Antiviral Research 254 (2026) 106495

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Susceptibility of clinical isolates, laboratory-adapted and drug-resistant herpes simplex virus strains to helicase-primase inhibitor adibelivir (IM-250) and its synergy in combination therapy with acyclovir, penciclovir and foscarnet

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ARTICLE INFO

Keywords:
Adibelivir
IM-250
Antiviral
Plaque reduction assay
PRA
Susceptibility
Acyclovir
Resistance
Synergy
Combination therapy

ABSTRACT

Herpes simplex virus (HSV) infections are widespread and cause a significant global health burden. Clinical manifestations range from herpes labialis and genital herpes to life-threatening herpes simplex encephalitis or neonatal herpes. Current treatment relies on nucleoside analogs such as acyclovir, valacyclovir or famciclovir. The emergence of resistance to these drugs is of concern in transplant recipients and immunocompromised patients. Next-generation helicase-primase inhibitors are under clinical development to provide more efficacious treatments and address resistance to current therapies. Adibelivir (IM-250) is a potent HSV helicase-primase inhibitor (HPI) with nanomolar activity against both HSV1 and HSV2. In the present study, we assessed the susceptibility of more than 50 clinical isolates and laboratory strains against adibelivir. Across all strains tested, adibelivir inhibited HSV replication in cell culture with mean EC_{50} values of 21 nM (HSV1) and 35 nM (HSV2), compared to up to 42-fold higher EC_{50} values for acyclovir (883 nM for HSV1 and 1456 nM for HSV2). No preexisting variants with a lack of susceptibility were identified. Furthermore, no cross-resistance was observed between nucleoside analogs and HPIs and finally, the antiviral activity depended on serum concentration and infectious dose. Collectively, these data establish the baseline susceptibility profile of HSV clinical isolates to adibelivir prior to its broader clinical introduction. Adibelivir retains activity against nucleoside-resistant HSV strains and exhibits synergistic antiviral effects when combined with acyclovir, penciclovir and foscarnet *in vitro*. These results support clinical evaluation of adibelivir as a therapeutic option for HSV infections and as a candidate for combination therapy.

1. Introduction

Herpesviruses are highly prevalent human pathogens that establish lifelong infections and impose a substantial disease burden worldwide. The majority of the global population is infected with at least one member of the *orthoherpesviridae* family. Among these, the α-herpesviruses – herpes simplex virus 1 (HSV1 or HuAHV1), herpes simplex virus 2 (HSV2 or HuAHV2), and varicella-zoster virus (VZV or HuAHV3) – are neurotrophic. In the general population, the seroprevalence of HSV1 exceeds 60%, while HSV2 infection rates range from 15 to 30% (Howley et al., 2021). Clinical manifestations of HSV infections include herpes labialis (cold sores), genital herpes, sight-impairing keratitis, and, less commonly, severe or life-threatening conditions such as herpes simplex encephalitis (HSE) and disseminated viremia encompassing hepatitis and pneumonia. These severe presentations occur predominantly in immunocompromised individuals, e.g. transplant recipients, cancer patients, HIV infected individuals, patients with inherited immunodeficiencies and neonates. Following primary infection, HSV establishes

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https://doi.org/10.1016/j.antiviral.2026.106495
Received 9 June 2026; Received in revised form 24 July 2026; Accepted 29 July 2026
Available online 30 July 2026

lifelong latency in the neurons of sensory and autonomic ganglia – such as trigeminal or dorsal root sensory ganglia and superior cervical and major pelvic autonomic ganglia – with the viral genomes persisting episomally within neuronal nuclei. At least 30% of infected individuals experience periodic reactivations triggered by various physiological or environmental stressors (UV light), resulting in contagious viral shedding and recurring disease (Howley et al., 2021).

Idoxuridine and trifluridine were the first antiviral drugs used clinically (Kaufman, 1962; Kaufman and Heidelberger, 1964; De Clercq and Li, 2016). Introduced in the 1960s and 1980s for the topical treatment of herpes keratitis, these nucleosides demonstrated limited target specificity and posed safety concerns. Vidarabine subsequently received regulatory approval for systemic treatment of herpes simplex encephalitis (Whitley et al., 1977). A landmark advance in antiviral therapy was the introduction of acyclovir (ACV), which combined an excellent safety profile with selective antiviral activity, albeit of moderate efficacy (Elion et al., 1977; Schaeffer et al., 1978; reviewed in De Clercq and Li, 2016; Kleymann, 2005). Since its approval in 1981, systemic ACV has remained the standard of care for HSV and VZV infections. To address the pharmacokinetic limitations of ACV – in particular its poor oral bioavailability and requirement for three-to five-times daily dosing – the prodrugs valacyclovir (VACV), (Perry and Faulds, 1996) and famciclovir (FCV), (Jarvest et al., 1998), derived from ACV and its congener penciclovir (PCV), respectively – were developed during the 1990s, enabling once- or twice-daily dosing. Intravenous ACV is required in cases of herpes simplex encephalitis or neonatal herpes to ensure therapeutic drug exposure in the central nervous system (CNS). Although nucleoside analogs reduce the frequency of recurrences and viral shedding, their effects are incomplete and transient, and thus insufficient to fully suppress disease or prevent transmission (Corey et al., 2004; WHO, 2016; CDC, 2022). Depending on the clinical manifestation of HSV infections, antiviral resistance is uncommon in immunocompetent individuals (<1%, but can reach up to 8% in recurrent herpetic keratitis), it can emerge at clinically significant rates in immunocompromised patients (Bacon et al., 2003; Sallée and Boutolleau, 2024). Critically, no currently approved therapy has been shown to reduce the latent viral reservoir once it is established (Bryson et al., 1983; Wald et al., 1994; CDC, 2022).

In the absence of a licensed HSV vaccine, a novel class of antivirals targeting the viral helicase-primase (HP) entered clinical development in the early 21st century. This class includes amenamevir (Kawashima et al., 2022), pritelivir (Kleymann et al., 2002; Betz et al., 2002; Wald et al., 2014), adibelivir (IM-250) (Gege et al., 2021; Bernstein et al., 2023), HN0037 (Hou et al., 2022), ABI-1179/GS-1179 (Fletcher, 2026) and ABI-5366/GS-5366 (Fletcher, 2026) (Fig. 1).

HN0037, GS-5366, GS-1179 and adibelivir have completed Phase 1 clinical trials and have advanced into Phase 2 evaluation. Pritelivir is currently under New Drug Application (NDA) review for the treatment of acyclovir-resistant HSV infections in immunocompromised patients. To date, only amenamevir has received regulatory approval, initially in Japan for herpes zoster (VZV) in 2017 and subsequently for recurrent HSV infections in 2023. However, its limited distribution into nervous tissues has raised concerns regarding its therapeutic utility in herpes ganglionic or CNS disease (Tada et al., 2024). In contrast, adibelivir has been shown to reduce the reactivation competence of HSV1 and HSV2 in preclinical animal models (Gege et al., 2021; Bernstein et al., 2023).

Overall, effective treatment of neurotropic herpesviruses requires sufficient free and pharmacologically active drug concentrations at the neuronal sites of infection, particularly within sensory ganglia and the CNS. Achieving adequate target-site exposure has proven challenging for nucleoside analogs such as ACV, as well as for most helicase-primase inhibitors (HPIs), limiting their ability to attenuate chronically persistent, latent HSV infections (Whitley and Baines, 2018; Gege et al., 2025).

In the present study, we characterize the susceptibility of clinical and laboratory HSV strains – including acyclovir-resistant mutants – to adibelivir under varying serum protein concentrations and multiplicities of infection (MOI), and compare the results with those obtained for ACV as the current standard of care. Finally, we demonstrate that combination therapy of adibelivir with nucleoside analogs acyclovir and penciclovir as well as the phosphonate foscarnet, results in synergistic antiviral activity as observed also for pritelivir (Kleymann et al., 2001) and amenamevir (Chono et al., 2013).

2. Results

2.1. Susceptibility of clinical, laboratory-adapted and acyclovir-resistant herpes simplex virus strains to adibelivir

Susceptibility testing across a diverse panel of HSV strains demonstrated that adibelivir potently inhibits viral replication of clinical isolates, laboratory-adapted strains, and acyclovir-resistant variants. A set of 21 clinical isolates from the virus collection of the University of

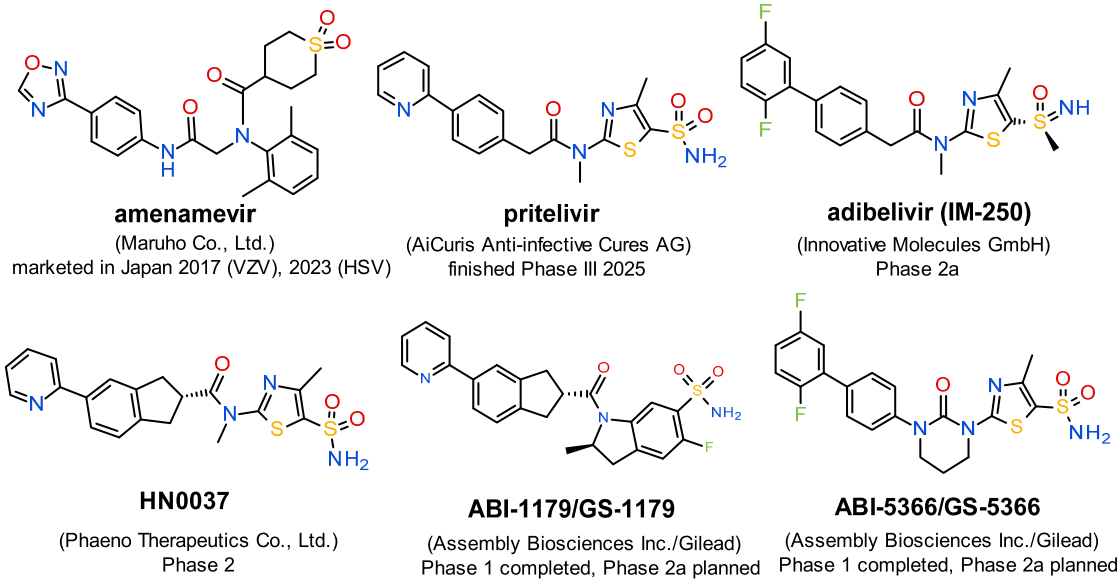


Fig. 1. Structures of helicase-primase inhibitors (HPIs) on the market and in clinical trials: amenamevir (ASP-2151), pritelivir (AIC-316, BAY 57-1293), adibelivir (IM-250), HN0037, ABI-1179/GS-1179 and ABI-5366/GS-5366.

Washington (Seattle, WA, USA) was evaluated using the plaque reduction assay (PRA) and found to be susceptible to adibelivir, with EC_{50} (half-maximal effective concentration) values ranging from 7 to 24 nM for HSV1 (Fig. 2A and B) and 23–98 nM for HSV2 (Fig. 2C and D). An independent panel of 20 clinical isolates from the virus collection of the University of Freiburg (Freiburg, Germany) was similarly susceptible to adibelivir with PRA-derived EC_{50} values of 11–104 nM for HSV1 (Fig. 3A and B) and 10–47 nM for HSV2 (Fig. 3C and D). A further selected subset of clinical isolates, together with laboratory-adapted and acyclovir-resistant strains evaluated at Innovative Molecules GmbH using the activity selectivity assay (AS-assay), yielded EC_{50} values of 1–20 nM for HSV1 and 8–28 nM for HSV2 (Fig. 4A). Across all strains tested, the mean EC_{50} (±SD) of adibelivir was 21 ± 23 nM for HSV1 and 35 ± 19 nM for HSV2 strains (Fig. 4B). In comparison, the corresponding mean EC_{50} values for acyclovir were 883 ± 312 nM for HSV1 and 1456 ± 332 nM for HSV2. These data indicate that adibelivir is approximately 42-fold more potent than acyclovir *in vitro*.

The selectivity index (SI = CC_{50}/EC_{50}) of adibelivir exceeded 2500 in all cases, reaching values of 10000–34000 for HSV1 and 2500–10800 for HSV2, indicating a favorable selectivity profile and broad tolerability in Vero cell culture, which is the standard cell line used in PRA and AS-assays.

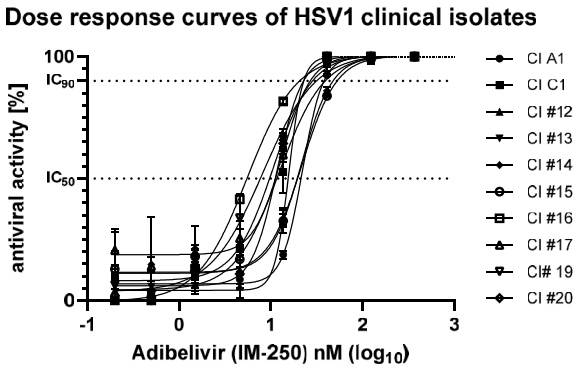
2.2. Acyclovir-resistant strains are susceptible to adibelivir and vice versa

Adibelivir-resistant HSV mutants were selected *in vitro* at low frequencies. For HSV1, resistant variants emerged in the presence of 1 μM adibelivir at a rate of 4.95×10^{-6} to 1.41×10^{-5}, whereas adibelivir-resistant-HSV2 viruses were observed at 10 μM with a frequency of 0.52×10^{-6} (Hagmaier, 2019; Gege et al., 2021). Sequence analysis identified mutations within the UL5 helicase gene in all resistant HSV1 isolates. Clones HSV1-IM-250-res #3 and #6 both had a single M355T mutation (UL5 nucleotide 1064 ATG→ACG) while clone HSV1-IM-250-res #8 harbored a M355I mutation (UL5 nucleotide 1065

A

HSV1 ID	EC_{50} (nM)	EC_{90} (nM)	SI
CI #19	7	35	33756
CI #17	8	30	30747
CI #20	9	22	26556
CI #13	9	31	28046
CI #16	11	27	23804
CI C1 R	11	42	22065
CI #12	13	47	18747
CI #14	22	51	11276
CI #15	22	57	11261
CI A1	24	39	10408

B



C

HSV2 ID	EC_{50} (nM)	EC_{90} (nM)	SI
CI #2	23	106	10837
CI F1	31	100	7972
CI #5	34	85	7404
CI #1	39	116	6453
CI E1	42	91	5979
CI H1 R	42	103	5926
CI #8	44	98	5737
CI #7	51	99	4868
CI #6	56	190	4464
CI G1 R	57	103	4405
CI #4	98	319	2542

D

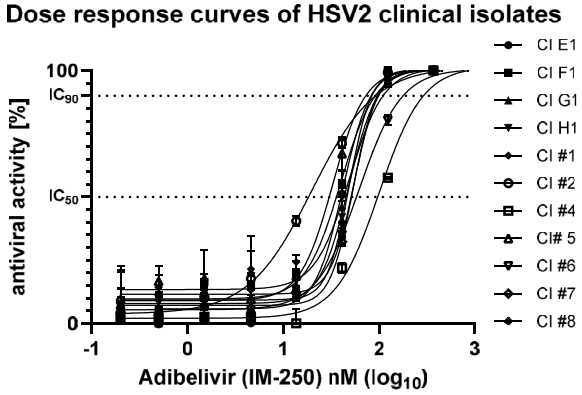
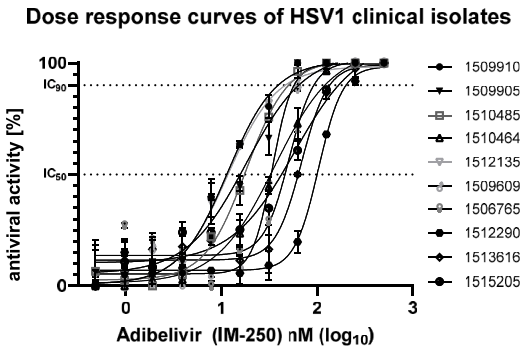


Fig. 2. Susceptibility (effective concentration EC_{50} and EC_{90}) of HSV1 and HSV2 clinical isolates (CIs, selected from the virus collection of the University of Washington, Seattle, USA) including acyclovir-resistant strains, to adibelivir determined with the plaque reduction assay (PRA). Acyclovir-resistant HSV strains are marked by an R, e.g. CI C1 R, CI G1 R and CI H1 R. The selectivity index (SI) was determined in addition or parallel to the antiviral activity.
A) EC_{50}, EC_{90} and SI values of adibelivir for HSV1 clinical isolates, determined by non-linear regression of log (inhibitor) vs normalized response (reduction of plaque counts).
B) Dose-response curves of adibelivir for HSV1 clinical isolates. Mean ± standard deviation (SD) of normalized plaque counts vs log (inhibitor).
C) EC_{50}, EC_{90} and SI values of adibelivir for HSV2 clinical isolates determined by non-linear regression of log (inhibitor) vs normalized response (reduction of plaque counts).
D) Dose-response curves of adibelivir for HSV2 clinical isolates. Mean ± standard deviation (SD) of normalized plaque counts vs log (inhibitor).
Number of test repetitions: n = 4 for #12, E1; n = 2 for #1, #5, #8, #16, #20; and n = 1 for all other strains.

A

HSV1 ID	EC_{50} (nM)	EC_{90} (nM)
1512135-S	11	40
1509910-S	12	42
1510485-R	18	55
1509905-R	19	109
1509609-S	34	58
1510464-S	36	170
1506765-S	51	99
1515205-S	58	307
1513616-S	69	147
1512290-R	104	196

B



C

HSV2 ID	EC_{50} (nM)	EC_{90} (nM)
1165008-S	10	44
1362444-S	16	71
1061619-S	24	71
1316658-S	27	66
1510715-S	34	127
1513749-S	37	85
1117044-R	37	162
1515929-S	40	102
1317049-S	41	121
1511009-R	47	199

D

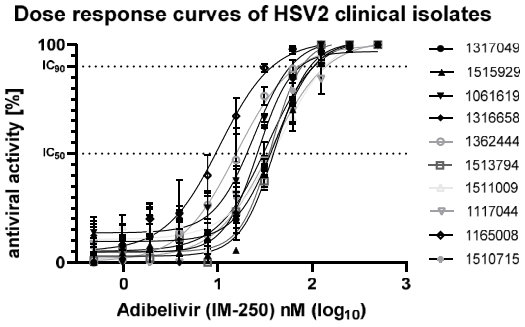


Fig. 3. Susceptibility (effective concentration EC_{50} and EC_{90}) of HSV1 and HSV2 clinical isolates (selected from the virus collection of the Institute of Virology, Freiburg, Germany), including acyclovir-resistant strains, to adibelivir as determined by plaque reduction assay (PRA). Acyclovir-resistant HSV strains are marked by an R, e.g. 1510485-R, 1509905-R, 1512290-R and 1511009-R, 1117044-R.
A) EC_{50} and EC_{90} values of adibelivir for HSV1 clinical isolates determined by non-linear regression of log (inhibitor) vs normalized response (reduction of plaque counts).
B) Dose-response curves of adibelivir for HSV1 clinical isolates. Mean ± standard deviation (SD) of normalized plaque counts vs log (inhibitor).
C) EC_{50} and EC_{90} values of adibelivir for HSV2 clinical isolates determined by non-linear regression of log (inhibitor) vs normalized response (reduction of plaque counts).
D) Dose-response curves of adibelivir for HSV2 clinical isolates, tested in duplicate. Mean ± standard deviation (SD) of normalized plaque counts vs log (inhibitor).

ATG→ATA). The corresponding dose-response curves for adibelivir and acyclovir against susceptible and resistant strains are shown in Fig. 5. Nucleoside prodrugs require activation by the viral thymidine kinase and ultimately after further cellular phosphorylation inhibit the HSV-DNA-polymerase, whereas the HPIs including adibelivir target the viral helicase-primase complex. While cross-resistance is commonly observed among drugs within the same class, no cross-resistance was detected between nucleoside analogs and HPIs. Accordingly, HPI-resistant HSV strains remained susceptible to nucleoside analogs, whereas nucleoside-resistant viruses are sensitive to HPIs. For example, adibelivir-resistant HSV1 strains #3 and #8, exhibiting resistance indices (RI = EC_{50} drug resistant/EC_{50} drug sensitive strain) of 125 and 660 for adibelivir, respectively, remained fully susceptible to acyclovir. In contrast, cross-resistance was observed with the structurally related HPIs amenamevir and pritelivir. For strains #3 and #8, the resistance indices were 3.2 and 3.1 for amenamevir and 8.4 and 11.7 for pritelivir, respectively. Similarly, the adibelivir-resistant HSV2 strain #2, carrying a K355N substitution (UL5 nucleotide position 1065, AAG→AAT), exhibited marked resistance to all three HPIs, with resistance indices of 11000 for adibelivir, 18000 for amenamevir, and 6000 for pritelivir. Patterns of cross-resistance among HPIs can be predicted from the genotypes of resistant viruses and are increasingly supported by structural studies, including cryo-electron microscopy analyses of inhibitor-bound helicase-primase complexes (Baranovskiy et al., 2025; Yu et al., 2026).

2.3. The antiviral activity of acyclovir and adibelivir is a function of serum concentration and viral load

The antiviral potency of a compound is determined not only by the intrinsic susceptibility of the viral strain but also by experimental variables such as the multiplicity of infection (MOI) and the concentration of serum proteins, which affect the free fraction of protein-bound drugs. The relationship of antiviral activity (EC_{50}) as a function of serum concentration and viral load are summarized in Fig. 6. HSV susceptibility assays (PRA or AS assay) are typically performed in the presence of 5%–10% FCS. Under these conditions, adibelivir exhibited EC_{50} values

A

HSV ID	EC_{50} (nM)	EC_{90} (nM)
HSV1 CI-4	1	11
HSV1 McKrae	2	7
HSV1 CI-5	5	19
HSV1 17syn+	6	32
HSV1 CI-2 R	10	19
HSV1 CI-1	19	65
HSV1 CI-3 R	20	36
HSV2 CI-8 R	8	24
HSV2 CI-7 R	10	40
HSV2 CI-6	20	63
HSV2 MS	28	58

B

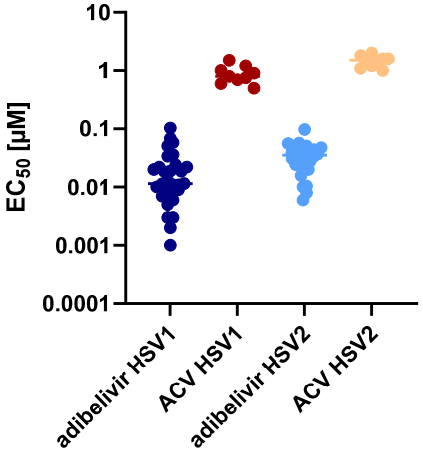


Fig. 4. Susceptibility (effective concentration EC_{50} and EC_{90}) of HSV1 and HSV2 clinical isolates, laboratory and acyclovir-resistant strains – tested at Innovative Molecules GmbH – to adibelivir determined using the activity-selectivity assay (AS).
A) Dose-response experiments with test item adibelivir were performed in the concentration range of 0.5 nM to 250 µM and the respective data analyzed by nonlinear regression with GraphPad Prism (IC_{50}: log (inhibitor) versus response (Variable slope (four parameters)), IC_{90}: log (inhibitor) versus response (Find EC Anything, least squares fit)).
B) Comparison of all EC_{50} values determined in Figs. 2–4 presented as a dot plot graph.

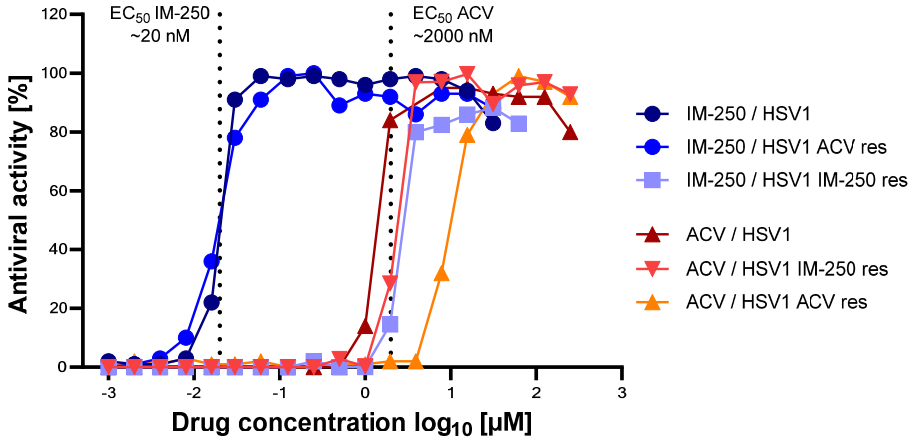


Fig. 5. Antiviral activity and dose-response curves were determined with the activity-selectivity assay.
Dose-dependent anti-HSV activity of adibelivir and ACV against sensitive and adibelivir and ACV-resistant HSV-strains is shown. Blue lines indicate treatment with adibelivir (HSV1 (CI-1) EC_{50} ~20 nM, HSV1 (CI-3, ACV-resistant) EC_{50} ~20 nM, HSV1 (M355T, adibelivir-resistant) EC_{50} ~3 µM). Red lines indicate treatment with ACV (HSV1 (CI-1) EC_{50} ~1.5 µM, HSV1 (CI-3, ACV-resistant) $EC_{50} \geq$ 10 µM, HSV1 (M355I, adibelivir-resistant) EC_{50} ~2 µM). The dotted lines indicate concentrations 20 nM and 2 µM. EC_{50} values were determined with the AS-assay. Representative results of duplicate experiments (mean) are shown.

of approximately 20 nM against HSV1 and 30 nM against HSV2. Increasing the FCS concentration resulted in a progressive reduction of antiviral activity. At 100% FCS, the EC_{50} of adibelivir increased to 694 nM (95% CI, 526.4–805.0 nM) for HSV1 strain Cl-1 and to 890 nM (95% CI, 694.5–1131 nM) for HSV2 strain MS. Similarly, the EC_{50} of acyclovir increased from approximately 1 µM under standard assay conditions to approximately 15 µM in the presence of 100% FCS. The corresponding EC_{90} values of adibelivir at 100% FCS were 1.9 µM for HSV1 and 2.2 µM for HSV2.

Regression analysis of the relationship between serum concentration and antiviral potency yielded exponential functions describing the increase in EC_{50} with increasing FCS concentration.

For HSV1, the relationship was described by:

$EC_{50} = 0.0198 + 0.0129 \cdot e^{0.039[FCS]}$ whereas for HSV2 the corresponding function was.

$EC_{50} = -0.0042 + 0.0677 \cdot e^{0.029[FCS]}$

The effect of protein binding was further investigated using human serum albumin (HSA). In the presence of 100% HSA (44 g/L), adibelivir exhibited EC_{50} and EC_{90} values of 2.0 µM and 2.7 µM, respectively, against HSV1. At an HSA concentration of 22 g/L, the EC_{50} values for HSV1 and HSV2 were 1.5 µM and 0.6 µM, respectively, while the corresponding EC_{90} values were 2.6 µM and 1.8 µM. The influence of viral inoculum on antiviral activity was also examined. Increasing the MOI to 1 resulted in a rise of the adibelivir EC_{50} to approximately 0.1 µM, whereas the EC_{50} of acyclovir increased to approximately 15 µM (Fig. 6B). These findings demonstrate that the apparent antiviral potency of both compounds is dependent on serum protein concentration and viral load, although adibelivir maintained substantially greater antiviral activity than acyclovir across all experimental conditions tested.

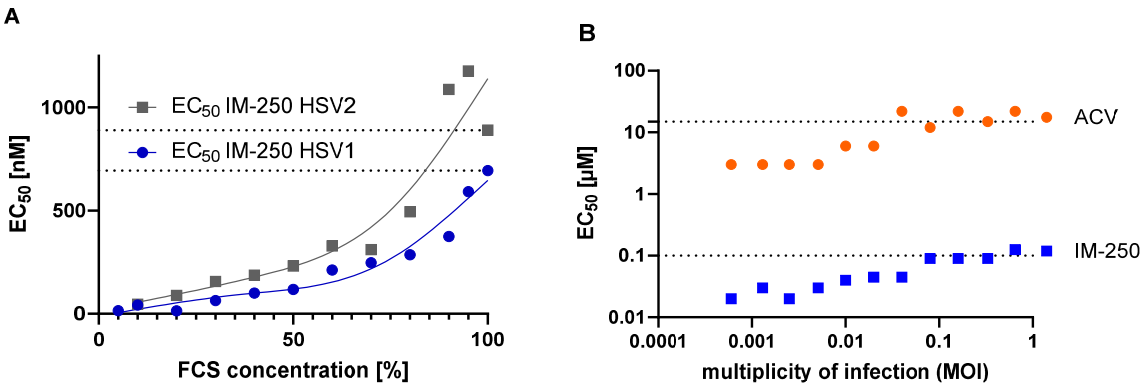


Fig. 6. The EC_{50} of drugs is a function of serum concentration and MOI in cell culture.
A) The EC_{50} of protein-bound drugs tested is a function of FCS concentration in cell culture. The EC_{50} values of adibelivir and ACV were analyzed in the presence of varying FCS concentrations with the AS assay and plotted against the concentration of FCS. The dotted line represents the EC_{50} of 694 nM for HSV1 and 890 nM for HSV2 at a FCS concentration of ~100%, respectively. Data are means of four independent experiments.
B) The EC_{50} is a function of the infectious HSV1 dose (multiplicity of infection (MOI)). The EC_{50} vakues of adibelivir and ACV were determined at varying infectious doses with the AS assay and plotted against the MOI. The dotted lines represent 0.1 µM (EC_{50} of adibelivir) and 15 µM (EC_{50} of ACV) at an MOI of ~1. Data are means of duplicates.

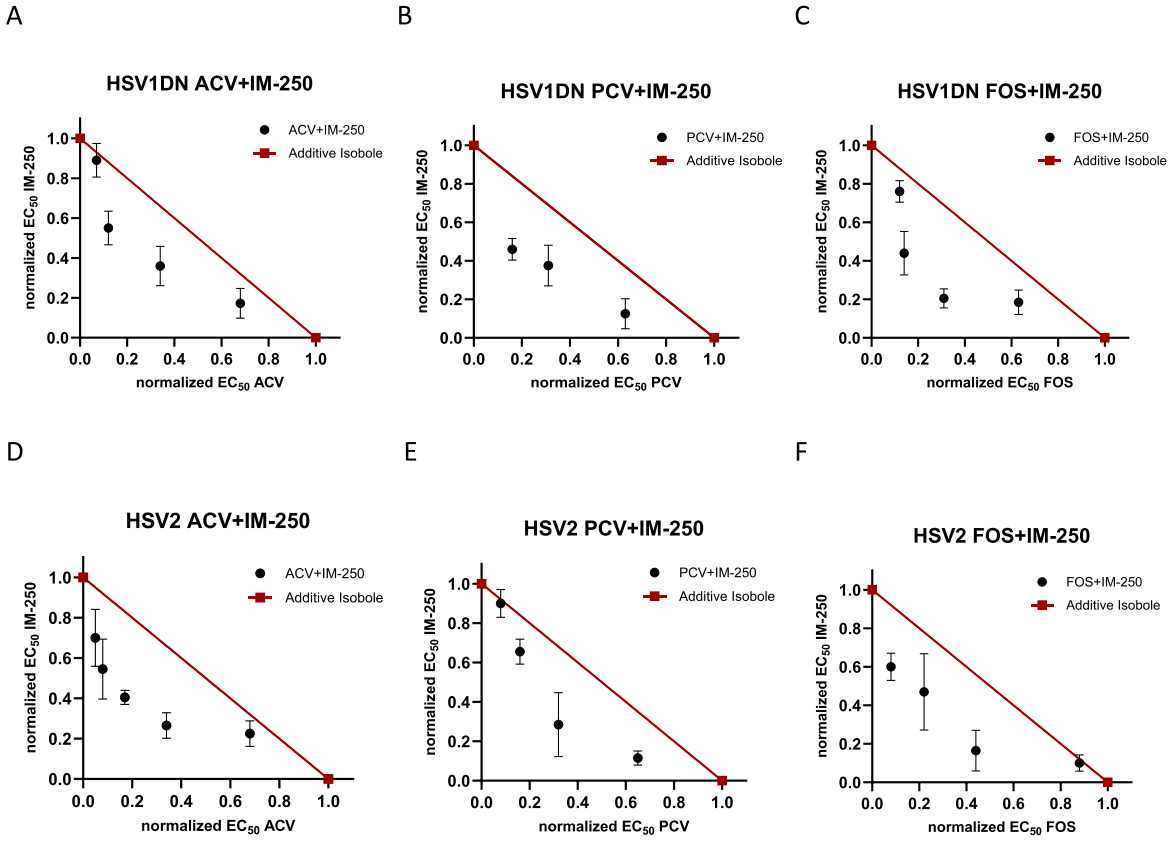


Fig. 7. Isobolograms for combination therapy of adibelivir with nucleosides and a phosphonate.
The isobolograms show the additive isoboles (red) and normalized concentration combinations of IM-250 and acyclovir, penciclovir or foscarnet (black) (Fig. 7A–C for HSV1(DN) upper and Fig. 7D–F for HSV2 (MS) lower isobologram, respectively).
A) Isobologram for combination therapy of adibelivir and acyclovir with HSV1 (DN) with $\alpha = 2.3 \pm 0.77$ (95% confidence interval 1.53–3.06) and p value of $\alpha = 0.0152$ and D) HSV2 (MS) with $\alpha = 6.05 \pm 1.81$ (95% confidence interval 4.24–7.86) and p value of $\alpha = 0.0066$.
B) Isobologram for combination therapy of adibelivir and penciclovir with HSV1 (DN) with $\alpha = 5.49 \pm 0.75$ (95% confidence interval 4.75–6.24) and p value of $\alpha = 0.0002$ and E) HSV2 (MS) with $\alpha = 3.44 \pm 1.19$ (95% confidence interval 2.24–4.64) and p value of $\alpha = 0.0184$.
C) Isobologram for combination therapy of adibelivir and foscarnet with HSV1 (DN) with $\alpha = 10.89 \pm 2.35$ (95% confidence interval of α 8.54–13.23) and p value of $\alpha = 0.0017$ and F) HSV2 (MS) with $\alpha = 4.37 \pm 2.42$ (95% confidence interval 1.95–6.79) and p value of $\alpha = 0.1050$.

2.4. Synergistic antiviral activity of adibelivir in combination with nucleoside analogs (ACV and PCV) and the phosphonate foscarnet (FOS)

The antiviral activity of adibelivir in combination with the nucleoside analogs ACV and PCV, as well as the pyrophosphate analog FOS, was evaluated against HSV1 (DN) = CI-5 and HSV2 (MS). The EC_{50} values of each antiviral agent were determined as described above. Dose-response data were analyzed by nonlinear regression using a sigmoidal Emax model implemented in GraphPad Prism, allowing estimation of EC_{50} values together with their corresponding 95% confidence intervals.

Drug interactions were assessed by isobologram analysis. The resulting isobolograms depict the theoretical additive isoboles (red lines) and the experimentally determined normalized concentration combinations of adibelivir with acyclovir, penciclovir, or foscarnet (black symbols). The upper panels (Fig. 7A–C) show the analyses performed with HSV1 (DN) = CI-5, whereas the lower panels (Fig. 7D–F) correspond to HSV2 (MS). The concentration combinations represent mean values derived from the antiviral susceptibility (AS) assay and were independently confirmed by microscopic evaluation of virus-induced cytopathic effects.

Drug interactions were quantified using the response-surface model of (Greco et al., 1990). According to this model, synergistic interaction is demonstrated when the interaction parameter α and the lower bound of its 95% confidence interval are both positive. The estimated positive α values, their corresponding positive lower 95% confidence limits, and the associated p-values are summarized in Fig. 7. Based on the analysis, adibelivir exhibits synergistic antiviral activity against HSV1 (DN) and HSV2 (MS) in combination therapy with acyclovir, penciclovir and foscarnet.

3. Discussion

Over the past seven decades, antiviral therapy for herpesvirus infections has evolved from relatively non-specific nucleoside analogs to highly selective, target-directed antiviral agents (De Clercq and Li, 2016; Kleymann, 2005; Gege and Kleymann, 2026). Early compounds such as idoxuridine and trifluridine, introduced in the 1960s and 1980s, exhibit antiviral activity in the low micromolar range but lack adequate specificity, as reflected by their historical application in cancer therapy due to their cytostatic properties. In fact, many cytostatic agents show antiviral activity but lack selectivity. Subsequently, vidarabine was approved as the first intravenous therapy of herpes simplex encephalitis in 1977.

A major milestone in antiviral drug development was the introduction of ACV, which remains the cornerstone of HSV therapy. ACV combines favorable selectivity with relatively low plasma protein binding (10–20%) and antiviral activity in the low micromolar range. (EC_{50} 0.3–3 μM). Its selectivity derives from preferential activation in infected cells through phosphorylation by the viral thymidine kinase, followed by conversion to the active triphosphate by host-cell kinases. The resulting metabolite ACV-PPP acts as an obligate chain terminator of the viral DNA polymerase, thereby effectively suppressing viral replication. Despite its excellent safety profile, ACV is limited by moderate potency, short plasma half-life in humans (2.9 h), restricted oral bioavailability (10–30%), and incomplete penetration into the central nervous system (CNS; 10–20% of plasma levels). Consequently, – alongside the main oral and topical applications – intravenous administration remains necessary for severe manifestations such as herpes simplex encephalitis to achieve therapeutic CNS exposure (Whitley and Gnann, 1992).

To improve oral bioavailability and pharmacokinetics to enable once- or twice-daily dosing, the pre-prodrugs VACV and FCV – derived from ACV and its congener PCV, respectively – were developed in the 1990s and represent an improvement over ACV's three-to five-times daily regimen (Perry and Faulds, 1996; Jarvest et al., 1998). However, despite these advances, all currently established nucleoside analogs ultimately target viral DNA synthesis but do not affect the lifelong HSV latency in sensory ganglia.

In the early 21st century, HPIs targeting the mechanistically distinct helicase-primase (HP), an essential component of the viral replication (Crute et al., 1989), entered clinical development (Gege et al., 2021; Gege and Kleymann, 2026). Helicase-primase inhibitors (HPIs) display nanomolar potency in standard *in vitro* assays. However, their high plasma protein binding (PPB ~75% amenamevir, > 90% other HPIs) increases the effective *in vivo* EC_{50} required for therapeutic efficacy considerably. Moreover, in contrast to adibelivir, most HPIs exhibit less than 10% CNS and ganglionic penetration (Gege et al., 2025; Tada et al., 2024). The findings presented here are consistent with earlier efficacy reports on adibelivir in animal models of primary and recurrent infections (Gege et al., 2021; Uhlig et al., 2021). Adibelivir showed significant potency in the lethal challenge model (ED_{50} = 0.5 mg/kg once daily) and reduced disease severity, viral shedding and the frequency of recurrences compared to standard nucleoside therapy. In addition, intermittent treatment during latency in established guinea pig HSV2 and ocular mouse HSV1 models reduced subsequent recurrences and reactivation over prolonged follow-up in the absence of treatment, suggesting that a latent neuronal infection can be modulated by antiviral therapy under appropriate exposure conditions (Bernstein et al., 2023). Together, the *in vivo* studies and the broader low-nanomolar activity against clinical isolates observed here, including acyclovir-resistant strains, substantiate the pharmacologic relevance. Importantly, adibelivir retained activity against acyclovir-resistant isolates, while reciprocal susceptibility of HPI-resistant viruses to nucleoside analogs confirmed the absence of cross-resistance between these mechanistically distinct antiviral classes. This finding supports the therapeutic potential of HPIs in settings where resistance to conventional nucleoside analogs compromises treatment efficacy. In addition, these assays are widely used to evaluate combination therapies to establish optimized treatment regimens. Simultaneous inhibition of multiple viral functions may enhance antiviral efficacy and potentially reduce the emergence of resistance. Based on the synergistic antiviral activity observed in combination therapy presented in this study, together with preclinical evidence of activity affecting the latent viral reservoir in animal models (Gege et al., 2021; Bernstein et al., 2023) and the favorable exposure of adibelivir in the CNS (Gege et al., 2025), phase 1 clinical trials were initiated for the parenteral formulation of adibelivir targeting the herpes simplex encephalitis indication and potentially neonatal herpes at a later stage in the context of combination therapy with acyclovir. Nevertheless, it should be emphasized that synergistic interactions observed *in vitro* do not necessarily translate into improved clinical outcomes and require confirmation in appropriately designed clinical studies.

For several decades, plaque reduction and activity selectivity assays, representing the smallest unit of infection in cell culture, provided the EC_{50} and EC_{90} values. The assays have served as the standard methods for profiling antiviral compounds and characterizing viral strains with respect to susceptibility and resistance. The mean EC_{50} values of 21 and 35 nM for HSV1 and HSV2 for clinical isolates determined in this study are comparable with the EC_{50} values of 19 nM and 30 nM originally reported for selected viral strains (Gege et al., 2021). However, the number of tested clinical isolates is still small considering broad application in the clinic.

Generally speaking, the EC_{50} represents a necessary but insufficient criterion for successful drugs. Both serum concentration and multiplicity of infection significantly influenced the apparent EC_{50} values of adibelivir and acyclovir. For *in vivo* pharmacodynamic predictions, the EC_{50} value must be considered a clinically relevant function of protein binding and viral load. These observations emphasize that *in vitro* susceptibility data represent only one component of antiviral drug evaluation and must be integrated with pharmacokinetic and tissue-distribution characteristics when predicting clinical efficacy. Accordingly, although comparable EC_{50} values may be achieved for different HPIs targeting the same binding pocket (Baranovskiy et al., 2025; Yu

et al., 2026) using diverse chemical scaffolds but related pharmacophores (Gege and Kleymann, 2026), the overall preclinical profile can differ substantially regarding the viral spectrum, plasma-protein binding, metabolic stability, pharmacokinetics, target tissue distribution and penetration and safety related to off-target effects e.g. carbonic anhydrase activity observed for some HPIs. For example, EC_{50} values in the range of 10–30 nM have been reported for amenamevir, HN0037, pritelivir, adibelivir and GS-5366 on HSV, whereas GS-1179 appears to exhibit even greater potency against HSV, likely due to optimization for reduced human serum protein binding. Amenamevir is also licensed to treat varicella-zoster virus (VZV) in Japan. The pharmacokinetic profile of HPIs in humans at a 100 mg oral dose varies considerably, with half-lives $t_{1/2}$ from 0.3 to 22 days and plasma exposures ranging from 0.5 to 5.0 μg/mL. For example, reported half-lives are approximately 0.3, 2, 3, 4, 5 and 22 days for amenamevir, HN0037, pritelivir, GS-1179, adibelivir and GS-5366, respectively. Corresponding plasma exposures are 0.5, 1.4, 1.6, 2.3, 3.8 and 5.0 μg/mL for amenamevir, GS-5366, pritelivir, adibelivir, HN0037 and GS-1179. Therapeutically relevant *in vivo* EC_{50} values are achieved with daily doses of up to 1200 mg for amenamevir, a 200-300 mg daily dose of HN0037, a 400 mg loading dose followed by 100 mg daily pritelivir, and weekly doses of approximately 350 mg and 20-50 mg for GS-5366 and GS-1179, respectively. The brain-to-plasma exposure ratios are below 10% for amenamevir, HN0037, pritelivir, GS-1179 and GS-5366, whereas adibelivir achieves CNS exposure levels comparable to plasma concentrations (Kusawake et al., 2017; Hou et al., 2022; Kropeit et al., 2023; Fletcher, 2026; Gege et al., 2025; Kleymann, 2026).

Phase 2 clinical trials will show whether HPIs will be more effective than the standard of care and which indications among herpes labialis, herpes genitalis, herpes simplex encephalitis or neonatal herpes and mucocutaneous herpes or herpes keratitis will be targeted with oral, topical or parenteral formulations. Currently, non-inferior amenamevir has been launched in Japan, pritelivir has been submitted as a new drug application (NDA) for the treatment of acyclovir-resistant HSV infections in immunocompromised patients. In parallel, HN0037, GS-5366, GS-1179 and adibelivir are undergoing or have completed Phase 2 trials for genital herpes. Additionally, adibelivir is also developed as an intravenous formulation for therapy of severe disease including herpes simplex encephalitis.

In conclusion, antiviral drug development for herpes viruses has progressed from relatively non-specific cytotoxic agents with a low selectivity index such as idoxuridine, trifluridine, and vidarabine to highly specific, well-tolerated antiviral agents such as acyclovir followed by pharmacokinetic optimizations through valacyclovir and famciclovir. Expansion of antiviral drug research beyond the viral polymerase toward alternative targets, including the HP, has opened new opportunities for improving treatment efficacy and addressing unmet medical needs. The broad activity of adibelivir against clinical and drug-resistant HSV isolates, its synergistic interactions with established antivirals, and its unique CNS exposure profile support further clinical investigation of this compound across a range of HSV-associated diseases. Ongoing and future clinical trials will ultimately determine whether the promising preclinical characteristics of adibelivir and other HPIs translate into meaningful therapeutic advantages over current standards of care.

4. Material and methods

4.1. Antiviral drugs, cells and viruses

The hydrochloride of adibelivir (IM-250 HCl) was synthesized based on patent WO2023/135303. Acyclovir, penciclovir and foscarnet were obtained from Sigma-Aldrich product no 1012065 or PHR1254, O0035 and PHR1436, respectively.

Vero (African green monkey kidney) cells and virus strains, including the HSV1 McKrae or strain 17syn+ and HSV2 strain MS (ATCC VR-540) were obtained from ATCC (American Type Culture Collection) or DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and cultivated according to standard procedures provided by the supplier. Vero Cell Line (ATCC® CCL-81™) was obtained from the University of Washington Clinical Virology Laboratory.

Clinical isolates (CIs) sensitive [wild type (wt)] or resistant to ACV were obtained from stem cell transplant patients.

HSV1: CI-1 (161447, throat swab, wt); CI-2 (H2521, ACV res, UL23 G240E, UL30 wt), CI-3 (H1850, ACV res, $EC_{50} \sim 25$ μM, UL23 R222H), CI-4 (Hl 118), CI-5 (H2219) = DN; HSV2: CI-6 (H2541, wt), CI-7 (H3285, ACV res, $EC_{50} > 40$ μM, UL23 add G 433 to 439) and CI-8 (H2414, ACV res $EC_{50} > 10$ μM, UL23 delC nt 586 to 590, UL30 wt) were obtained from the viral collection of K. Hamprecht at Institute of Medical Virology, University Hospital Tübingen according to standard operating procedure SOP AM-VI-316M, DAkkS-Deutsche Akkreditierungsstelle: D-ML-1313 0-03-00.

21 HSV strains, including acyclovir-resistant (R) and sensitive (S) strains, HSV1: GDMAB#12 (S), #13 (S), #14 (S), #15 (S), #16 (S), #17 (S), #19 (S), #20 (S) and A1 (S), C1 (R) and HSV2: GDMAB#1 (S), #2 (S), #4 (S), #5 (S), #6 (S), #7 (S), #8 (S), E1 (S), F1 (S), G1 (R) and H1 (R) were obtained and selected for testing their susceptibility against IM-250 HCl in a plaque reduction assay (PRA) at the University of Washington, Seattle, USA.

20 HSV strains, including acyclovir-resistant (R) and sensitive (S) strains, HSV1: VIRO-FR #1509905 (R), #1509910 (S), #1509609 (S), #1506765 (S), #1510485 (R, single frame shift mutation SFS185 (TK)), #1510464 (S), #1512290 (R, stop mutation Q185 (TK)), #1512135 (S), #1513616 (S), #1515205 (S), and HSV2: VIRO-FR #1515929 (S), #1061619 (S), #1317049 (S), #1316658 (S), #1362444 (S), #1513794 (S), #1511009 (R), #117044 (R), #1165008 (S), #1510715 (S) were obtained and selected for testing their susceptibility against IM-250 HCl in a PRA at the University of Freiburg, Germany.

HSV1 and HSV2 clinical isolates were cultured, viral stocks generated and titrated to determine the concentration of plaque forming units (PFU).

4.2. Activity-selectivity (AS) assay

A total of 1×10^4 Vero cells per well of a microtiter plate (MTP) were infected with 25 PFU of herpes simplex virus (HSV laboratory strains or clinical isolates of HSV1 or -2; MOI = 0.0025) in a total volume of 200 μL of media [Dulbecco's modified Eagle medium (DMEM) high glucose (4.5 g/liter) supplemented with 10% (range 5–100% in the respective experiments) fetal calf serum (FCS), 2 mM glutamine, penicillin (100 IU/mL), and streptomycin (100 μg/mL)] in the presence or absence of drug and incubated for 5 days at 37°C and 5% CO_2. The wells of the MTP were washed with phosphate-buffered saline (PBS) (250 μL) and then filled with 200 μL of PBS containing fluorescein diacetate (10 μg/mL; Sigma-Aldrich). After incubation for 45 min at room temperature, fluorescence was measured at 485-nm excitation and 538-nm emission wavelengths (Kleymann et al., 2002; Kleymann and Werling, 2004). Data (antiviral activity [%] = (*RFU* sample - *RFU* virus control)/(*RFU* cell control - *RFU* virus control) * 100) were evaluated as described in analysis.

4.3. Plaque reduction assay (PRA, freiburg, Germany)

Vero cells were seeded in 24-well plates and infected 24 h later with different HSV1 or HSV2 isolates at a MOI of 0.0025 using centrifugal enhancement (30 min at 2300 rpm). After one additional hour of incubation at 37°C, the infected cells were overlaid with 1.5% Avicel®/5% FCS DMEM solution containing serial 2-fold dilutions of IM-250 HCl. The compound was tested in duplicates at 11 concentrations ranging from 0.48 nM to 500 nM, alongside a virus control without treatment. Following 4 days of incubation, cells were fixed with 3.7% formaldehyde and stained with ethyl violet. Plaques were counted, normalized to

determine antiviral activity (%), and data were analyzed as described in the analysis section.

4.4. Plaque reduction assay (PRA, Seattle, USA, based on Swierkosz, 2004)

Vero cells (ATCC® CCL-81™) 5×10^5 were grown in 6-well plates in high glucose DMEM with 2 mM L-glutamine, without sodium pyruvate, 10% FCS (range 10–100% depending on experiment), 100 U/mL Pen/Strep at 37°C, 5% CO_2 and infected with 30–260 PFU (MOI 0.00006–0.005) per well, and then overlaid with a serial dilution series of IM-250 HCl in a 1.5% carboxymethylcellulose solution. Cells were treated with 1:3 dilution series of IM-250 HCl concentrations ranging from 0.2 nM to 1000 nM for a total of 11 concentrations plus a virus-only control. Plaques were stained with 0.1% crystal violet in 20% MeOH/Dulbecco's phosphate-buffered saline, image files of plaques counted with Image J, and data analyzed with GraphPad Prism version 10.1.2.

4.5. Combination therapy of antiviral agents (Elis, 2019)

Synergy tests of combination therapy experiments were performed using the AS assay for checkerboard experiments. A horizontal 2-fold dilution gradient (start concentrations 320 nM for HSV1 and 10 μM for HSV2) of the antiviral agent IM-250 was combined with a vertical 2-fold dilution gradient of the nucleoside analogs acyclovir (start concentrations 16 μM for HSV1 and 20 μM for HSV2), penciclovir (start concentration 32 μM for HSV1 and 80 μM for HSV2) and the pyrophosphate analog foscarnet (start concentration 160 μM for HSV1 and 320 μM for HSV2). In parallel to the checkerboard experiments, a control plate was prepared for each experiment in which a 4-fold EC_{50} determination was carried out for each of the two antiviral substances to be tested. The plates were incubated for 4 days at 37°C and 5% CO_2 and then analyzed using the antiviral AS assay by adding the fluorescein diacetate. On day 4, a microscopic analysis of the assay was also performed, in which the EC_{50} value was estimated with respect to the cell control as well as the virus control.

4.6. Cytotoxicity assay

Cytotoxicity of IM-250 HCl in Vero cells was tested after 48 h incubation by staining with fluorescein diacetate and read out was measured at 480 nm excitation and 530 nm emission wavelength (Kleymann et al., 2002; Kleymann and Werling, 2004). The tolerability in cell culture was determined as $SI = CC_{50}/EC_{50}$ (selectivity index = half-maximal cytotoxic concentration/half-maximal effective concentration) for all strains tested against IM-250 in 10% FCS.

4.7. Analysis

EC_{50} values were determined by a nonlinear plot of antiviral activity as a function of drug concentration (IC_{50}: log (inhibitor) versus response (variable slope (four parameters)), IC_{90}: log (inhibitor) versus response (find EC90, least squares fit)).

For the statistical analysis of the combined antiviral activities, a response surface model according to (Greco et al., 1990) (formula 4) was used.

4.8. Generation and sequencing of resistant HSV-strains

ACV and IM-250 resistant strains were generated and sequenced according to (Hagmaier, 2019).

Funding

This research did not receive any specific grant from funding agencies in the public or not-for-profit sectors. Life Science Partners, now acquired by EQT Life Sciences, invested in Innovative Molecules GmbH.

CRediT authorship contribution statement

Christian Gege: Formal analysis, Methodology, Validation, Visualization, Writing – original draft. **Valeria Falcone:** Investigation, Methodology, Validation, Visualization, Writing – original draft. **Haiying Zhu:** Investigation, Methodology. **Julia Elis:** Investigation, Methodology. **Timo Hagmaier:** Investigation, Methodology. **Bhanupriya Madarampalli:** Investigation, Methodology. **Ailyn C. Pérez-Osorio:** Investigation, Methodology, Validation, Visualization, Writing – original draft. **Nathalie Pretzsch:** Investigation, Methodology. **Keith R. Jerome:** Formal analysis, Methodology, Supervision, Validation, Writing – original draft. **Daniela Huzly:** Conceptualization, Formal analysis, Methodology, Supervision. **Gerald Kleymann:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CG and GK are inventors of patent families WO2017/174640 (Title: Aminothiazole derivatives useful as antiviral agents) and WO2019/068817 (Title: Enantiomers of substituted thiazoles as antiviral compounds). CG is a consultant to Innovative Molecules GmbH. GK is shareholders of Innovative Molecules GmbH. All other authors declare that they have no competing interests.

Acknowledgements

We thank Thomas Hoffmann for proofreading of the manuscript.

Data availability

Data will be made available on request.

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