Superior efficacy of helicase-primase inhibitor BAY 57-1293 for herpes infection and latency in the guinea pig model of human genital herpes disease

Judith Baumeister¹, Ruediger Fischer, Peter Eckenberg, Kerstin Henninger, Helga Ruebsamen-Waigmann* and Gerald Kleymann²

AiCuris GmbH & Co KG, Wuppertal, Germany
¹Present address: Tibotec, Mechelen, Belgium
²Present address: University of Tübingen, Interfakultäres Institut für Biochemie IFIB, Tübingen, Germany

*Corresponding author: Tel: +49 202 31763 1111; Fax: +49 202 31763 1177; E-mail: helga.ruebsamen-waigmann@Aicuris.com

The efficacy of BAY 57-1293, a novel non-nucleosidic inhibitor of herpes simplex virus 1 and 2 (HSV-1 and HSV-2), bovine herpesvirus and pseudorabies virus, was studied in the guinea pig model of genital herpes in comparison with the licensed drug valaciclovir (Valtrex™). Early therapy with BAY 57-1293 almost completely suppressed the symptoms of acute HSV-2 infection, and reduced virus shedding and viral load in the sacral dorsal root ganglia by up to three orders of magnitude, resulting in decreased latency and a greatly diminished frequency of subsequent recurrent episodes. In contrast, valaciclovir showed only moderate effects in this set of experiments. When treatment was initiated late during the course of disease after symptoms were apparent, that is, a setting closer to most clinical situations, the efficacy of therapy with BAY 57-1293 was even more pronounced. Compared with valaciclovir, BAY 57-1293 halved the time necessary for complete healing. Moreover, the onset of action was fast, so that only very few animals developed new lesions after treatment commenced. Finally, in a study addressing the treatment of recurrent disease in animals whose primary infection had remained untreated BAY 57-1293 was efficient in suppressing the episodes. In summary, superior potency and efficacy of BAY 57-1293 over standard treatment with valaciclovir was demonstrated in relevant animal models of human genital herpes disease in terms of abrogating an HSV infection, reducing latency and the frequency of subsequent recurrences. Furthermore, BAY 57-1293 shortens the time to healing even if initiation of therapy is delayed.

Keywords: antiviral agent, BAY 57-1293, guinea pig, helicase-primase, HSV

Introduction

Herpes simplex viruses type 1 and 2 (HSV-1 and HSV-2) are members of the α-subfamily of human herpesviruses. HSV-1 and HSV-2 are large enveloped DNA viruses with double-stranded linear genomes of approximately 150 kbp, which encode more than 80 open reading frames (ORFs) that share extensive homologies. Upon infection of mucous membranes or abraded skin, these neurotropic viruses replicate in permissive cells and travel retrogradely to sensory nerve dorsal root ganglia (DRG), where latency is established. After reactivation, virus becomes evident at the innervated dermatome as skin vesicles or ulcers. HSV-1 and HSV-2 differ in their predilection for the site of infection and recurrent disease. Most cases of genital herpes are caused by HSV-2, but the frequency of this disease due to HSV-1, which usually infects oropharyngeal sites, is increasing (Kleymann, 2006; Whitley, 2006; Yeung-Yue et al., 2003).

Genital herpes constitutes one of the most prevalent sexually transmitted diseases worldwide. It has reached epidemic levels in the US, with an estimated 25% of the population aged over 20 years infected with HSV-2 (Fleming et al., 1997). Whilst the disease initially was believed to be generally asymptomatic in immunocompetent patients, more recent data indicate that genital herpes is mostly symptomatic and that virus shedding occurs during periods with no signs of the disease as well as during symptomatic episodes (Diamond et al., 1998; Wald et al., 1995). Disease symptoms range from mild discomfort to serious pain with a negative impact on quality of life and, in particular, personal relationships (Patel et al., 2001). Genital HSV-2 infection is the major cause of neonatal herpes, a life-threatening disease with an incidence ranging from 1 in 2,500 to 1 in 20,000 live births. Without treatment,
mortality may be as high as 70%. Treated survivors may suffer from serious sequelae, such as retardation, blindness and skin scarring (Scott et al., 1997). In addition, HSV-2 infection predisposes HIV acquisition (Waldr & Link, 2002) and, therefore, plays an important role in AIDS epidemics. Therapy for herpesviruses has constantly been refined during the last 50 years (Kleymann, 2006). During the mid-1950s the first antiviral was licensed. Idoxuridine was used as a topical treatment for herpetic eye disease and was soon replaced by less toxic topical trifluorothymidine. In 1978, vidarabine, the first systemic antiviral drug, was approved for treatment of HSV encephalitis. Since its launch in 1981, the selective and safe antiviral agent acyclovir has become the standard therapy for HSV infections. During the mid-1990s, valaciclovir and famciclovir, produgs of acyclovir and penciclovir with more convenient dosing schedules, were marketed. Current Centers of Disease Control and Prevention guidelines call for episodic treatment of primary disease for 7–10 days and for recurrent episodes for 1–5 days with valaciclovir, acyclovir or famciclovir, to shorten the duration of the episode (Workowski & Berman, 2005; Kleymann, 2006). The Varecilla-Zoster virus drug brivudine is active against HSV-1, but is less potent against HSV-2. Foscarnet and cidofovir (off label) are used as second line therapeutics for acyclovir resistant viral infections.

At present, launched drugs are developed as novel formulations and, more importantly, for new indications. Recently, suppression therapy with valaciclovir in a placebo-controlled trial has prevented transmission of genital herpes in discordant couples (Corey et al., 2004). This study indicates that therapy influencing virus shedding might impact the epidemic.

The challenge to discover new anti-herpes compounds for improved therapy of herpes disease has been reviewed (Kleymann, 2005). BAY 57-1293 (N-[5-(aminosulfonil)-4-methyl-1,3-thiazol-2-yl]-N-methyl-2-[4-(2-pyridinyl)phenyl]acetamide) is a new non-nucleosidic inhibitor of the HSV helicase-primase complex (Kleymann et al., 2002). Based on a novel mode of action, helicase-primase inhibitors are active against HSV-strains resistant to current medications. Activity was also shown against the helicase-primases of bovine herpesvirus and pseudorabies virus (Kleymann et al., 2002; Kleymann, 2006).

BAY 57-1293 showed superior antiviral activity in vitro and in vivo compared with all marketed compounds (Betz et al., 2002; Kleymann et al., 2002). It is at least two orders of magnitude more potent in blocking viral replication in cell culture and at least 10-fold more potent in relevant animal models, such as the mouse and rat lethal challenge, and mouse zosteriform spread models of herpes disease.

HSV-2 infection in guinea pigs is a well-established and predictive model for genital herpes in humans (Lukas et al., 1974; Whitley, 2006). It closely resembles human disease due to the following aspects: it uses the natural route of infection, the disease is self-limiting, and recurrent episodes occur spontaneously. The model has been widely used to study the pathogenesis of the disease and the influence of treatment on disease outcome.

The potency of BAY 57-1293 in diverse animal models has briefly been described (Kleymann et al., 2002). This report presents the antiviral activity of BAY 57-1293 in the guinea pig model of human genital herpes in detail and correlates the efficacy of antiviral treatment with the reduction in ganglionic viral DNA levels. Therefore, the aim of this study is to link potency and in particular efficacy of anti-herpes therapy with the seeding of ganglia in guinea pigs, and the resultant recurrence rates as well as the severity of disease. The additional information of viral DNA half-life in HSV-infected guinea pigs supports this finding based on latency.

Materials and methods

Virus and cell culture

The laboratory strain HSV-2 MS (ATCC No. VR-540) was propagated in Vero cells (African green monkey-kidney cells, ATCC No. CCL-81). Cells were cultivated in Dulbecco’s modified Eagle medium (DMEM, Gibco No. 41966-052; Invitrogen, Karlsruhe, Germany) supplemented with 100 U/ml penicillin G-Na salt, 100 μg/ml streptomycin-sulphate in physiological saline (Gibco No. 15140-114) and 10% fetal calf serum (FCS, Biochrome No. 80115 [Biochrome KG, Berlin, Germany] heat inactivated at 56°C for 30 mins).

Virus preparation and plaque assay

Eighty percent confluent Vero cell monolayer in T75 cell culture flasks were infected with a multiplicity of infection (M.O.I.) of 0.001-0.01. Briefly, medium was removed and the cell monolayer was washed twice with phosphate buffer saline (PBS). The appropriate volume of virus stock was added to a final volume of 2 ml DMEM containing penicillin/streptomycin. After 60 min of incubation at 37°C and in 5% CO₂, the inoculum was discarded and 12 ml DMEM containing penicillin, streptomycin and 10% FCS was added. After incubation for 3–5 days at 37°C and 5% CO₂, 80% of the cells showed cytopathic effects and viral stocks were prepared by three cycles of freezing and thawing. Culture supernatant containing cell-free virus was collected after centrifugation at 800×g. Virus stock was stored at -80°C. Titres were determined using the following plaque assay (Bernstein et al., 1986; Landry et al., 1982): 0.1 ml of serial dilutions of the virus stock was plated onto confluent Vero cell monolayers in 24-well tissue culture plates. Medium was removed from the cells, the virus inoculum was added and after 1 h of adsorption at 37°C in 5% CO₂, cells were overlaid with DMEM containing 1%
methylcellulose (Fluka Methocel® MC No. 64630; Fluka, Buchs SG, Switzerland), penicillin/streptomycin and 10% FCS. After 3–4 days, when plaques were clearly visible, the cells were fixed in 5% formaldehyde in distilled water for 30 min at room temperature and stained with a 1% crystal violet solution w/v in 50% ethanol (Fluka No. 61135). Visible plaques were counted and the virus titre was determined as plaque forming units (PFU)/ml.

**Guinea pig model of genital herpes – experimental animals and husbandry**

All animal experiments were registered with authorities in accordance to German law. Female Dunkin Hartley guinea pigs Crl:HA (Charles River Wiga, Sulzfeld, Germany), weighing 200–250 g, were purchased. After arrival, the animals were acclimatized to conditions in the animal laboratory for at least 5 days before the start of treatment or infection. Animals were housed in pairs or groups of three according to European guidelines of animal housing 86/609/EWG. Guinea pigs were kept in high temperature polysulphone cages type IV in separately ventilated units (Tecniplast, Hohenpeibenberg, Germany). Diet consisted of fixed-formula standard diet for guinea pigs (NAFAG Nr. 845, Eberle Nafag AG, Rickenbach-Will, Switzerland), irradiated hay and tap water. Food and water were available for consumption ad libitum.

After infection, animals were assigned to dose groups using random lists and earmarked for identification using a staining solution (Carbol-Fuchsin Fluka No. 21820).

**Infection of animals**

Guinea pigs were infected intravaginally using a modified procedure described previously (Bernstein *et al*., 1986; Kern *et al*., 1981). Guinea pigs weighed 250–300 g at the time of infection. Animals were anesthetized prior to the procedure using a mixture of 40 mg/kg ketamine (Ketavet®, Pfizer, Karlsruhe, Germany) and 5 mg/kg xylazine (Xylazine 2%, aniMedica, Senden-Bössensell, Germany). After disruption of the vaginal closure membrane with a sterile cotton-tipped swab, the mucous membrane was swabbed dry using a fresh cotton-tipped swab. HSV-2 strain MS (2.5×10⁵ PFU in a volume of 500 μl) was instilled into the vaginal vault using a tuberculin syringe with a 22-gauge plastic catheter attached (Introcan-W, B. Braun Melsungen, Melsungen, Germany).

**Test substance formulation and treatment of animals**

Valaciclovir (Valtrex™, GlaxoSmithKline, Middlesex, UK) was extracted from commercially available Valtrex film tablets. BAY 57-1293 was used as a micronized substance. Prior to use, compounds were ground with 2% dimethyl sulfoxide (DMSO, Fluka No.41639) and suspended in 0.5% methylcellullose/PBS (Clariant No.700586, MH 300 P2, Tylose; Clariant GmbH, Sulzbach, Germany). Methylcellulose/PBS (0.5%) was used for placebo controls. All suspensions were made fresh and were sonicated for 5 min prior to use. For purified compounds, the concentrations were verified by high pressure liquid chromatography analysis. Purity of valaciclovir (≥99%) was determined by LC/MS and 1H-NMR.

Compounds were administered orally by means of oral gavage twice or three times daily, as indicated, with an application volume of 0.2 ml for BAY 57-1293 and placebo, and 0.5 ml for valaciclovir. Each animal was treated with 5 mg/kg gentamycin (Ratiopharm, Ulm, Germany) from day 1 to day 14 post-infection (p.i.) to prevent secondary bacterial infections. Treatment with test compounds started as indicated. Length of treatment varied according to the treatment schedule used.

**Assessment of clinical disease**

Animals were examined daily for external genital skin lesions, which were graded on a modified 0–4 scale with 0.5 increments as previously described (Kern *et al*., 1978; Myerson & Hsiung, 1983; Stanberry *et al*., 1982). The lesion scores were defined as follows: lesion score 0 = no vesicle or healed vesicles (crusts fallen off); lesion score 1 = ≤ 2 vesicles or crusted vesicles; lesion score 2 = 2–10 vesicles or crusted vesicles; lesion score 3 = ≥ 11 vesicles or crusted vesicles; and lesion score 4 = confluent vesicles, ulceration or large crusts over many vesicles. Additional symptoms such as redness and swelling of the vagina, loss of bladder control or hind limp paralysis (usually temporary) were noted daily, but did not contribute to the disease score. Body weight of each individual was measured daily and noted.

**Virus isolation**

Cervicovaginal viral cultures were obtained daily from day 1 to day 4 p.i. using a sterile dry cotton-tipped swab (modified from Stanberry *et al*., 1982). Swabs were placed in tubes containing 1 ml of DMEM (Gibco No. 41965) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco No. 15070-063), and 2.5 μg/ml amphotericin B (FungizoneR, Gibco No. 15290-018). Swabs were removed after 30 min incubation at 4°C with vigorous shaking. Samples were freeze-thawed, cell debris removed by centrifugation at 800g and cleared supernatants were stored at -80°C. Virus titres of swab samples were determined using the plaque assay described above.

**Preparation of dorsal root ganglia**

Animals were necropsied after a T61 (Hoechst, Frankfurt, Germany) injection under deep anesthesia with pentobarbital-sodium (Narcoren, Rhone-Merieux [Merial], Hallbergmoos, Germany). The sacral bones were collected and processed immediately. The encased six
sacral dorsal root ganglia (sDRG) of each animal were extracted using a stereomicroscope.

**Isolation of DNA from tissue**

sDRG of individual animals were pooled. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen No. 51304; Qiagen, Hilden, Germany) according to the manufacturer’s instructions (Qiagen No. 13323).

**Preparation of HSV DNA from virus particles**

Vero cells were infected with HSV-2 strain MS. Cells were harvested 3–4 days p.i. After three cycles of freeze-thawing, the supernatant was harvested and cell debris was removed by low-speed centrifugation. Virus particles were collected by ultracentrifugation for 1 h at 45,000 × g at 4°C. Pellets were resuspended using a Potter-Elvehjem homogenizer and purified by centrifugation through a 30% sucrose cushion (150,000 × g, 1 h, 4°C). DNA was extracted using the Qiagen Blood and Cell Culture DNA Kit according to the manufacturer’s instructions (Qiagen No. 13323).

**Real time quantitative PCR using a TaqMan assay**

DNA was quantified by real-time 5′-exonuclease PCR (TaqMan) assay with primer-probe sets selected with Primer Express software (PE Applied Biosystems, Darmstadt, Germany). The primers gp-tq-1 (gtgttctggaagccaactgct) and gp-tq-2 (caccagtcaaagagtcccacc), and the probe (6-carboxyrhodamine) recognize HSV-2 glycoprotein H sequences. Primers were used at 300 nM and probes at 200 nM in a 50 μl reaction volume. Reactions were performed using the TaqMan® PCR Core Reagent kit (PE Applied Biosystems No. 402930) with standard cycling conditions (45 cycles consisting of 15 sec at 95°C and 1.5 min cycle at 60°C) as single reporter assays with an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). Standard DNA was used as follows: DNA purified from virus particles was diluted in 10-fold increments to contain 100–1000 copies of genomic HSV DNA/μl. DNA isolated from non-infected guinea pig tissue was diluted in 10-fold increments to contain 100,000–100 pg genomic DNA/μl. Results were analysed with the standard curve method and given as copies of HSV DNA/ng of guinea pig DNA.

**Read out and statistics**

Mean lesion scores represent the average of the total of the above described disease scores over the specified time. Clinical recurrences were reported as the total number of days with any lesion (lesion days), lesion score or number of recurrences (a separate recurrence was scored when a new lesion appeared). Recurrence rates were calculated as the number of recurrences over 10 days. Cumulative disease scores (mean cum scores) are the mean scores added over time. Time to healing is the sum of days from the day of infection until complete re-epithelization of lesions. The mean increase in disease score 24 h after onset of therapy was determined as the increase between day 4 (before treatment) and day 5 (24 h after treatment start) for each animal, guinea pigs with no increase in disease score or a decrease in disease score were counted as 0. Swab titres are virus titres determined from swab cultures given as PFU/ml. Standard deviations (±) are given in brackets. Data were evaluated for statistical significance using one-way analysis of variance (ANOVA) with Bonferroni’s post-test. Data were log-transformed where indicated. Student’s t-test was used in some instances for verification of results of the post-test. Tests were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA). Calculation of DNA half-life was carried out using the proprietary pharmacokinetic data evaluation program Kincalc version 2.50.02 (Bayer HealthCare, 1994, Wuppertal, Germany).

**Results**

After infection with HSV-2, genital herpes disease developed in untreated guinea pigs as described previously (Landry et al., 1992; Lukas et al., 1974; Stanberry et al., 1982; Stanberry et al., 1985; Stanberry, 1994). Vesicles first appeared on the external genitalia 3–4 days p.i. More vesicles developed up to day 5–8, when most animals showed ≥10 vesicles, confluent or even ulcerative lesions (lesion scores 2–4) accompanied by severe swelling and redness (Figure 1E). Crusts were forming after 8–10 days and healing continued until day 15–18, when loss of crust was seen and the external skin appeared unscarred. Most animals developed urinary retention between days 5–10; this symptom always resolved after a few days. Depending on the experiment, ≤70% of the infected animals showed hind limb paralysis. Animals were euthanized if neurological symptoms did not improve after 7 days. Usually, however, hind limb paralysis resolved after 7–10 days. In rare cases, sudden deaths occurred, probably due to herpesvirus-induced encephalitis. After the primary symptoms had resolved, recurrent vesicles appeared. Guinea pigs were examined daily up to 85 days p.i. for recurrent lesions, which lasted from 1 to 3 days and usually consisted of 1–2 vesicles (lesion score 1).

Potency and efficacy of drugs were studied using the following three different treatment regimens: early therapy, delayed therapy and suppression therapy.
Efficacy of BAY 57-1293 in guinea pig model

To determine the efficacy of therapy on disease symptoms of a primary HSV-2 infection, such as disease score, virus shedding and recurrence rate, treatment was initiated 6 h p.i. (once on the day of infection) and continued for 4 subsequent days (twice daily or three times daily as indicated). In dose-finding studies, BAY 57-1293 was orally applied at 10, 20 or 30 mg/kg twice daily. Dissolving BAY 57-1293 or valaciclovir in DMSO prior to adding methylcellulose suspension to prepare a standard formulation had no significant effect on potency or efficacy of oral applications (data not shown). Treatment with BAY 57-1293 reduced or blocked primary signs of disease in a dose-dependent manner (Figures 1A and B), whereas treatment

\[\text{Figure 1. Early therapy regimen of BAY 57-1293 and valaciclovir in HSV-2-infected guinea pigs}\]

(A & B) BAY 57-1293 aborts herpes simplex virus (HSV)-2 infections in guinea pigs in a dose-dependent manner, and shows superior potency and efficacy in suppressing symptoms of primary disease at up to 15 times lower doses compared with valaciclovir. Ten HSV-2-infected guinea pigs per group were treated with the doses indicated for 5 days (days 0–4), starting 6 h after infection. Figure 1A and B show curves from two independent experiments. (C–E) Disease symptoms of the animal experiment shown in Figure 1B were photographed at day 6. (C) BAY 57-1293 (disease score 0), (D) valaciclovir (disease score 2) and (E) placebo-treated animals (disease score 4). Redness, swelling and herpetic vesicles are observed only in placebo- and valaciclovir-treated animals. DMSO, dimethyl sulphoxide.
Dosing of valaciclovir was limited to 100 or 150 mg/kg per dose due to the maximum amount of substance that can be formulated in 500 μl, which is the maximum volume given orally. A daily dose of acyclovir of 50–100 mg/kg intraperitoneally or 250–400 mg/kg orally is considered effective (Bernstein et al., 1993; Kern, 1981; Landry et al., 1982; Pronovost et al., 1982; Rapp, 1984). The valaciclovir-treated groups showed only a moderate effect compared with the mean lesion scores of the placebo-treated group. One-way ANOVA of both studies revealed P-values <0.0001 using log-transformed data. The effect of BAY 57-1293 measured as the mean cumulative lesion score at day 12 p.i. was highly significant when compared with placebo (20 and 30 mg/kg P<0.001; 10 mg/kg P<0.01); the effect of BAY 57-1293 given at 20 or 30 mg/kg twice daily was also significantly superior compared with the valaciclovir-treated groups (P-values <0.001). The effect seen after treatment with valaciclovir was not statistically significant compared with placebo, although clearly recognizable.

The effect of an early therapy regimen on virus shedding was also studied in an experimental setting as described above. Without treatment, virus shedding was most pronounced on day 2 p.i. and decreased on day 3. Figure 2 shows the effect of treatment with BAY 57-1293 on virus shedding on day 1 through to day 3 p.i. in comparison with valaciclovir. BAY 57-1293 reduces virus shedding by three orders of magnitude on day 2 p.i. Statistical analysis was carried out with data from day 2 p.i. from two independent experiments. A significant reduction was shown for BAY 57-1293-treated groups in comparison with valaciclovir- and placebo-treated groups with P-values <0.001 using log-transformed data. The effect of valaciclovir treatment compared with placebo was not statistically significant.

After primary lesions had resolved completely, a representative number of 10 animals per treatment group were monitored for recurrent disease symptoms for ≤85 days. Recurrent vesicles were scored on a daily basis. Figure 3 shows the cumulative disease score from day 13 to day 75 p.i. of two independent experiments. BAY 57-1293-treated animals showed considerably and consistently less recurrence than valaciclovir- or placebo-treated animals. In one experiment, a slight effect on lesion severity was seen in the valaciclovir-treated group (150 mg/kg twice daily): the cumulative disease score was somewhat lower than in the placebo group. The recurrence rate for the valaciclovir group was the same in this experiment as in the placebo group until day 31 and clearly higher than for BAY 57-1293-treated animals (see Figure 3 for details). Note, the dose of BAY 57-1293 is lower in the second experiment (30 mg/kg versus 20 mg/kg twice daily). In agreement with a dose-dependent effect, the efficacy of BAY 57-1293 is lower in this experiment.
**Figure 3. Early therapy regimen – treatment of primary disease with BAY 57-1293 led to a reduction/suppression of recurrent episodes/symptoms in a dose-dependent manner**

A  

- Placebo
  - Valaciclovir 100 mg/kg three times daily
  - BAY 57-1293 30 mg/kg twice daily

B  

- Placebo
  - Valaciclovir 150 mg/kg three times daily
  - BAY 57-1293 20 mg/kg twice daily

Ten herpes simplex virus (HSV)-2 infected guinea pigs per group were treated for 4 consecutive days with the doses indicated, starting 6 h post-infection. After primary lesions had healed, animals were monitored daily for up to 85 days for recurrent vesicles. The mean cumulative disease score of the recurrent disease state was determined for each treatment group. Data from two independent experiments (A & B) are shown.

The mean recurrence rate after early onset of therapy was calculated. Results from two independent experiments were used for data evaluation. BAY 57-1293-treated animals experienced significantly fewer episodes than placebo- or valaciclovir-treated animals within a 10 day period (mean recurrence rate [10 days]: BAY 57-1293 20 mg/kg twice daily = 0.4 ± 0.3, 30 mg/kg twice daily = 0.3 ± 0.3; valaciclovir 100 mg/kg three times daily or 150 mg/kg twice daily = 0.9 ± 0.3; and placebo = 1.0 ± 0.3). The effect of BAY 57-1293 was slightly less at the lower dose and, therefore, seems to be dose-dependent. Statistical analysis using one-way ANOVA revealed a P-value of 0.0001. The effect of BAY 57-1293 in reducing the 10 day recurrence rate was significant compared with valaciclovir and with placebo in both dose groups (all P-values < 0.001). Treatment of the acute disease with valaciclovir had no effect in terms of the number of recurrent episodes. Effective inhibition of replication within the first days of infection due to treatment with BAY 57-1293, therefore, influenced the recurrence rate.

To explore the underlying mechanism of the reduction of recurrence rates, guinea pigs were treated as described above, sacrificed at day 7 p.i. and the six sDRG were explanted. DNA was isolated from pooled sDRG and HSV copy numbers were normalized to the mass of guinea pig DNA, as described in the Material and Methods section.

The results of the experiment are shown in Figure 4. Early therapy with BAY 57-1293 reduced the viral load, and thus seeding in sDRG, by nearly three orders of magnitude (4.2 × 10^3 ± 9.2 × 10^1 copies HSV-DNA/ng guinea pig DNA compared with 1.3 × 10^4 ± 1.8 × 10^1 copies HSV-DNA/ng guinea pig DNA in the placebo-treated group determined on day 7 p.i.), whereas following treatment with valaciclovir, viral load in the sDRG in this model decreased only 5-fold (2.9 × 10^3 ± 1.9 × 10^1 copies HSV-DNA/ng guinea pig DNA in the valaciclovir-treated group) at day 7. Statistical analysis revealed a P-value < 0.0001 (one-way ANOVA), with the effect of BAY 57-1293 being significant compared to placebo (P < 0.001) and to valaciclovir (P < 0.001). This result fits well with those reported above, for example, the decrease in the mean recurrence rate after BAY 57-1293 compared with placebo or valaciclovir treatment, or the marked decrease in the cumulative disease score in the recurrent disease stage (Figure 3) in BAY 57-1293-treated animals only.

Therefore, we conclude that due to early therapy fewer viral copies are deposited during acute disease in the sDRG and lead to less recurrences during the chronic phases of disease.

In order to study the amount of virus DNA in the sDRG over time, animals were sacrificed at day 25 and day 85 p.i. (end of the experiment), sDRG were collected and TaqMan assays performed. The results are summarized...
in Figure 4 (HSV genome copies/ng DNA on day 7 & 25: BAY 57-1293 30 mg/kg twice daily = 4.2 x 10^5 ± 9.2 x 10^4 & 1.6 x 10^4 ± 1.9 x 10^4; valaciclovir 150 mg/kg twice daily = 2.9 x 10^4 ± 1.9 x 10^4 & 1.3 x 10^5 ± 1.3 x 10^4; and placebo = 1.3 x 10^4 ± 1.8 x 10^4 [for placebo-treated animals, only data for day 7 and 85 are available]. HSV genome copies/ng DNA on day 85: BAY 57-1293 20 mg/kg twice daily = 2.6 x 10^3 ± 3.9 x 10^2; valaciclovir 100 mg/kg three times daily = 1.6 x 10^3 ± 2.3 x 10^2; and placebo = 3.1 x 10^3 ± 3 x 10^3). Interestingly, the numbers of viral copies decrease over time in this model (Figure 4). BAY 57-1293-treated animals harbour significantly fewer viral genome copies in their sDRG throughout the observation time compared with placebo and valaciclovir-treated animals. All curves show a similar slope. The half-life of virus DNA in sDRG was calculated to have a mean value of 250 ± 27 h (10 days) in all cases.

**Efficacy of delayed therapy regimens**

Generally, patients will see a doctor when a florid primary or secondary disease is present. To evaluate the efficacy of BAY 57-1293 in a clinically relevant setting, treatment was delayed to day 4 p.i., when vaginal lesions were apparent, and continued to day 14 p.i. The time course of disease score after delayed treatment start is depicted in Figure 5. In BAY 57-1293-treated animals (20 mg/kg twice daily), redness and swelling disappeared within 24 h of treatment. This effect was striking, but was not accounted for in the lesion score system (see Material and Methods). Only very few guinea pigs developed new lesions after the onset of therapy with BAY 57-1293. The increase between day 4 (before treatment) and day 5 (24 h after treatment start) was calculated for each animal. Guinea pigs with no increase in disease score or a decrease in
Suppression therapy of recurrent disease

In a clinical setting, patients frequently present recurrent lesions rather than primary herpes virus disease. Some patients may have more than 10 recurrent episodes a year and are treated continuously to alleviate symptoms and reduce the number of clinically apparent episodes — the so-called suppression treatment regimen. In order to study recurrent herpesviral disease and suppression therapy with BAY 57-1293, guinea pigs were infected as described. Primary disease was allowed to develop unchecked. Treatment (BAY 57-1293 30 mg/kg twice daily, valaciclovir 150 mg/kg twice daily) was started at day 20, after the healing of primary vesicles was complete, and continued for 20 days until day 40. The animals were monitored daily for recurrent vesicles and lesion scores were noted. The observation period ended 79 days after infection.

Curves depicting the development of the cumulative disease score are shown in Figure 6. Placebo-treated animals developed recurrent vesicles on a regular basis. Consequently, the resulting curve is diagonal. During treatment from day 20 to day 40, both treatment groups show nearly horizontal curves (Figure 6). This means that very few recurrent vesicles were developing. The values of the treatment groups at the end of the experiment (mean lesion scores overall [day 20–79]: placebo =12.7 ±6.7; valaciclovir 150 mg/kg twice daily =4.8 ±4.7; and BAY 57-1293 30 mg/kg twice daily =4.9 ±3.8) were statistically significantly different from the placebo group (ANOVA P=0.0032; with post-test P-values of <0.001 [Bonferroni’s], and t-test P-values of 0.006 for BAY 57-1293 and 0.008 for valaciclovir). There was statistically no significant difference between the treatment groups.

In order to assess the treatment effects in more detail, the mean cumulative disease scores of the treatment period (mean lesion scores day 20–40: placebo =4.4 ±3.3; valaciclovir 150 mg/kg twice daily =0.9 ±1.5; and BAY 57-1293 30 mg/kg twice daily =0.5 ±0.9) and the observation time after treatment was stopped (mean lesion scores day 40–79: placebo =8.3 ±3.8; valaciclovir 150 mg/kg twice daily =3.9 ±3.7; and BAY 57-1293 30 mg/kg twice daily =4.4 ±3.3) were calculated. At the end of the treatment period (day 40), the effect of BAY 57-1293 was significant compared with placebo (ANOVA P<0.0001; post-test [Bonferroni’s] P<0.05; t-test verification P=0.002), but not compared to valaciclovir. After treatment was discontinued,
recurrence rates in both treatment groups increased but, interestingly, were lower than those of the placebo-treated group. After treatment was discontinued (day 40–79), again, the effects of both treatment groups were significant compared with placebo (ANOVA P-value <0.0001; post-test P-values <0.05 [Bonferroni’s]; t-test P-values =0.002 for BAY 57-1293 and 0.008 for valaciclovir). There was no significant difference between the treatment groups.

**Discussion**

BAY 57-1293 has been shown to be a potent inhibitor of herpesvirus replication *in vitro* and *in vivo* (Betz et al., 2002; Kleymann et al., 2002; Kleymann, 2003a,b; Kleymann, 2004). Here, an in-depth evaluation of the efficacy of BAY 57-1293 was performed in the guinea pig model of genital herpes. In the past it proved difficult to link efficacy of antiviral therapy with a significant reduction of viral genomes in neuronal tissue of HSV-infected animals due to the lack of potency of most of the antiviral compounds tested. The present study clearly correlates the reduction in ganglionic viral DNA levels with the outcome of treatment regarding a reduction in frequency and severity of HSV disease. Abrogation of seeding of neuronal tissue during primary infection and the information on severity of HSV disease. Abrogation of seeding of neuronal tissue of HSV-infected animals due to the lack of potency of most of the antiviral genomes in neuronal tissue of HSV-infected guinea pigs. This result is in accordance with findings in a very different viral infection, disease in HSV-infected guinea pigs. This result is in accordance with findings in a very different viral infection, which also results in latency – the SIV-infected monkey. Studies (for example, Mori et al., 2000) have shown that early therapy of the primary infection was beneficial for long-term outcome by reducing the number of infected target cells and by allowing a more vigorous immunological response against the virus. Whether an immunological component is also adding to the beneficial effects of early treatment of HSV remains to be determined.

The guinea pig model is a clinically relevant animal model to evaluate compounds in development and for use in the clinic (Bernstein et al., 2000; Bernstein et al., 2001; Bourne et al., 1992; Bourne et al., 1999a,b; Bravo et al., 1993; Jennings et al., 1999; Millet et al., 1999). This model has been shown to be predictive for the efficacy of acyclovir in humans (Kern, 1984). Valaciclovir, the prodrug of acyclovir with improved bioavailability, was chosen as the treatment control in the studies described, because it represents the current therapeutic standard. Valaciclovir is the L-valin ester of acyclovir and has to be hydrolysed *in vivo* to release the drug acyclovir. Oral bioavailability of valaciclovir in mice, rats and monkeys is 59–84% compared with the 10–15% oral bioavailability of acyclovir (Szczech, 1996).

Compared with BAY 57-1293, valaciclovir is clearly less potent and efficacious. This is in accordance with data in the literature, where an acyclovir treatment is described to alleviate, but not abolish, disease symptoms (Bernstein et al., 1993; Kern, 1981; Landry et al., 1982; Pronovost et al., 1982; Rapp, 1984). The dose of valaciclovir chosen for this study was the maximum dose possible in the guinea pig model because of technical constraints. It is known that acyclovir shows species differences in terms of absorption, metabolism and elimination of the drug (de Miranda & Good, 1992). Tissue distribution of the drug seems not to be significantly different in the species examined. Guinea pigs are known to metabolize a significant proportion of the drug (acyclovir recovery in urine ranges from 48% to 73%), unlike mice, dogs and humans (acyclovir recovery in urine ranges from 71% to 99%). Thus, the efficacy of valaciclovir may be underestimated in the guinea pig model (de Miranda & Good, 1992). However, the superior efficacy of BAY 57-1293 in the guinea pig model shown here is in accordance with previous data from mice and rats (Betz et al., 2002).

In the first set of experiments, treatment was started soon after HSV-2 infection of the guinea pigs. This experimental design aimed to generate results comparable with data from the literature and to identify the optimal dose of BAY 57-1293 in this animal model. Treatment with BAY 57-1293 led to the complete suppression of symptoms of acute disease, whereas treatment with valaciclovir only alleviated the course of disease (Figure 1). This in accordance with data from literature, where treatment with acyclovir at doses up to 100 mg/kg four times daily or 125 mg/kg twice daily resulted in reduction, but not in complete suppression, of symptoms. Virus titres obtained from vaginal swabs were not altered following acyclovir treatment (Kern, 1981; Kern, 1984). In this study, there was no significant reduction of virus shedding after valaciclovir treatment, thereby confirming data in the literature. In contrast, treatment with BAY 57-1293 reduced shedding by three orders of magnitude (Figure 2). It has been shown recently that treatment with valaciclovir reduces transmission of genital herpes between discordant couples (Corey et al., 2004). Given the results mentioned above, BAY 57-1293 can be expected to be more effective in preventing virus transmission than valaciclovir.

We also found that early therapy with BAY 57-1293 greatly reduced the amount of herpesvirus DNA in the DRG at day 7 p.i. (Figure 4). It has been proposed for some time that the frequency of recurrences is determined in part by the number of neurons harbouring virus. In the guinea pig model, the quantity of latent virus DNA in the DRG correlates with the reactivation rates of HSV-1 and HSV-2 (Efstathiou et al., 1999; Lekstrom-Himes et al., 1998). Therefore, effective treatment of primary disease should reduce consecutive recurrent episodes. This has been shown to some extent for treatment of HSV-infected animals with acyclovir at high doses and with a treatment
start earlier than 12 h p.i. In the clinical setting, treatment with acyclovir is usually not reported to lead to a significant reduction of recurrence rate or time to recurrence (Bryson et al., 1983; Efstathiou et al., 1999; Roizman & Knipe, 2006). Only one study reported a reduction of recurrence rate after treatment of true primary episodes with oral acyclovir (Bryson et al., 1985). In the guinea pig model of genital herpes, treatment with 60 mg/kg acyclovir given intraperitoneally twice daily led to a significant reduction of the number of latently infected DRG (Efstathiou et al., 1999), a finding which we were able to reproduce for valaciclovir in this study. However, BAY 57-1293 led to a significantly greater reduction of viral load in the DRG compared with treatment with acyclovir. In guinea pig studies reported in the literature, treatment of the primary disease with acyclovir had no effect on the recurrence rate (Bernstein et al., 1986; Efstathiou et al., 1999) – a result we were also able to confirm. In contrast, treatment with BAY 57-1293 had a significant effect on the ensuing recurrence rate (Figure 3). It may be concluded that fewer viral copies deposited during acute disease in sDRG lead to less frequent recurrences during later disease stages and that seeding of sDRG can be reduced by efficacious therapy, such as treatment with BAY 57-1293. It would be interesting to determine whether treatment started as late as 4 days after infection would also affect the recurrence rate. Treatment reduces replication of the virus at both the peripheral and ganglionic sites, and the reduction of clinical symptoms, which is correlated with the viral load in the DRG in this model. Therefore, it is tempting to speculate that the successful treatment of primary disease, with treatment starting as late as 4 days p.i., may reduce viral load and, therefore, the recurrence rate. A reduction in the number of latently-infected neurons in ganglia cannot be expected with acyclovir when treatment is delayed for 4 or more days p.i. (Efstathiou et al., 1999).

Using samples from this study, we were able to trace herpesviral DNA in the DRG from treated or untreated animals over the entire study time (85 days). It could be shown that latent herpesvirus DNA is lost in guinea pigs over time with a half-life of approximately 10 days, irrespective of whether the animals were treated or not. In earlier studies, the reservoir of latent DNA was reported to be stable over 4 months, although this finding has since been challenged (Efstathiou et al., 1999). The loss of HSV DNA in the ganglia was suggested to be a consequence of the destruction of latently-infected neurons following reactivation. This finding would explain why the number and severity of recurrent episodes in intravaginally infected guinea pigs decline over time (Landry et al., 1992; Stanberry et al., 1985). In most patients, recurrence rates and severity also decrease after 1 year (Benedetti et al., 1999). The guinea pig model of genital herpes can be proposed as a model to elucidate the mechanism of DNA loss, given the results discussed above.

In a clinical setting, patients usually present with most of their symptoms already apparent. Therefore, we attempted to treat acute herpesvirus disease when vesicles were formed in all animals 96 h after virus inoculation. The therapeutic effect of acyclovir in the guinea pig model, when treatment was started late, has been described as modest. The difference in the mean lesion score between untreated animals and animals treated with 50 mg/kg acyclovir per day intraperitoneally was only significant after day 6, even when treatment was started 72 h p.i. (Pronovost et al., 1982). In other studies, a minor reduction of mean lesion scores was seen after acyclovir treatment was started 72 h p.i., with up to 100 mg/kg per day given intraperitoneally (Landry et al., 1982). Again, others reported no effect if acyclovir was given 72 or 96 h p.i. (Kern, 1981; Myerson & Hsiung, 1983).

In our studies, valaciclovir had only a minor effect. The dose-response curve observed in our study after treatment with valaciclovir resembled those described in the literature for acyclovir, when treatment is started 72 h p.i. (Landry et al., 1982; Pronovost et al., 1982). The superiority of BAY 57-1293 was most pronounced in this treatment schedule (Figure 5). BAY 57-1293 is the most potent anti-HSV compound in vivo and acts very quickly, as shown by the very minor increase in disease score and the very small numbers of animals developing new lesions after the onset of treatment. Most striking was the resolution of inflammatory symptoms within 24 h, although this was not accounted for in our disease scoring system. The time necessary to complete the healing of the exacerbation was cut in half with BAY57-1293 treatment, whereas valaciclovir only achieved a 15% reduction. It should be noted that the exceptional activity of BAY 57-1293 in delayed therapy was achieved with a dose of 20 mg/kg twice daily, which had been inferior in efficacy in experiments using an early treatment start. Clinically, oral acyclovir shortened the mean healing time by 25% in patients with primary and non-primary episodes. Valaciclovir has been shown to be as effective, but not superior (Whitley & Roizman, 2001). Again, this is matched nicely by our study results, where valaciclovir only has a minor effect.

Many patients with recurrent genital herpes need continuous treatment (suppression therapy) to alleviate symptoms and reduce the number of recurrent episodes. Recommended regimens include 400 mg acyclovir twice daily, 1 g valaciclovir once daily or 250 mg famiclovir (Famvir™) twice daily (Kleymann, 2006). With these treatments, a significant reduction of clinically apparent recurrent episodes, but not complete suppression of symptoms and shedding, may be achieved (Corey et al., 2004).
In the suppression therapy experiment described in this report, a significant reduction in the number of recurrent vesicles per group was achieved during the treatment interval with BAY 57-1293 and valaciclovir (Figure 6). Withdrawal of the compounds, however, led to increased recurrence rates in both treatment groups. This mirrors the clinical situation, where discontinuation of treatment leads to relapse and the reappearance of symptoms (Fife et al., 1994). It is thought that this is due to latent herpesvirus that serves as a reservoir for recurrent symptomatic and asymptomatic episodes. Interestingly though, the recurrence rate after the treatment interval was significantly lower in both treatment groups compared to the placebo group. As the primary disease was not treated in the described suppression experiment, the DRG of the guinea pigs were already seeded at the start of treatment, as has been shown in experiments analysing the DNA contents of the DRG at day 7 (Figure 4). Therefore, it may be suggested that either latent virus is affected by antiviral treatment, that is, the seeding of ganglia is not completed by day 20, or deposition of new virus DNA is blocked in the guinea pig model. The same phenomenon has been observed in patients that come off long term therapy with acyclovir, a majority of whom may experience lower recurrence rates than before treatment. This effect may be due to the natural decline of recurrence rates, but because a correlation with the clinical response to suppressive therapy was shown, it may also be linked to the efficacy of the treatment (Fife et al., 1994). Both valaciclovir and BAY 57-1293 were equally effective in this experiment. We believe that this is due to the low virus load during recurrent disease in guinea pigs. It has been shown that BAY 57-1293 is more effective than acyclovir when the viral load is increased in vitro (Kleymann et al., 2002). Apparently, the dose administered to the valaciclovir group was sufficient to suppress the minor symptoms of recurrent disease (Figure 6), but this was not the case in the treatment of acute disease, where a much higher viral load is suspected (Figure 1). It should be noted that recurrent disease in humans is much more severe than in the guinea pig model; therefore, the effectiveness of BAY 57-1293 in suppressing herpesviral disease in the clinic may be superior to standard therapies.

In conclusion, helicase–primase inhibitors are the most potent antiviral agents of HSV infection and disease in all animal models tested. A significant advantage of BAY 57-1293 was shown over the licensed drug valaciclovir in the guinea pig model of genital herpes. This is most probably due to the superior potency of the compound based on its binding mode to the viral target together with its favourable pharmacokinetic profile in experimental animals. In particular, the capacity of BAY 57-1293 to inhibit HSV replication at high viral loads (that is, at high M.O.I.) may be an advantage in vivo (Betz et al., 2002; Kleymann et al., 2002; Kleymann, unpublished data) especially regarding its superior ability to prevent the seeding of ganglia. Since valaciclovir has been proven to be efficacious in treating genital herpes in man and shows efficacy in the guinea pig model, it can be expected that BAY 57-1293 will show efficacy in a clinical setting, assuming favourable pharmacokinetic properties in humans.

Acknowledgements

We like to thank Marion Heidtmann, Dorothea Hoecker and Petra Koch for excellent technical assistance, and Prof. Harald Labischinski for discussions. We would also like to acknowledge the contributions of Wolfgang Bender, Dieter Haebich, Gabriele Handke-Ergüden, Martin Hendrix and Udo Schneider in preparing the compound used in this study. We are grateful to Prof. Tim Harrison and the reviewers for significantly improving the manuscript.

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