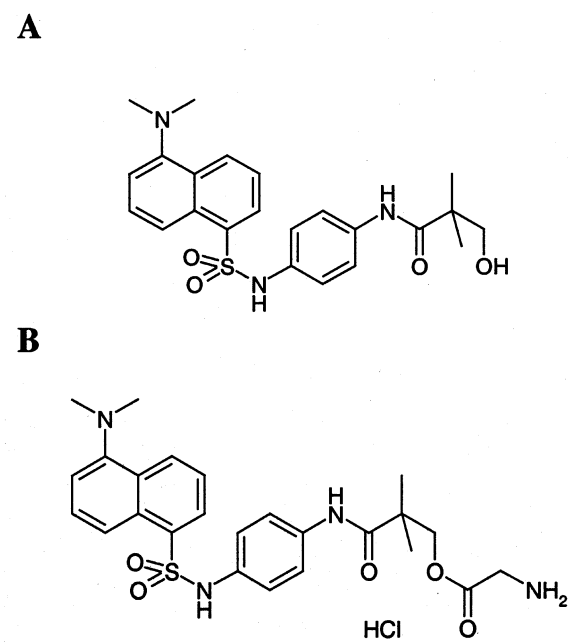

Fig. 1. Structural formulae of BAY 38-4766 (A) and BAY 40-1007 (B).

Table 1
Reduction of MCMV-DNA in salivary glands, livers and kidneys of MCMV-infected NOD-SCID mice after treatment with either BAY 38-4766 or ganciclovir[a]

Dose (mg/kg)	Ganciclovir MCMV-DNA (%)			BAY 38-4766 MCMV-DNA (%)		
	Salivary gland	Liver	Kidney	Salivary gland	Liver	Kidney
0	100 ± 12	100 ± 21	100 ± 9	100 ± 12	100 ± 21	100 ± 9
3	20 ± 12.1*	7 ± 2.8**	10 ± 12**	83 ± 56	21 ± 25*	30 ± 12*
10	16 ± 19.7*	6 ± 3.6**	10 ± 13**	40 ± 16*	12 ± 8.4**	13 ± 2.5**
30	2 ± 2.2**	3 ± 1.5**	10 ± 11**	23 ± 6*	10 ± 2.3**	9 ± 2.6**
100	2 ± 2.2**	3 ± 3.8**	8 ± 9.7**	8 ± 6.3**	11 ± 5.4**	2 ± 2.5**

[a] The compounds (in 0.5% Tylose) were administered per os using gavage twice daily for eight consecutive days at dosages of 0 (placebo), 3, 10, 30 and 100 mg/kg b.w. per application in a total application volume of 10 ml/kg per application ($n = 6$ animals per group). Treatment was initiated 20 h after infection. The placebo group was set as 100% MCMV-DNA. * $P < 0.05$, ** $P < 0.001$; variance analysis with post-hoc comparison. Means and standard deviations (S.D.) are indicated.

cant influence on the survival of MCMV-infected mice in a dose-dependent manner (Fig. 2). Long-term antiviral therapy of MCMV-infected NOD-SCID mice resulted in a significant prolongation of survival in both ganciclovir and BAY 38-4766-treated animals (Fig. 3). However, there were no significant differences between the BAY 38-4766-treated group and the ganciclovir-treated group. This result demonstrates that viral clearance cannot be achieved under these conditions. The compounds were very well tolerated by the animals in all dosages. No clinical signs of toxicity have been observed and uptake of food and water was similar to uninfected mice. In addition, pathohistological examination of a variety of tissues in uninfected drug-treated satellite animals did not show any differences to uninfected untreated animals.

3.2. Antiviral activity of BAY 38-4766 in the hollow fiber model

Antiviral in vivo activity of BAY 38-4766 could also be demonstrated in the hollow fiber mouse model (Fig. 4). The relative reduction of the number of plaque forming units compared with the placebo-treated group was recorded. BAY 38-4766 reduced the viral titer in a dose-dependent fashion in the SCID-tube mouse model and showed comparable efficacy to ganciclovir under these conditions.

3.3. Treatment of cytomegalovirus disease in mice with prodrug BAY 41-1007

In the previous experiments, therapy was initiated approximately 20 h after infection, a time point when viral replication is still ongoing. In this experiment, treatment was started 5 days after infection when the virus has already spread to different organs and viral replication is intensive, as demonstrated in preliminary experiments (data not shown). Any effects that might have been due to the moderate solubility of BAY 38-4766 were avoided by using the glycine ester prodrug BAY 41-1007 (Fig. 1B). BAY 40-1007 was then used to demonstrate curative potential of this class of compounds. The results are depicted in Fig. 5. The data clearly show that BAY 40-1007 reduced viral DNA levels at least as effectively as ganciclovir in severely infected animals. The differences between BAY 40-1007-treated and ganciclovir-treated animals were not significant (variance analysis with post-hoc comparison). When treatment was initiated, the mice showed signs of a clinically apparent disease but during the treatment period, a clinical cure was observed (not shown).

4. Discussion

A novel class of non-nucleosidic inhibitors of cytomegalovirus replication has been identified by

us in a cytopathogenicity screen on human embryonic lung fibroblasts (Reefschlaeger et al., 1999). These inhibitors target virus-specific proteins known to be involved in the cleavage and packaging of viral DNA (Hallenberger et al., 1999). Ganciclovir-resistant clinical isolates were sensitive to our non-nucleosidic inhibitors (not shown) indicating that this class of antiviral agents might be used to treat resistant mutants or might be combined with nuclosididic inhibitors to prevent or delay the emergence of drug resistance. With BAY 38-4766 (3-hydroxy-2,2-dimethyl-*N*-[4({[5-(dimethylamino)-1-naphthyl]sulfonyl} amino)-phenyl]propanamide) as a representative of this class, we were able to demonstrate a strong antiviral activity of this class in vivo in two different models of cytomegalovirus infection.

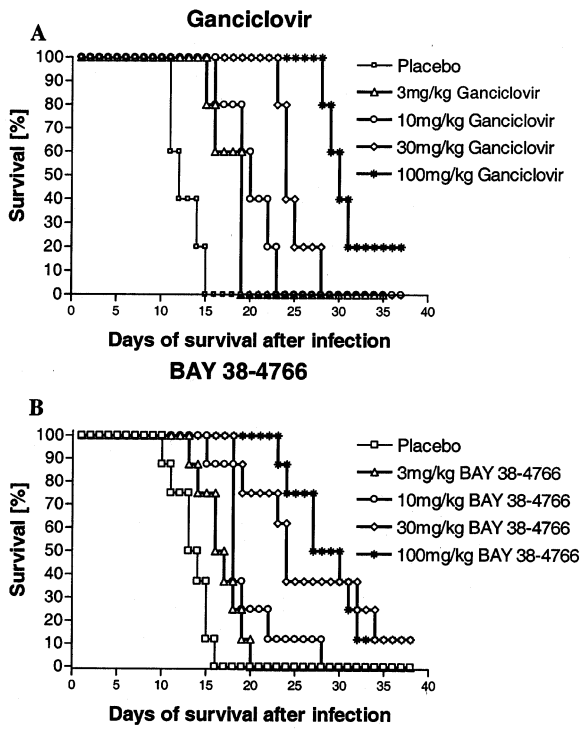

Fig. 2. Treatment with either ganciclovir or BAY 38-4766 had significant influence on survival of MCMV-infected mice in a dose-dependent manner. Male NOD-SCID mice (25g body weight b.w.) were treated per os twice daily for eight consecutive days with 3, 10, 30 or 100 mg/kg b.w. per application ganciclovir ($n = 5$ animals per group) or BAY 38-4766 ($n = 8$ animals per group). Treatment was initiated 20 h after infection. In the BAY 38-4766-treated groups, significance was observed in all dosages ($P = 0.02223$ in the 3 mg group and $P = 0.00375$ in the 100 mg group; log-rank analysis). In the ganciclovir-treated animals, significance was also observed in all dosage groups ($P = 0.01425$ in the 3 mg group and $P = 0.00217$ in the 100 mg group).

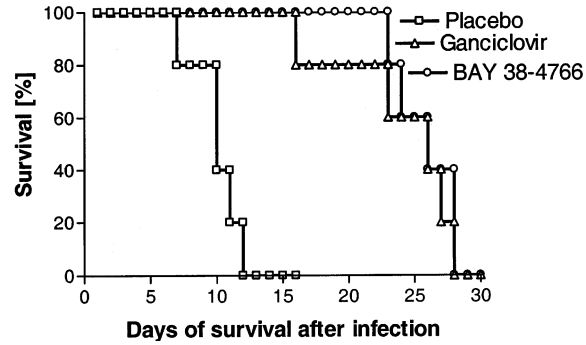

Fig. 3. The influence of a long-lasting BAY 38-4766 therapy on the survival of MCMV-infected NOD-SCID mice in comparison with ganciclovir or placebo-treated animals. Five animals per group were infected as already described and treated at a dosage of 25 mg/kg b.w. per application twice daily for 3 weeks. Treatment was initiated 20 h after infection. Antiviral therapy resulted in a significant prolongation of survival in both ganciclovir ($P = 0.00435$) and BAY 38-4766-treated animals ($P = 0.00361$).

In the first experiments, we investigated the antiviral activity of BAY 38-4766 in MCMV-infected immunodeficient mice. We measured viral DNA as a surrogate marker since this clinically proven method represents an efficient, sensitive and fast approach for estimation of viral load in a significant number of samples. Moreover, in preliminary studies, we found an excellent correlation between amount of viral DNA, viral titers and survival in immunodeficient mice. The therapeutic schedule of an 8-day treatment was chosen since we could observe highest viral titers and significant mortality of infected untreated animals after day 9. We could clearly demonstrate equal antiviral efficacy of BAY 38-4766 in vivo in comparison with ganciclovir as measured by viral DNA. The slightly lower reduction of viral DNA in BAY 38-4766 compared with ganciclovir may be due to the different mode of action. In contrast to BAY 38-4766, ganciclovir triphosphate is a competitive inhibitor with respect to dGTP for DNA polymerase (Biron et al., 1985; Freitas et al., 1985)

and inhibits de novo synthesis of viral DNA. However, treatment was initiated approximately 20 h after infection in our experiments and, therefore, the results should not only reflect effects on viral replication but also on spread of infection.

In the next experiment, we investigated the influence of antiviral treatment on survival of infected mice. SCID mice cannot be cured of the infection but the mean day of death can be prolonged (Neyts et al., 1992; Smee et al., 1992). Indeed, treatment with either BAY 38-4766 or ganciclovir resulted in significantly prolonged survival of infected animals in comparison with the control groups. In all the experiments already described, therapy was initiated 20 h after infection, a time point when a round of viral replication has not yet finished. Now, treatment was started 5 days after infection when the virus has already spread to different organs and viral replication is intensive as demonstrated in pilot studies (data not shown). We used C.B-17/Icr *scid*/*scid* (SCID) mice in these experiments, since these mice show, despite to their lack of functional T and B lymphocytes, very robust NK activity. Virus infection allows NK cells to proliferate, which, in turn, is important for clearance of MCMV infection (Welsh et al., 1991; Polic et al., 1996). In preliminary infection studies, we could demonstrate a prolonged survival and a slightly milder disease of these mice in comparison with NOD-SCID mice (not shown) and, therefore, we expected to better monitor differences between ganciclovir and BAY 38-4766. Using this model of MCMV disease, BAY 40-1007, the glycine ester prodrug of BAY 38-4766 reduced viral DNA levels at least as efficiently as ganciclovir. The prodrug was chosen for its improved solubility. Taken together, BAY 38-4766 shows a significant anti -MCMV activity under the conditions described herein. However, MCMV may differ from HCMV in drug susceptibility and compounds that inhibit HCMV but not small animal cytomegaloviruses and vice versa have been identified (Kern, 1991).

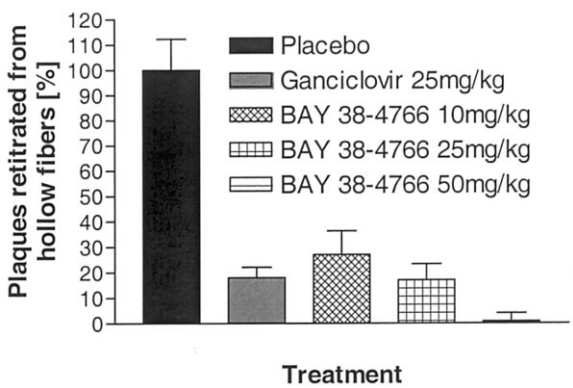

Fig. 4. Antiviral activity of BAY 38-4766 in the hollow fiber mouse model. The animals were treated with dosages of 10, 25 and 50 mg/kg b.w. per application BAY 38-4766 in a 0.5% Tylose suspension ($n = 8$ animals per group). Means and standard deviations (S.D.) are indicated. Treatment was initiated 6 h after transplantation and maintained for 4 days (b.i.d., per os). After extraction of the fiber tubes, the viral titer was determined in a plaque reduction assay. Plaque counts from hollow fibers received from placebo-treated animals were significant above all treatments and dosages ($P < 0.0001$; variance analysis with post-hoc comparison). In addition, the number of plaques from the BAY 38-4766 50 mg group-hollow fibers was significant below that of the 10 mg group ($P < 0.02$; variance analysis with post-hoc comparison).

Due to the strict species specificity of HCMV and the absence of an animal model, it has been difficult to evaluate antiviral compounds directed exclusively against HCMV in a natural virus–host cell system. Recently, a hollow fiber system for entrapment of tumor cells and subsequent transplantation of these hollow fibers into mice has been described (Hollingshead et al., 1995a,b; Weber 2000) in order to assess anti-cancer activity of drugs. Using this model, we found that the anti-HCMV activity of BAY 38-4766 was comparable with that of ganciclovir. BAY 38-4766 is about ten times more efficacious against MCMV than against HCMV in vitro. In addition, the compound is about 600-fold more active against MCMV as ganciclovir in vitro. In contrast, both compounds have displayed a similar in vivo efficacy in mice. This discrepancy was not monitored in our in vivo experiments for several reasons. First, we used two complete different systems. In the MCMV model, we have monitored viral spread, in contrast, in the hollow fiber model we monitored viral replication. Second, different readouts were chosen. In the hollow fiber model, we have estimated viral titers in tissue cultures. Because a substantial number of cells were in-

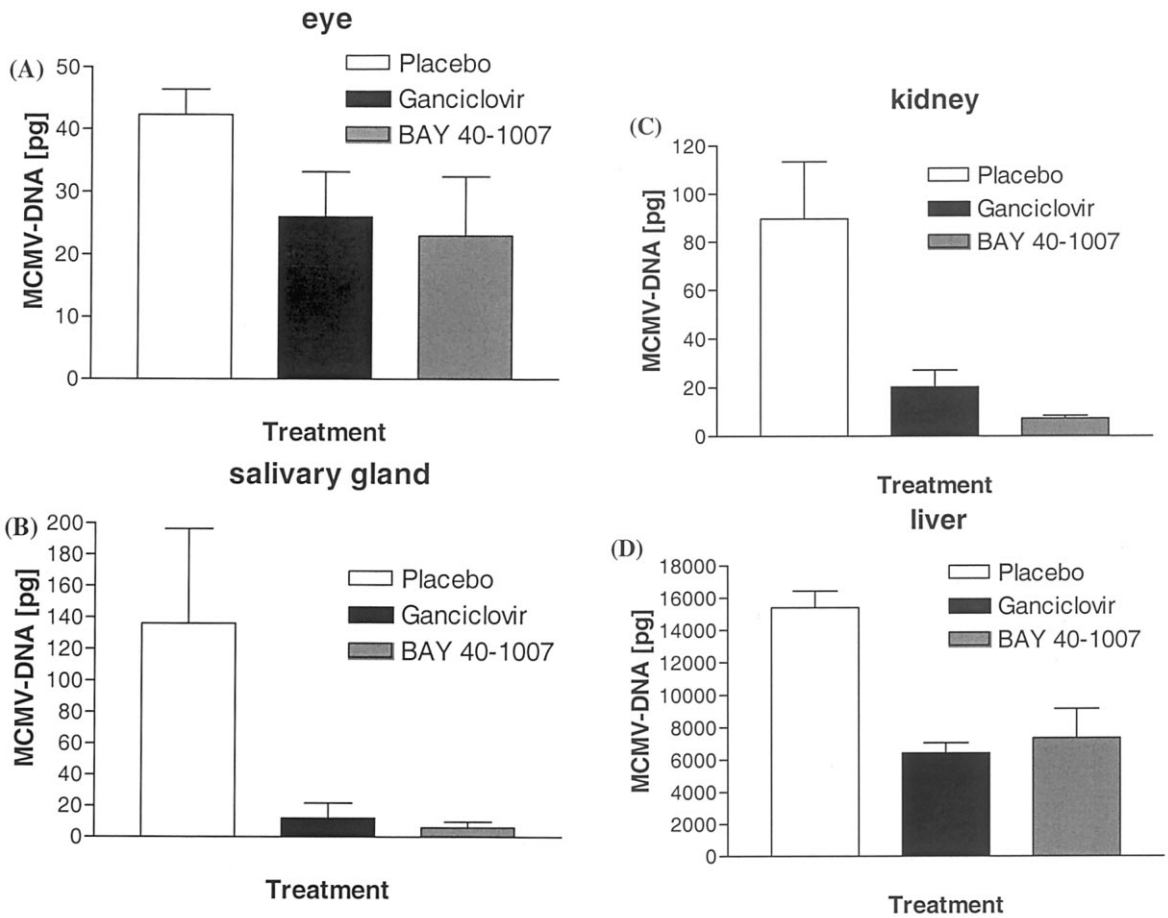

Fig. 5. Female Fox Chase SCID mice ($n = 7$ per group), 20 g b.w., were infected with 5×10^5 PFU in vivo passaged MCMV strain Smith i.p., as described. Treatment of MCMV-infected SCID-mice was initiated 5 days post infection. After this period of time, viral infection of the animals eyes could be detected in pilot studies (not shown). Treatment was maintained for 4 days at a dosage of 25 mg/kg per application b.i.d. per os. The animals were sacrificed and viral DNA levels were determined in eyes (A), salivary gland (B), kidneys (C) and livers (D). In both ganciclovir and BAY 40-1007-treated animals, reduction of viral DNA was significant in all organs in comparison with the placebo (0.5% Tylose)-treated animals ($P < 0.05$; variance analysis with post-hoc comparison). Means and standard deviations (S.D.) of picograms of MCMV DNA per tissue are indicated.

fected before treatment was initiated, we wanted to monitor both viral replication and production of novel infectious particles. In the MCMV model, viral DNA was measured. Using this parameter, the mode of action that is inhibition of processing of viral DNA (Hallenberger et al., 1999) but not inhibition of de novo synthesis of viral DNA as in the case of ganciclovir could have effected the results. Pharmacokinetics also contribute to the discrepancy. Ganciclovir triphosphate has an extremely long intracellular half-life, which may contribute to its efficacy in vivo (Smee et al., 1985). On the other hand, due to the pronounced protein binding of BAY 38-4766 (the free fraction of compound (f_u) in the cell culture medium was estimated to be 29%, f_u in SCID mouse plasma was 0.8%) total plasma concentrations of 0.62 mg/l are needed to meet the IC_{50} in vitro (0.039 μM, 0.017 mg/l). In fact, plasma concentrations of the compound obtained after a single peroral dose of 25 mg/kg as suspension in Tylose to female SCID mice exceeded this level for not more than 4 h after administration (C_{max}, 11.7 mg/l; C_{4h}, 1.3 mg/l). An active metabolite M1

of BAY 38-4766 contributes to the in vivo efficacy as well. Further details with regard to animal pharmacokinetics of BAY 38-4766 will be described elsewhere (Henninger, in preparation).

In summary, with the experiments described, BAY 38-4766 and its prodrug, BAY 40-1007, were identified as potent representatives of a novel class of non-nucleosidic antiviral compounds. Further assessment of these novel non-nucleosidic inhibitors of cytomegalovirus replication will clarify whether these compounds will overcome the problems associated with the currently available anti HCMV-drugs.

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