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Novel inhibitors of HSV-1 protease effective *in vitro* and *in vivo*

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) is a widespread human pathogen known to cause infections of diverse severity, ranging from mild ulceration of mucosal and dermal tissues to life-threatening viral encephalitis. In most cases, standard treatment with acyclovir is sufficient to manage the disease progression. However, the emergence of ACV-resistant strains drives the need for new therapeutics and molecular targets. HSV-1 VP24 is a protease indispensable for the assembly of mature virions and, as such, constitutes an interesting target for the therapy. In this study, we present novel compounds, KI207M and EWDI/39/55BF, that block the activity of VP24 protease and consequently inhibit HSV-1 infection *in vitro* and *in vivo*. The inhibitors were shown to prevent the egress of viral capsids from the cell nucleus and suppress the cell-to-cell spread of the infection. They were also proven effective against ACV-resistant HSV-1 strains. Considering their low toxicity and high antiviral potency, the novel VP24 inhibitors could provide an alternative for treating ACV-resistant infections or a drug to be used in combined, highly effective therapy.

1. Introduction

Herpes simplex virus type 1 (HSV-1) is a prevalent human pathogen, estimated to infect 67% of the global population (Looker et al., 2015). The infection is commonly associated with 'cold sores' and, in some cases, more severe diseases, i.e., stromal keratitis, a leading cause of blindness in developing countries, and potentially fatal viral encephalitis (Bradshaw and Venkatesan, 2016; Farooq and Shukla, 2012). The virus is easily transmitted through contact, such as kissing or sexual intercourse. Primary infection begins in epithelial cells in the oral, ocular, or genital area and enables the virus to reach the synapses of the sensory neurons. Next, virus particles, hijacking the retrograde cellular transport, make their way up to the neuron's body to enter the cell nuclei and establish a dormant infection of the ganglia, known as latency. A life-long latent infection, a hallmark of the *Herpesviridae* family, is a complex process related to the inefficient axonal transport of viral regulating factors, which leads to a blockade of immediate early gene transcription (De Regge et al., 2010; Wilson and Mohr, 2012). The infection may be reactivated by triggers such as UV light or in periods of decreased immunity, such as menstruation, fever, or immunosuppressive treatment (Arvin et al., 2007).

Approved therapeutic strategies are based on nucleoside analogs, such as acyclovir (ACV). When built into newly synthesized viral DNA, these compounds inhibit its elongation and consequently block virus replication (Mubareka et al., 2010). However, as almost all approved drugs interact with the same molecular target, the viral DNA

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polymerase, reports on the viral strains resistant to this treatment are frequent. Currently, only foscarnet is approved to treat ACV-resistant herpes simplex infection. While foscarnet is a structural mimic of pyrophosphate and not a nucleoside analog, its activity is also based on the inhibition of viral DNA polymerase, and resistance to this drug has already been reported (Blot et al., 2000). Despite many trials, no effective HSV vaccine is available (Johnston et al., 2016; Whitley and Baines, 2018).

Proteases are encoded in many viral genomes, as they are used for post-translational modification of viral proteins and their maturation. Usually, these enzymes are characterized by high selectivity and unique substrate specificity, which ensures tight regulation of the process (Steinkühler, 2008). For the same reason, these enzymes are convenient targets for antiviral therapy. Several protease inhibitors are already marketed, most notably as components of highly active antiretroviral therapy (HAART) against HIV-1 (Ghosh et al., 2016). Another clinically relevant example is the hepatitis C (HCV) NS3/4A protease inhibitors. The third generation of these drugs, with improved potency and HCV genotype coverage, is currently used in therapy (de Leuw and Stephan, 2018; McCauley and Rudd, 2016). Similarly, an inhibitor of SARS-CoV-2 main protease, paxlovid, in combination with ritonavir, is used to treat COVID-19. Administered orally, the drug significantly reduced hospital admissions and deaths among patients (Mahase, 2021).

Proteases encoded in the *Herpesviridae* genomes have previously been considered antiviral therapy targets. A series of peptidomimetic inhibitors were developed to target human cytomegalovirus protease (HCMV), but they have never made it to clinical trials (Tong et al., 1998). An attempt was also made to block the activity of Kaposi's sarcoma-associated herpesvirus (KHSV) protease with a peptide designed to disrupt the formation of the dimer. Still, it was not shown to inhibit virus replication (Shimba et al., 2004).

The formation of HSV-1 capsid is a complex process involving an interplay of various viral components. It begins in the cytoplasm with the aggregation of triplexes of capsid proteins VP5, VP23, and VP26, which are then transported to the nucleus, where the capsid formation occurs. A fully formed HSV-1 capsid is an icosahedral sphere of ~120 nm in diameter and consists of 162 capsomeres, 150 hexons, 12 pentons, and a portal (Grünewald et al., 2003). The VP21 protein is essential for the formation of the capsid. It forms a scaffold on which the structural proteins associate to form the capsid, and later on is cleaved, removed from the core, and replaced by the viral DNA. Lack of VP21 results in the formation of abortive, non-functional capsids. However, inhibition of the VP24 activity also blocks capsid formation, as the scaffold may not be released from the capsid, and consequently, the DNA may not be loaded (Knipe and Howley, 2013). A set of recombinant viruses produced in the baculovirus system proved that a virus deprived of UL26.5 gene product may still produce infectious virions. However, functional capsids are not formed when the UL26 gene product is missing (Gao et al., 1994). Here we present novel highly active inhibitors of HSV-1 protease, EWDI/39/55BF, and KI207M, selected via screening of diverse, core-structurally similar molecules.

Since HSV therapeutic options are limited, we have decided to adopt a screening approach focused on a few series of novel, structurally diverse compounds to identify lead structures that might potentially prompt the development of a new drug. Tested heterorganic compounds contained various biologically relevant structural features such as a chiral bicyclic skeleton (**1–3**) with one or two nitrogen atoms (amine/imine functionality) substituted with aminophosphonic or sulfonamide function. These structural components can be found in several antiviral drugs, including beclabuvir, dasabuvir, or maraviroc, as well as in many other compounds manifesting diverse biological activity, including selective inhibition of cancer cell growth (Iwanejko J; Iwanejko J and J, 2017; Samadaei et al., 2020). Two of the tested compounds showed promising activity, and their efficacy and mechanism of action were studied. The obtained results clearly show that the compounds inhibit the protease of the HSV-1 virus, which means the virus replication *in vitro* and *in vivo*.

2. Methods

2.1. Active compounds and proteins

Active compounds were synthesized as described in detail in the Supplementary Material 1 - Chemistry. The structure of the compounds is shown in Fig. 1.

The N-terminal, 306 amino acids long, region of HSV capsid scaffolding protein (UniProt accession number: P10210) containing the serine protease domain was amplified based on a codon-optimized synthetic gene using PCR and oligonucleotides containing NcoI and KpnI restriction sites. The gene was ligated into a plasmid coding for the sequences of His_6 tag followed by a cleavage site of tobacco etch virus protease (TEV) at the N-terminal end of the VP24 protease. The expression of the protein was carried out in *E. coli* BL21 *lysY* (New England Biolabs) in the LB medium (Amrane et al., 2014). Initially, the culture was grown at 37 °C until OD_{600} ~0.6, expression of the protein was induced with 0.25 mM IPTG, and the culture was incubated overnight at 25 °C. The cells were harvested by centrifugation (4500×*g*, 30 min, 4 °C), suspended in 20 ml of Tris buffer pH 7.5 (50 mM Tris, 500 mM NaCl, 5 mM imidazole, 5% (*m/v*) glycerol) and lysed with lysozyme followed by sonication (20 min, 3s on 3s off, amplitude 80%). After the second centrifugation (20 000×*g*, 30 min, 4 °C) the supernatant was collected, and the VP24 protease was purified using Ni^{2+} affinity chromatography (Bio-Rad, Warsaw, Poland) and Tris buffers with increasing imidazole concentration (5, 25 and 500 mM). Subsequently, the elution fractions with the highest protein concentration were combined, the buffer was changed (10DG columns, BioRad) to the Tris buffer with 5 mM of imidazole, TEV protease was added, and the sample was incubated overnight at 4 °C. The second Ni^{2+} affinity column allowed us to obtain VP24 protease with the removed His_6 tag in the flowthrough fraction. The protease autoproteolysis at position 247 and the protein purity were analyzed by SDS-PAGE (4–12%, Tris-glycine) and visualized by Coomassie blue R250 staining.

2.2. Biochemical activity assay

A kinetic assay was carried out for 6h at 37 °C. The reaction mixture consisted of 5 μM of HSV-1 VP24 protease, 0.1–50 μM of inhibitors, and 5–20 μM of fluorogenic substrate resembling HSV-1 protease R site (dabcyl-TYLQASEKFK-Glu(edans)-NH_2) in a buffer containing 50 mM

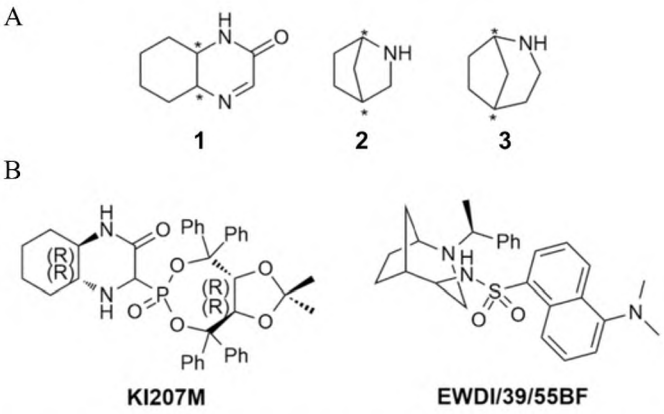

Fig. 1. Structures of tested compounds. A - chiral bicyclic scaffolds found in compounds used in the study: hexahydroquinoxalin-2(1H)-one (1), 2-azabicyclo[2.2.1]heptane (2), and 2-azabicyclo[3.2.1]octane (3). Stereogenic centers are shown by asterisks (substitution can result in the formation of additional chiral centers). B - structures of the most active VP24 inhibitors selected in the presented study.

Tris, 500 mM NaCl, 5 mM imidazole, 5% glycerol pH 7.5. The reaction was monitored at $\lambda_{EX} = 336$ nm and $\lambda_{EM} = 455$ nm using Tecan M200 plate reader. The range of inhibitor concentration was adjusted considering the limited solubility of the compounds in the reaction buffer.

2.3. Cells

Vero E6 cells (African green monkey kidney epithelial) purchased from ATCC (CRL-1586) were maintained in Dulbecco-modified Eagle's medium (DMEM, high glucose, Corning) supplemented with 3% heat-inactivated fetal bovine serum (FBS, Life Technologies), penicillin (100 U/ml), streptomycin (100 μg/ml), and ciprofloxacin (5 μg/ml) at 37 °C in an atmosphere containing 5% CO_2. Primary human skin fibroblasts (HSF) were cultured in DMEM (high glucose, Corning) supplemented with 10% heat-inactivated FBS (Life Technologies), 1% nonessential amino acids (Life Technologies), penicillin (100 U/ml), streptomycin (100 μg/ml), and ciprofloxacin (5 μg/ml) at 37 °C in an atmosphere containing 5% CO_2.

2.4. Viruses

HSV-1 strain 17+ (0104151v) and HSV-2 strain HG52 (0104152v) were acquired from Public Health England. Clinical strains 920, 452/01, and 2473/01 were isolated from patients at the Department of Microbiology, Collegium Medicum, Jagiellonian University. Acyclovir-resistant clinical strains RAT-2, MS-1, and MC were isolated from immunosuppressed patients hospitalized at the Department of Hematology, Oncology, and Internal Medicine, Medical University of Warsaw (Cieśluk et al., 2008).

Virus stocks were generated by infecting fully confluent Vero E6 cells with the virus at $TCID_{50}$ of 400/ml. Cells were cultured until the appearance of the cytopathic effect (CPE). The cultures were lysed by freezing at −80 °C for at least 2 h, thawed, aliquoted, and stored at −80 °C. Mock samples were produced in the same manner using uninfected cells. The virus titers were determined according to Reed and Muench formula (Reed and Muench, 1938). The number of CPE-positive wells was scored two days post-infection (p.i.).

2.5. Virus replication assay

Cells in 96-well plates were overlaid with media containing tested compounds and incubated for 30 min at 37 °C. Subsequently, the medium was discarded, and cells were infected with the virus at $TCID_{50}$ = 400/ml or mock-infected for 2 h at 37 °C in the presence or absence of the inhibitor. Following the incubation, cells were washed thrice with phosphate-buffered saline (PBS), and overlaid with fresh media supplemented with the inhibitors or reference samples. Media samples for qPCR and plaque assay were collected two days p. i.

2.6. qPCR

The viral yield was assessed using quantitative real-time PCR. Viral DNA was isolated from cell culture media with a Viral DNA/RNA Isolation Kit (A&A Biotechnology). The reaction volume was 10 μl, and it consisted of Kapa Probe Fast qPCR Master Mix, a specific probe labeled with 6-carboxyfluorescein (FAM) and Black Hole Quencher 1 (BHQ1) (sequence 5′-FAM CCG CCG AAC TGA GCA GAC ACC CGC GC BHQ1-3′, 100 nM), and primers (450 nM each; sense primer 5′-CAT CAC CGA CCC GGA GAG GGA C-3′, antisense primer 5′-GGG CCA GGC GCT TGT TGG TGT A-3′) and 2.5 μl of the template DNA. The thermal profile was 3 min at 95 °C followed by 37 cycles of 2 s at 95 °C and 20 s at 60 °C, and the reaction was conducted in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

DNA standards were prepared to calculate the amount of viral DNA particles in samples. A fragment of the gene coding for viral DNA polymerase was amplified and cloned into pTZ57 R/T plasmid (Thermo Scientific) using InsTAclone PCR cloning kit (Thermo Scientific). The plasmid was amplified in *E. coli* TOP10 (Life Technologies), isolated with GeneJET Plasmid Miniprep Kit (Thermo Scientific), and linearized with *Kpn*I restriction enzyme. Then, DNA concentration was measured by NanoDrop (Thermo Scientific), and the number of DNA particles per milliliter was calculated using the Avogadro constant. Serial dilutions of the DNA standard (10 μl each, 10^{12}–10^{6} DNA copies/ml) were used to produce a standard curve.

2.7. Plaque assay

Fully confluent Vero E6 cells in 24-well plates were infected with 10-fold serial dilutions of infectious samples for 1 h at 37 °C. Subsequently, cells were washed with PBS and cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 1% methylcellulose (Sigma-Aldrich) for three days. Then, the methylcellulose medium was removed, and the cells were stained with 0.1% crystal violet solution in 50:50 water/ethanol mixture for 10 min at room temperature, washed with tap water, and the plaques were counted.

2.8. Microscopy

Experiments were performed on Vero E6 cells growing on coverslips in 12-well plates. After the respective experiment, the cells were fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 12 min, and nonspecific binding sites were blocked with 5% bovine serum albumin (BSA) in PBS overnight at 4 °C. For virus staining, cells were incubated for 2 h with primary rabbit anti-HSV antibody (20-HR50, Fitzgerald Industries) diluted 1:500 in PBS with 1% BSA followed by 1-h incubation with secondary goat anti-rabbit antibody conjugated with Atto 488 (A11001, Invitrogen) diluted 1:200. VP5 was stained using VP5 antibody (6F10) (sc-13525, Santa Cruz Biotechnology) diluted 1:100 for 2 h followed by 1 h incubation with rabbit anti-mouse antibody conjugated with Alexa Fluor 488 (A11059, Invitrogen). F-actin was stained during 1-h incubation with AlexaFluor 647-conjugated phalloidin (Invitrogen) diluted at 1:50. For counterstaining of nuclear DNA. Slides were incubated with 0.1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 20 min. Preps were then mounted on glass slides using ProLong Diamond Antifade Mountant (Thermo Scientific). Confocal images were acquired under a ZEISS LSM 710 (release version 8.1) confocal microscope using ZEN 2012 SP1 (black edition, version 8.1.0.484) software. Stack acquisition parameters: frame size 2048 × 2048 pixels, pixel size 0.13 μm. Fluorescent images were acquired using EVOS Cell Imaging System (Thermo Scientific). ImageJ FIJI version was used for image processing. For the imaging of infection inhibition by protease inhibitors, the cells were stained on 16 × 16mm glass slides with the field of view as wide as possible to see the outlines of the cells. To quantify capsid egress from the nucleus, the fluorescence intensity and the ratio between the nucleus and the whole cell area were assessed in 50 infected cells using ImageJ FIJI software.

2.9. In vivo toxicity

For *in vivo* studies, formulations of protease inhibitors were prepared by dissolving the compounds in DMSO at 200 mM, then slowly mixing them with PEG (400 Da) to achieve a concentration of DMSO of 2% and 4 mM of the inhibitor. The toxicity of the compounds was tested in 8-week-old male BALB/c mice purchased from The Center for Experimental Medicine of the Medical University of Bialystok. The mice were healthy and SPF-certified. Animals were housed individually at a temperature of 22 °C ± 2 °C with a relative humidity of 55% ± 5% and 12-h light and dark cycles. The ventilation was sufficient to ensure no less than 20 air changes per hour. Inhibitor formulations were prepared by

adding 200 mM inhibitor solution in DMSO to PEG (400 Da) to a final concentration of 4 mM inhibitor and 2% DMSO. Control formulation was prepared in the same manner using pure DMSO. Forty-five animals divided into three groups (n = 15) received formulations containing protease inhibitors or control formulations without the compounds. The mice were shaved at the lateral dorsal side, 1 cm^2 of the skin was scratched with a needle, and 100 μl of the formulation was applied to the site, equal to 0.27 mg/cm^2 for KI207M and 0.19 mg/cm^2 for EWDI/39/55BF. The treatment was administered every 12 h for four subsequent days. Each day for 14 days, the animals were weighed and examined for skin abnormalities and general condition.

2.10. In vivo antiviral activity

Eight-week-old male BALB/C mice were shaved at the lateral dorsal side, the skin was scratched with a needle, and 100 μl of the formulation (containing the inhibitor or the vehicle only) was applied to the site. Next, a patch containing 1×10^7 PFU of the virus was placed on the scarified area for 2 h. Subsequently, the patch was removed. The treatment was administered every 12 h for four subsequent days. Each day for 14 days, the animals were weighed and examined for skin abnormalities and general condition. Health was assessed using the following score: 0 – no signs; 1 – formation of blisters; 2 – slight ulceration; 3 – large patches of ulceration; 4 – a confluent band of ulceration; 5 – hind limb paralysis; 6 – death. The study was approved by the 2nd Local Ethics Committee for Animal Experiments in Cracow (approval no. 145/2017).

2.11. Statistical analysis

All experiments were performed in triplicate, and results are presented as mean ± SEM unless otherwise indicated. One-way ANOVA with Tukey HSD post-hoc test was used to assess the statistical significance of acquired results. When the parametric test assumptions were violated, the nonparametric Kruskal-Wallis with Dunn's post-hoc test was used. P values of 0.05 and less were considered significant. Values statistically significant are indicated by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, or ns – nonsignificant.

3. Results

3.1. Biochemical activity assay

The recombinant HSV-1 VP24 protease's enzymatic activity and the inhibitory potential of compounds KI207M and EWDI/39/55BF were analyzed by the spectrofluorimetric assay. In order to verify if obtained recombinant protease was catalytically active, the previously described substrate of HSV-1 VP24 protease spanning positions 242–254 of the natural autoproteolytic cleavage site (Darke et al., 1994; Hall and Darke, 1995) was incubated with the enzyme. The same conditions were applied to analyze compounds' inhibitory potency towards the enzyme. The substrate processing at the VP24 protease-specific cleavage site (H-HTYLQA↓SEKFKMW-NH_2) was confirmed by mass spectrometry (see Supplementary material 2 – Biochemistry and Biology Figs. S2 and S3).

A kinetic assay was carried out to determine the activity of the novel compounds against HSV-1 VP24 protease. Both tested compounds were found to significantly inhibit the activity of HSV-1 VP24 protease in the kinetic assay. Calculated IC_{50} (half-maximal inhibitory concentration) values were 2.88 ± 0.49 μM for KI207M and 10.31 ± 0.92 μM for EWDI/39/55BF (Fig. 2).

3.2. Inhibition of HSV-1 replication

In order to determine the antiviral activity of the compounds, a virus replication assay was performed in Vero E6 cells. Cells were treated with non-toxic concentrations of the inhibitors (10 μg/ml) (see Supplementary Material 2 – Biochemistry and Biology Fig. S4). The qPCR results are presented in Fig. 3. The most potent effect was recorded for KI207M and EWDI/39/55BF; therefore, these two compounds were selected for subsequent studies. Unfortunately, neither of the tested compounds exhibited activity against HSV-2. Even though HSV-1 and HSV-2 are closely related, their proteases differ significantly (90% similarity on the amino acid sequence level).

Subsequently, effective concentrations of the two selected compounds were determined using qPCR and plaque assay. The latter method was used to assess the amount of infectious virus released from cells. Dose-response was evaluated, and results are presented in Fig. 4. Control cells were treated with 0.1% DMSO, an amount equal to present in the highest concentration of the inhibitor. Calculated IC_{50} values were 1.31 ± 0.50 μM for EWDI/39/55BF and 0.53 ± 0.21 μM for KI207M, as determined by qPCR, and 2.35 ± 2.38 μM for EWDI/39/55BF and 0.93 ± 0.35 μM for KI207M, as determined by plaque assay.

3.3. Mechanism of antiviral activity

A set of functional assays was conducted to determine the mechanism of action. Each assay aims to indicate inhibition of a different stage of viral infection. First, the compounds' ability to directly inactivate HSV-1 virions was investigated (test I), but no decrease in viral yield was observed. Then, the possibility that the compounds make the cells resistant to the virus (assay II) was checked and some inhibition was recorded. Subsequently, the influence of the compounds on viral adhesion to the cell surface (test III) was ruled out. Finally, a significant reduction of viral yield was observed with the compounds acting on the late stages of infection (test IV) in cell culture supernatant as well as a cell lysate, which indicates that the inhibitors prevent viral replication or assembly of new virions inside the cell. Fig. 5 demonstrates the results of the functional assays. The mechanism of action for both compounds

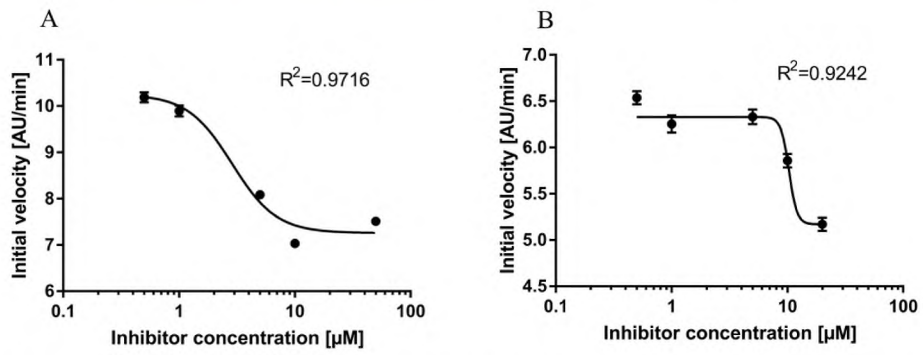

Fig. 2. Inhibition of HSV-1 VP24 protease by KI207M (A) and EWDI/39/55BF (B). The reaction was performed at 37 °C for 6h. The results are presented as mean ± standard error of the mean (SEM). Similar results were obtained in at least three replicates in three independent experiments.

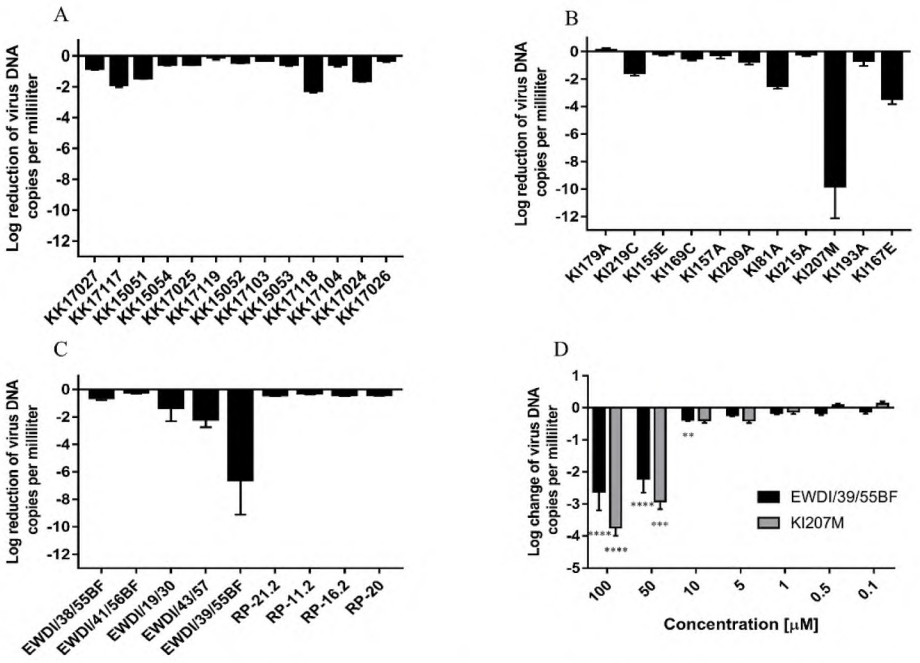

Fig. 3. Inhibition of HSV-1 replication in cell culture. Vero E6 cells (A, B, C) and primary human skin fibroblasts (D) were infected with HSV-1 at TCID50 = 400/ml in the presence of the inhibitors or control samples. After two-day incubation, the media was collected, and virus yields were assessed using qPCR. The results are presented as mean ± standard error of the mean (SEM).

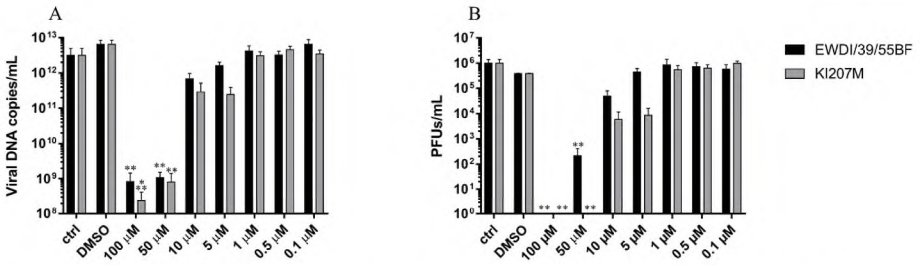

Fig. 4. Dose-dependent inhibition of HSV-1 replication by VP24 protease inhibitors. Viral yield in samples was assessed by qPCR (A) or plaque assay (B). Results are presented as the mean ± SEM.

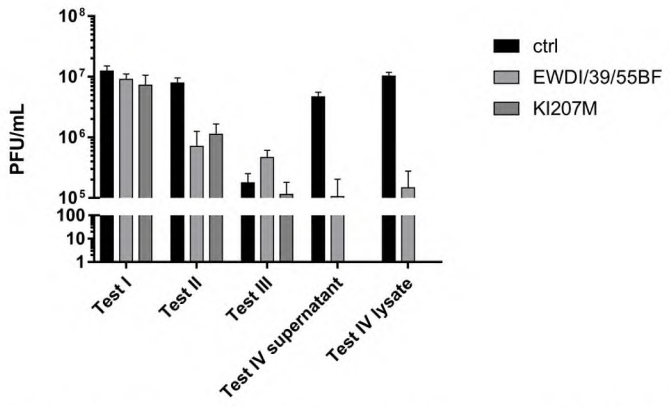

Fig. 5. Mechanism of antiviral activity. Functional tests involving the inhibitor presence (10 μM) at different stages of infection were performed. Samples were analyzed by plaque assay. Test I: inactivation of virion; test II: cell protection; test III: virus attachment; test IV: inhibition of virus replication, assembly, and release. Results are presented as the mean ± SEM.

depends on the inhibition of viral protease and, consequently, virus assembly and egress of infectious virus particles. These results further confirm the presumed mechanism of action.

3.4. Inhibition of infection spread

To visualize the effect of VP24 protease inhibitors on HSV-1 infection, confluent Vero E6 cells were infected with the virus at $TCID_{50}$ (50% tissue culture infectious dose) of 10 000/ml for 1 h, then cultured for 16 h in the presence of the inhibitors. The slides were then fixed and stained for HSV-1, f-actin, and nuclear DNA. Single focal planes of the acquired images are presented in Fig. 6.

Without inhibitors, large patches of infected cells with a plaque forming in the middle were noticeable. For EWDI/39/55BF a significant reduction of virus transmission between cells was observed, while in the presence of KI207M the spread was blocked, limiting the infection to a single cell.

3.5. Retention of HSV-1 major capsid protein inside cell nuclei

As the activity of VP24 protease is indispensable for the formation of mature HSV-1 capsids, and only mature capsids can leave the cell nucleus, disruption of the protease activity should cause accumulation of

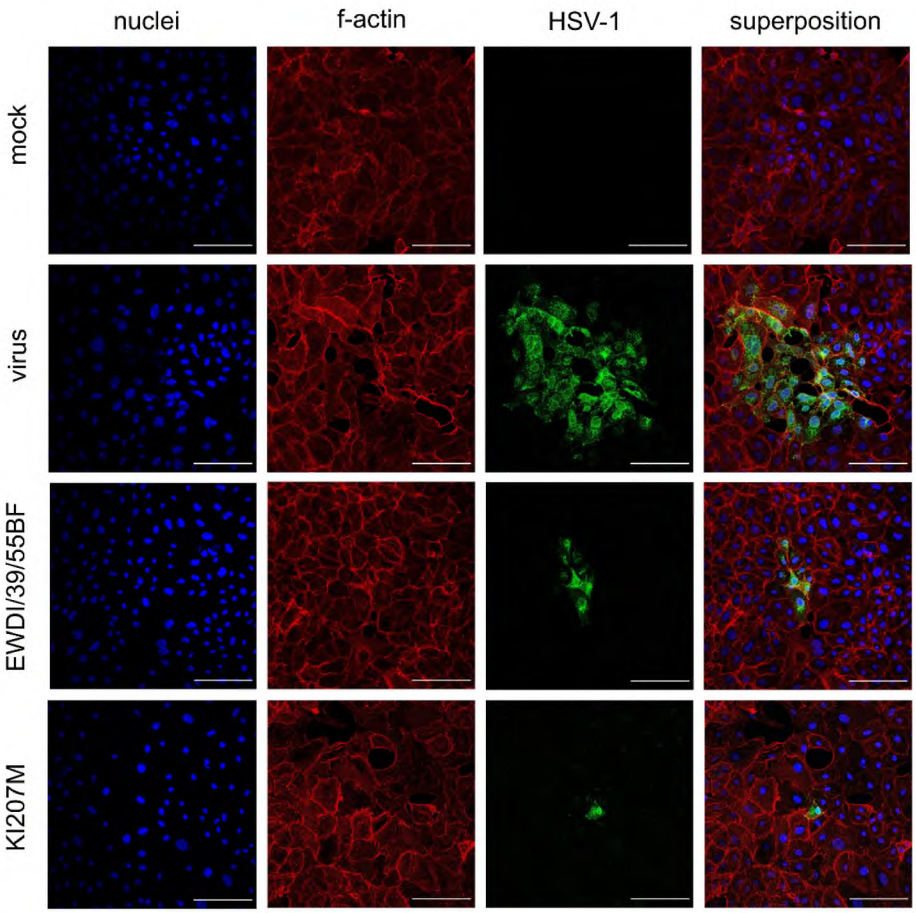

Fig. 6. Inhibition of HSV-1 infection spreading by protease inhibitors. Infected cells were treated with the inhibitors at 10 μM for 16h. Blue—DNA, red—f-actin, and green—HSV-1. Representative single focal planes are presented. Scale bar 100 μm.

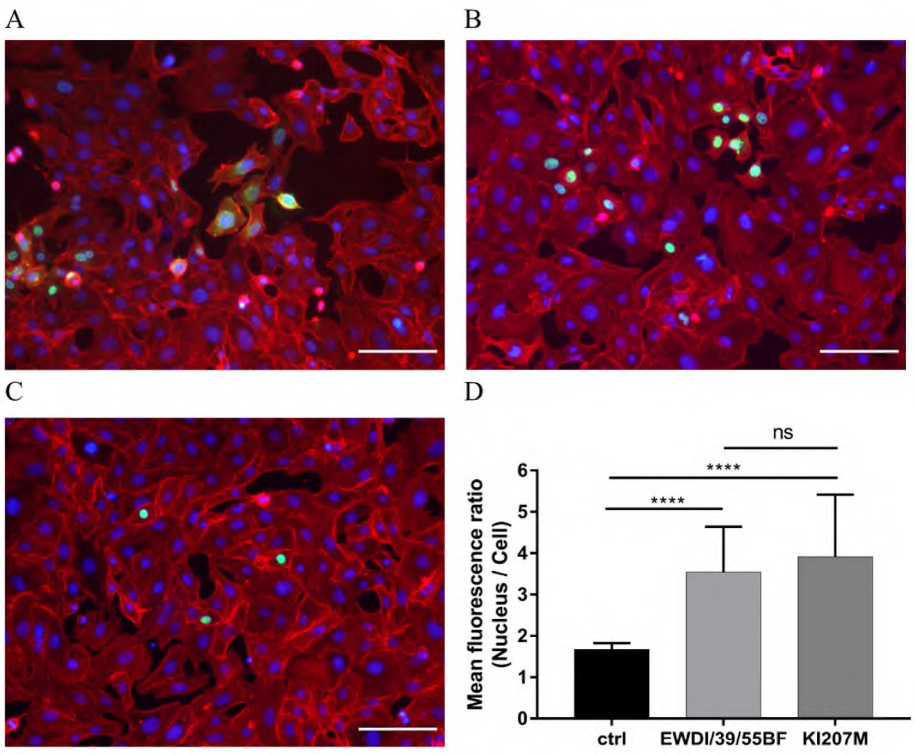

Fig. 7. Retention of VP5 inside cell nuclei. A, B, C – Vero E6 cells were infected with HSV-1 at $TCID_{50}$ = 10 000/ml for 16 h in the absence of inhibitors (A) and in the presence of EWDI/39/55BF (B) or KI207M (C) at 10 μM. Blue—DNA, red—F-actin, and green—VP5. Representative fluorescent images are presented. Scale bar 100 μm. D - mean fluorescence ratio was calculated from 50 different cells for each model, the experiment was repeated thrice with three technical repeats for each biological repeat.

the capsids inside the nucleus. To investigate this, Vero E6 cells were infected with HSV-1 at $TCID_{50}$ = 10 000/ml for 16 h in the presence of protease inhibitors. Then, slides were fixed and stained for major capsid protein VP5, f-actin, and nuclear DNA. Subsequently, the ratio of fluorescence intensity in the nucleus to the intensity in the whole cell area was determined. As indicated in Fig. 7, the viral capsids were retained in the nucleus in the cells treated with protease inhibitors, as compared to the untreated cells.

3.6. Antiviral activity against clinical strains of HSV-1

To assess the potential value of VP24 protease inhibitors in clinical applications, their antiviral activity was tested against six clinical strains of HSV-1. Three of the selected strains, 920, 452/01, and 2473/01, exhibited normal sensitivity to ACV, while the other three, RAT-2, MS-1, and MC showed significant resistance to this drug (Pachota et al., 2019). Results presented in Fig. 8 indicate, that protease inhibitors hamper the replication of all clinical HSV-1 strains.

3.7. In vivo toxicity and antiviral activity

Protease inhibitors were applied to the scarified skin in a hydrogel formulation at a concentration of 4 mM. The inhibitors were administered to the scarified skin twice daily for 4 days for toxicity studies. None of the tested inhibitors exhibited systemic or local toxicity during the observation. The results of *in vivo* toxicity studies are shown in Supplementary Material 3 – *In vivo* toxicity. For antiviral activity analysis, both inhibitors were administered in two settings: before and after HSV-1 infection, or only after the infection, in which case a placebo formulation was applied prior to the infection, then the treatment was repeated twice daily for 4 following days, and the animals were observed for 14 days in total. A commercially available ointment containing 5% (w/v) ACV was used as a positive control. The results are presented in Fig. 9. On day 9, all the infected animals receiving no treatment were dead. Animals receiving protease inhibitors before and after the infection were the last to develop disease symptoms. By the final day of the observation, 46.7% of mice treated with EWDI/39/55BF before and after the infection were still alive and did not develop any disease symptoms, compared to 66.7% survival achieved by treatment with ACV. Significant differences in health scores were observed between the negative control group (no treatment) and both groups receiving protease inhibitors before and after the infection. A statistically significant difference in survival was recorded between the negative control group and all the groups treated with protease inhibitors. Interestingly, there was no statistically significant difference between the groups treated with protease inhibitors and those treated with ACV.

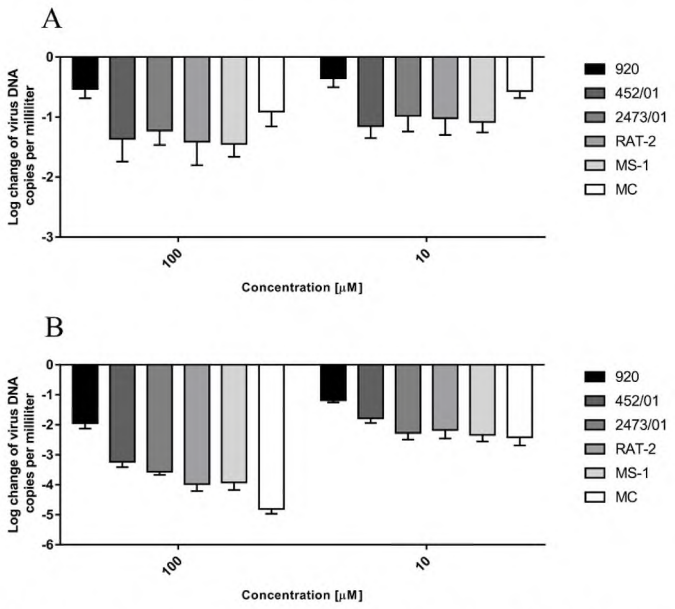

Fig. 8. Inhibition of clinical strains of HSV-1 by VP24 protease inhibitors. Replication assay (assay 0) was conducted for six clinical strains of HSV-1 in the presence of EWDI/39/55BF (A) and KI207M (B). Two days p. i. cell culture supernatants were collected, and viral yields were assessed by qPCR. Results are presented as the mean ± SEM.

4. Discussion

Although currently used nucleoside analogs are sufficient to control herpes simplex type 1 infections in most cases, there is a need for novel therapeutics as resistant mutants emerge, and in some cases, conventional drugs display limited effectiveness. This is especially dangerous for patients progressing to more systemic infections, including encephalitis, which is often fatal and almost invariably has severe and unmanageable sequelae (Akahoshi et al., 2017; Harris and Holmes, 2017; Jiang et al., 2016; van Velzen et al., 2013). The only other class of antiherpetic drug candidates currently undergoing clinical trials are helicase-primase inhibitors: pritelivir and amenamevir (Katsumata et al., 2018; Kawashima et al., 2017; Wald et al., 2016). Both have been proven to significantly increase animal survival in *in vivo* models in doses as low as 0.3 mg/kg (Katsumata et al., 2011; Quenelle et al., 2018). N-docosanol, a viral entry inhibitor (IC_{50} ~1300 μM), is available as on over-the-counter cream for the treatment of oral HSV outbreaks. (Pope et al., 1998). Some recently proposed HSV-1 inhibitors targeting various stages of infection are presented in Table 1. Other methods of HSV infection management have been suggested, including CRISPR/-Cas9 (van Diemen et al., 2016), but these are far from the clinic yet. Several attempts have been made to develop an HSV vaccine, but no effective vaccine candidate has been identified thus far (Bernstein et al., 2019; Cohen, 2017; Johnston et al., 2016).

From the series of 2-azabicyclo[3.2.1]octane derivatives only compound with N,N-dimethylnaphtalen-1-amine substituents displayed antiviral activity. Interestingly similar compounds with 2-azabicyclo [2.2.1]heptane did not show any activity in a cell-based assay. Moreover, compounds with 2-azabicyclo[3.2.1]octane moiety with substituents other than N,N-dimethylnaphtalen-1-amine did not show antiviral activity. This suggests the importance of the two molecule scaffold of the inhibitor: 2-azabicyclo[3.2.1]octane and N,N-dimethylnaphtalen-1-amine.

Among the compounds with hexahydroquinoxalin-2(1H)-one moiety only derivative with aminophosphonate group showed inhibition of HSV-1 protease as well as antiviral activity in cell-based studies. One possible explanation for the exclusive activity of KI207M is that the aminophosphonate moiety creates an irreversible bond with catalytic serine residue and reacts with the protease in a way similar to the diaryl esters of α-aminophosphonates, well-known inhibitors of serine proteases. The extensive phosphonate moiety of KI207M could activate the phosphorous atom, making it susceptible to attack by catalytic serine residue of the protease.

The two most promising compounds were proven to inhibit the activity of the enzyme as well as the replication of HSV-1 in cell culture at low micromolar range while being well-tolerated by cells even at high concentrations. The inhibitors blocked the virus's spread between cells, effectively limiting the infection. It is, however, worth mentioning that one of the selected compounds, EWDI/39/55BF, was significantly less effective against the clinical strains of HSV-1 compared to the laboratory strain, though the difference was not dependent on the strains' susceptibility to ACV. Most importantly, VP24 protease inhibitors were able to protect animals in a lethal HSV-1 challenge. Also, no signs of systemic or local toxicity were observed in the animal model. Further animal studies would be necessary to assess the compounds' suitability for oral delivery and, thus, their potential against systemic HSV-1 infection. Described

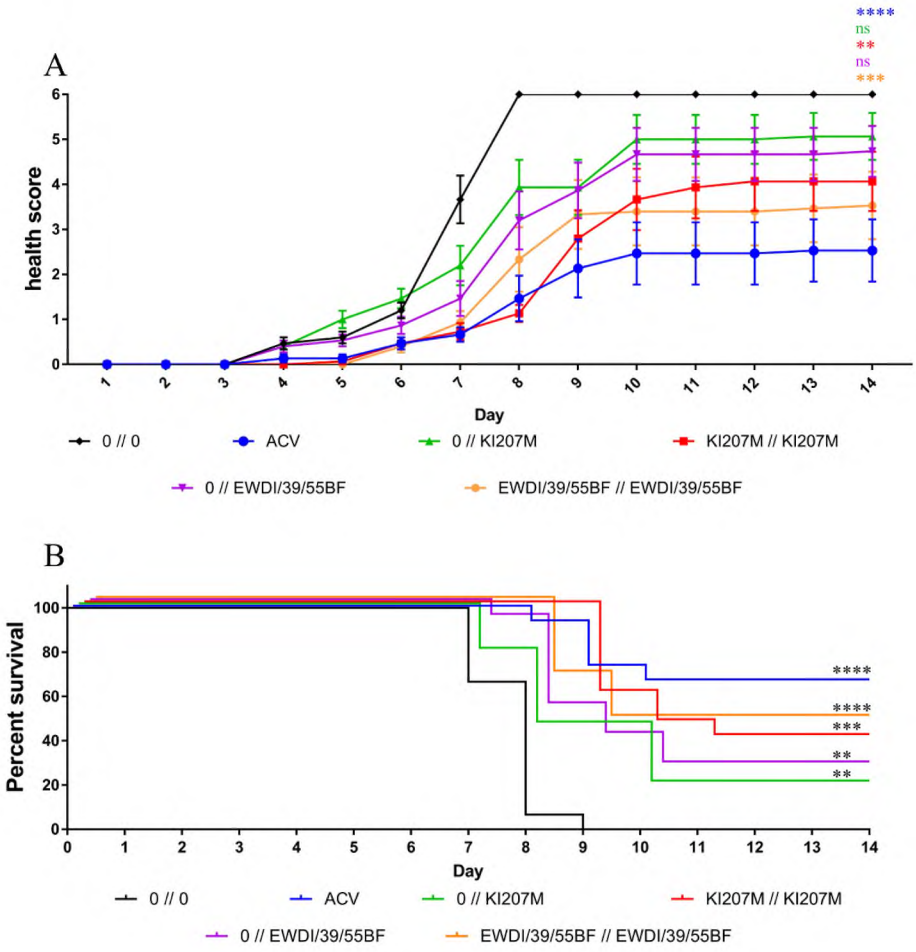

Fig. 9. In vivo antiviral activity of VP24 protease inhibitors. Mice were infected with HSV-1 through dermal scarification and treated with protease inhibitors or ACV every 12 h or no treatment for 4 days, then observed for 10 following days. Assigned groups were: 0 – no treatment, ACV – acyclovir treatment, 0//KI207M – KI207M only after infection, KI207M//KI207M – KI207M before and after infection, 0//EWDI/39/55BF - EWDI/39/55BF only after infection, EWDI/39/55BF//EWDI/39/55BF - EWDI/39/55BF before and after infection. A - health was assessed using the following score: 0—no signs; 1—formation of blisters; 2—slight ulceration; 3—large patches of ulceration; 4—a confluent band of ulceration; 5—hind limb paralysis; 6—death; B – Survival curve. Survival is given as a percent of animals alive per group (n = 15).

Table 1
Selected HSV-1 inhibitors.

Name	Target	IC_{50}	Reference
IM-250	Helicase-primase	20 nM	Uhlig et al. (2021)
PEG_{45}-b-$PMAPTAC_{52}$	Cellular HSV-1 receptors	360 nM	Pachota et al. (2019)
nelfinavir	Envelope proteins	5 μM	Gantt et al. (2015)
MBZM-N-IBT	Unknown/multiple	3.62 μM	Kumar et al. (2021)
AT-533	UL42-Hsp90 complex	50 nM	Qin et al. (2021)
Compound A	ICP0	9.6 μM	Deschamps et al. (2019)
Nootkatin	Nucleotidyltransferase family enzymes	240 nM	Tavis et al. (2014)

compounds constitute a novel class of antiherpesviral drugs that may offer protection in monotherapy, especially if strains resistant to the current treatments become more frequent. However, these may also increase the potency of the therapy and delay the emergence of resistance if used in combination therapy.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The described inhibitors are covered by granted patents PL237651B1 and PL237652B1 and other related patent applications worldwide. The patents are owned by Wroclaw University of Science and Technology and Jagiellonian University.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2023.105604.

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