https://doi.org/10.31925/farmacia.2022.6.14 **ORIGINAL ARTICLE**

INFECTIVITY AND REPLICATION INHIBITION EFFECT OF 5-FLUOROURACIL ON HERPES SIMPLEX VIRUS TYPE-1 ASSOCIATED WITH MUTATIONS IN THYMIDINE KINASE GENE

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Abstract

Two pandemics over the first two decades of the 21st century have shown that viruses with epidemic and pandemic potentials constitute a major threat to human health due to the lack of effective antivirals. Developing new antivirals and treatment strategies is also as important as vaccines play a crucial role in the first line of preventing viral infections. Using base analogues that directly target viruses and understanding their action mechanisms can present essential alternative treatment strategies to combat viral diseases. The current study investigated the effects of a base analogue on herpes simplex virus type-1 (HSV-1) replication in a cell culture system using the pyrimidine analogue 5-fluorouracil (FU). After that, the full-length UL-23 gene encoding thymidine kinase (TK) of HSV-1 was sequenced to detect induced mutations. The results showed a diminishing viral titre and viral load at 2 logs and 663 times, respectively, at the end of 10 consecutive passages with 5-FU. Furthermore, two mutations substitute amino acids in the non-conserved region of the TK gene, which confers drug resistance, were also identified. The current research is a feasibility study to investigate the antiviral effects of 5-FU on DNA viruses and has reinforced the fact that 5-FU can have an antiviral effect on HSV-1. However, drug resistance for viruses should not be underestimated.

Rezumat

Pandemiile din primele decenii ale secolului XXI au arătat că virusurile cu potențial epidemic și pandemic constituie o amenințare majoră pentru sănătatea umană din cauza lipsei de medicamente antivirale eficiente. Dezvoltarea de noi antivirale și de noi strategii de tratament este la fel de importantă ca și vaccinurile care joacă un rol important în prima linie de prevenție a infecțiilor virale. Utilizarea analogilor de baze care țintesc direct virusurile și înțelegerea mecanismelor de acțiune ale acestora pot reprezenta strategii de tratament alternative esențiale pentru combaterea bolilor virale. Studiul de față a investigat efectele unui analog de bază asupra replicării virusului herpes simplex de tip 1 (HSV-1) într-un sistem de cultură celular, și anume analogul pirimidinic 5-fluorouracil (FU). Ulterior, a fost secvențiată gena UL-23 de lungime completă care codifică timidin kinaza (TK) a HSV-1 pentru a detecta mutațiile induse. Rezultatele au arătat o scădere a titrului viral și a încărcăturii virale de 2 log și, respectiv, de 663 de ori, la sfârșitul a 10 treceri consecutive cu 5-FU. În plus, au fost identificate și două mutații care înlocuiesc aminoacizii din regiunea neconservată a genei TK, ce conferă rezistență la medicamente.

Keywords: 5-fluorouracil, antiviral, Herpes simplex virus, thymidine kinase, UL-23

Introduction

Herpes simplex virus type-1 (HSV-1) is a member of the genus *Simplex virus*, classified under subfamily *α-herpesviriniae* and within the family *Herpesviridae*, that is commonly found and is endemic worldwide [1]. The two phases of the HSV-1 life cycle alternate and are known as "latent" and "lytic" [2]. The clinical manifestations of HSV-1 infection oscillate from mild to severe symptoms, resulting in keratitis, corneal blindness and encephalitis, in addition to recurrent oral and peroral lesions [3].

HSV-1 infection may be severe in immunocompromised patients having deficiencies in cell-mediated immunity. The standard treatment protocol of HSV-1 is based widely on using acyclovir (ACV) and its derivatives [4, 5]. However, long-term treatment with ACV and

its derivatives can lead to drug resistance, especially in immunocompromised patients who are diagnosed with severe HSV-1 infections [3]. Thymidine kinase (TK) of HSV-1, which is encoded by the UL-23 gene, is a viral enzyme composed of 376 amino acids having six active or highly conserved gene regions in different positions. HSV-1 TK plays a vital role in virus characterization as it influences acute and latent HSV-1 infection, particularly the reactivation of virus latency [6]. Mutations in these critical regions of the UL-23 gene can cause drug resistance [7], which is an important concern not only for HSV-1 infections but also other viral infections.

Throughout history, most viruses that have epidemic or pandemic potential, such as influenza, HIV, small-pox, Ebola, Zika, MERS-CoV and SARS-CoV, were considered a threat to human health due to the absence of efficient treatments; which resulted in the continuity of those viral outbreaks. In a public health emergency, vaccine development plays a crucial role in controlling, protecting and preventing the surge of epidemics or pandemics. However, concerns about the production, efficacy and side effects of new vaccines can affect their approval and even halt progress in defeating the pathogens [8]. In terms of public health, developing broad-spectrum antiviral molecules and new treatment strategies are at least as necessary as vaccine development to successfully combat viral diseases.

Antivirals target either the host-cell equipment or the viruses [8, 9]. Base analogues with modified heterocyclic fragments are mutagenic chemicals that work against viruses because they substitute RNA or DNA bases causing altered base pairings and structural changes in ways that obstruct DNA replication or transcription [10]. Base analogues can also show antiviral effects by inhibiting DNA synthesis [11]. 5-fluorouracil (5-FU), currently used to treat some cancers as a thymine base analogue, is one of the mutagens that blocks thymidylate synthases, which are key enzymes in thymine synthesis, and thereby inhibit DNA synthesis [11]. Thus this drug can suppress or inhibit the replication of DNA viruses in host cells [12]. Additionally, 5-FU, with its chemical structure $C_4H_3FN_2O_2$, can be transformed by the uracil transport system in the targeted cells into final active metabolites; those mainly are fluorouridine triphosphate (FUTP), fluoro-deoxyuridine triphosphate (FdUTP) and fluorodeoxyuridine monophosphate (FdUMP), which may alter RNA function and processing, induce DNA damage or suppresses the function of thymidylate synthase (TS) in the ternary complex, all this lead to a profound effect on RNA and DNA, triggering cell death in virally infected or tumour cells [13].

In the current study, we aimed to investigate the effects of base analogue 5-FU on HSV-1 replication by implementing consecutive passages in Vero cell culture in its presence. Then, we partially sequenced the TK gene to identify changes that could confer viral resistance.

Materials and Methods

Cell line, viruses and mutagen

The African Green Monkey (Vero) cell line and the KOS strain of HSV-1 employed in this study were obtained from stock collections of the virology department in the Veterinary Medicine Faculty of Ondokuz Mayis University, Turkey. Vero cells were cultivated in Dulbecco's Minimal Essential Medium (DMEM, Sigma, UK) supplemented with 10% foetal calf sera (FCS, Gibco, UK) and 1% antibiotic solution consisting of penicillin and streptomycin (Sigma-Aldrich, USA).

The KOS strain of HSV-1 was produced in freshly prepared Vero cells by using DMEM supplemented with 1% FCS and stored at -80ºC until use. 5-fluoro-uracil (2,4-dihydroxy-5-fluoropyrimidine) ($C_4H_3FN_2O_2$) (Sigma-Aldrich F6627) was commercially purchased, dissolved in dimethylsulfoxide (DMSO) at 384 mM, and stored at -20ºC until use as 1 mL aliquots.

Cell viability assay

An *in vitro* viability assay used the trypan blue exclusion staining method to determine the minimally toxic drug concentration for cells. Briefly, 3.5×10^5 Vero cells were seeded in a 12-well plate at 24 hours and checked to ensure they reached 85% monolayer status on the day of the experiment. The different changing concentrations of 25, 50, 100, 200, 400, 600, 800 and 1000 µM were prepared from stocked 5-FU in DMEM. The medium was removed from wells and replaced in duplicate using 500 µL of each drug concentration. The plates were incubated at 37ºC for 24 hours in a humidified incubator with 5% CO_2. After that, each 5-FU concentration was removed from corresponding wells. 100 µL of 0.25% trypsin were added to each well to detach cells and incubated for 1 minute at 37ºC. The detached cells were collected and gently pelleted using low-speed centrifugation. Pelleted cells were resuspended in 20 µL of DMEM. Following, 10 µL of resuspended cells were mixed with an equal volume of 0.4% trypan blue, loaded in cell counting slides and then read using an automated cell counter (TC20, Bio-Rad, UK). The viability of cells for each 5-FU concentration was found as percentages based on the rate of live cells to total cells. The cell viability assay was repeated three times on different days, and the mean of these three repeats was obtained to determine the cytotoxicity effect of the drug.

Drug treatment and 50% tissue culture infective dose ($TCID_{50}$) assay

To demonstrate the antiviral effect of 5-FU on HSV-1, we synchronously carried out 10 consecutive passages with HSV-1 under 5-FU pressure (▲HSV-1) and without 5-FU pressure (ΔHSV-1) in different

plates. Briefly, two 12-well plates were coated with 350,000 Vero cells *per* well and incubated at 37ºC for 24 hours. Optimal non-toxic drug concentrations by diluting in DMEM were also freshly prepared on the day of viral inoculation. In one of the plates, the medium in all plate wells was aspirated. Then cells were treated with 1000 µL of optimal non-toxic drug concentration and left at 37ºC for 2 hours for drug uptake; then medium containing the drug was removed from all wells. After that, cells were infected with 100 µL 3.14 x 10^5/mL $TCID_{50}$ starting titre of the virus and incubated at 37ºC for 2 hours, and then inoculum was removed from the cells after viral incubation. The virus was placed under pressure by adding a medium containing optimal non-toxic drug concentration to all wells at 37ºC in humidified 5% CO_2 for 72 hours with daily checking. As a positive control, the virus without the drug, with the same starting titre was also passed in the growth medium in another plate *via* the same procedure and conditions above.

At the end of each passage, we carried out an infectivity assay using the 50% tissue culture infective dose ($TCID_{50}$) method to compare the infectivity of ▲HSV-1 and ΔHSV-1. For this purpose, we first added 100 µL of DMEM supplemented with 2% FCS to every well of a 96-well plate. 11 µL of both viruses, under drug pressure and control viruses, were added to the top wells in quadruplicate, followed by a ten-fold dilution descending to the end of the plate. After the dilution, 50 µL of Vero cells were added to each well, resulting in 3 x 10^4 cells/well. The plates were incubated for 72 hours at 37°C before reading cytopathogenic effect (CPE) and calculating the titre according to the method of Reed and Muench [14]. Titres were expressed as $\log_{10}$ $TCID_{50}$/mL.

Nucleic acid extraction and polymerase chain reaction (PCR) assays

Viral DNA was extracted from HSV-1-infected cells by using the PureLink Genomic DNA Mini Kit (Invitrogen, Cat No: K182002), according to the instruction manual. To confirm the presence of HSV-1 grown in Vero cells, real-time (qPCR) described by Namvar *et al*. was performed using the BioRad CFX Connect device [15]. Briefly, the iTaq Universal Probes One-Step Kit (BioRad, Cat No: 1725141) was used for the qPCR reaction. In each sample, 12.5 µL 2× iTaq buffer, 320 µM forward primer (5'-GCAGTTTACGTACAACCACATACAGC-3'), 320 µM reverse primer (5'-AGCTTGCGGGCCTCGTT-3'), 160 µM probe (5'FAM- CGGCCCAACATATCGT TGACATGGC-TAM RA-3') and 5 µL samples were mixed. The temperature conditions for amplification were as follows: 3 minutes pre-denaturation at 95°C, 7 seconds at 95°C, 10 seconds at 60°C after 40 repeat cycles, and cooling at 12°C [15].

Cloning

HSV-1 glycoprotein B (gB) was subcloned to pGEM-T easy vector to establish standard samples for the absolute quantification of HSV-1 DNA copies in the infected Vero cells. The iTaq Universal Probes One-Step Kit (BioRad, Cat No: 1725141) was used in the PCR reaction for the gB of HSV-1 with forward (5'-GCAGTTTACGTACAACCACATACAGC-3') and reverse primers (5'-AGCTTGCGGGCCTCGTT-3'), given as above. For this purpose, a 25 µL mixture consisting of 12.5 µL 2× iTaq buffer, 320 µM of each primer and 5 µL template DNA was prepared, and then the PCR reaction profile was conducted as follows: a cycle for 3 minutes at 95°C; 40 cycles for 7 seconds at 94°C, 10 seconds at 55°C. 10 µL DNA product was run on 1% agarose gel containing 0.5 µg/mL ethidium bromide in 20 mM acetic acid and 1 mM EDTA buffer for 50 minutes. The products were evaluated under UV light. The 117 base pairs of gB gene amplicon obtained from PCR were cloned into the pGEM-T easy vector system (Promega, Cat No: A1380) employing the T4 DNA ligase *via* blunt-end ligation. JM109 *E. coli* cells were used for transformation. The cloning and transformation were completed according to the manufacturer's manual. Briefly, a 10 µL ligation mixture was prepared as 5 µL 2× rapid ligation buffer (400 mM Tris-HCl, 100 mM $MgCl_2$), 2 µL T4 DNA ligase (3 Weiss U/µL), 100 ng purified HSV-1 gB amplicon, 50 ng pGEM-T Easy plasmid. The mixture was incubated overnight at 4°C. For the transformation process, 50 µL of JM109 competent *E. coli* (1 × 10^8 CFU/µg DNA) was carefully pipetted into a 10 µL volume of ligation mixture and incubated for 30 minutes on ice. The cells were shocked at 42°C for 50 seconds, then placed again on ice and incubated for 2 minutes. 150 µL of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) at room temperature was added into the mixture and incubated at 37°C at 150 rpm in a shaking incubator for 90 minutes. The broth was spread on an LB agar (10 g Bacto tryptone, 5 g Bacto-yeast, 10 g NaCl, 15 g agar and 100 µg/mL ampicillin) plate and incubated at 37°C overnight. White colonies containing gB insert were picked and cultivated in 2× YT liquid broth (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl and 100 µg/mL ampicillin) for 16 hours. Plasmids were extracted from 2× YT liquid broth using a GeneJET plasmid miniprep kit (Thermo, Cat No: K0503) and then stored at -20°C.

Calculation of viral loads

To calculate the viral loads, the level of the gB gene, which was inserted into the pGEM-T easy vector, was measured using a spectrophotometer. The number of viral copies *per* µL was calculated based on results using the formula below. The plasmid was reconstituted ten times in $\log_{10}$ dilutions. These

dilutions were used as the standard in qPCR to calculate the viral load in Vero cells.

$$\text{Viral copy (1 µL)} = \frac{\text{Amount}\left(\frac{\text{ng}}{\text{uL}}\right) \text{x } 6.022 \text{ x } 10^{23}}{[\text{Plasmid+insert (bp)}] \text{ x } 1 \text{ x } 10^{9} \text{ x } 650}$$

DNA was extracted from the cell culture then qPCR was performed using the aforementioned kits, device and probes with the same reaction conditions.

PCR for UL-23 gene of HSV-1 and sequencing

We did a PCR assay from 1, 3, 5, 7 and 10th passages of both ▲HSV-1 and ΔHSV-1 for the UL-23 gene of HSV-1 encoding thymidine kinase protein using forward (5'-TCCACTTCGCATATTAAGGT-3') and reverse primers (5'CTGTCTTTTTATTGCCGTCA-3') as previously described [16]. PCR was performed in 50 µL reaction volume consisting of 5 µL 10x Taq Buffer, 1 µL Taq polymerase, 5 µL of template DNA, 1 µL dNTP (10 mM), 2 µL of each primer, 5 µL of $MgCL_2$ (25 mM) and 29 µL RNAse free water. PCR amplification conditions included a denaturation stage of 10 minutes at 95ºC followed by 60 cycles of 1 minute at 95ºC, 1 minute at 50ºC and 1 minute at 72ºC with an extension stage of 10 minutes at 72ºC. After PCR amplification, 10 µL of PCR product were loaded on a 1.5% agarose gel stained with ethidium bromide and then visualized using Quantum gel imaging and documentation system (Vilber, Frankfurt, Germany) after running on 100 V for 40 minutes.

Amplified PCR products of selected passages were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and were commercially sequenced by BMlabosis (Ankara, Turkey, www.bmlabosis.com). All sequences obtained from selected passages were submitted to GenBank. The accession numbers, which correspond to 1, 3, 5, 7 and 10th passages, respectively, were OK180984, OK274284, OK274286, OK274288 and OK274290 for ▲HSV-1, as well as OK180985, OK274285, OK274287, OK274289 and OK274291 for ΔHSV-1.

Statistical analysis

One-way analysis of variance (one-way ANOVA) evaluated the difference between the means of cell viability percentages. Tukey's multiple comparison test evaluated the percentages of cell viability in different concentrations. The difference between viral titres and viral load values of sequential 10 passages under mutagen pressure and without mutagen pressure were analysed using the student t-test. GraphPad Prism software version 5.0 (GraphPad 5.0 software, USA) was used for all statistical analysis, and a $p < 0.05$ value was considered significant.

Results and Discussion

Determination of a non-cytotoxic dose for 5-FU on cultured cells

The trypan blue exclusion method in the Vero cell line was performed in duplicate and repeated three times by obtaining the relative values to find the non-cytotoxic dose of 5FU. The mean cell viability percentage for cell control was calculated as 97.16% ± 0.44. The percentages of cell viabilities in different concentrations between 25 and 1000 diminished from 95.0% to 57.23%. When the ratio of cell control was compared with one of the lower concentrations of 5-FU, such as 50 µM, the cell viability was 89.73% ± 0.36. On the other hand, the ratio was 57.23% ± 10.34 for 1000 µM and considered the highest concentration of 5-FU used for the current study. The moderate cellular viability of 81.66% ± 2.76 was found for 700 µM concentration and evaluated as a non-cytotoxic dose of 5-FU.

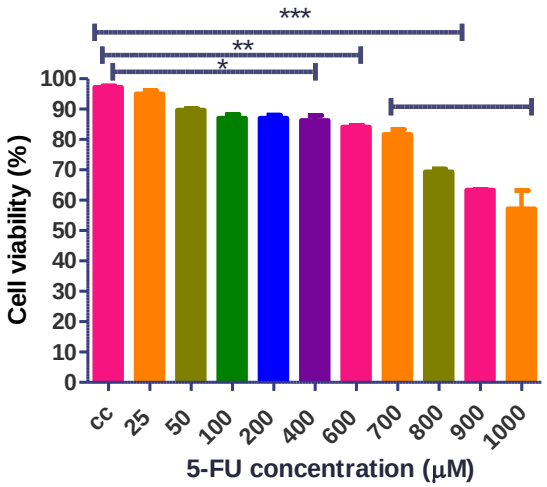

Figure 1.

Effect of 5-FU on Vero cells. Cytotoxicity was determined using a Trypan-blue exclusion assay after treatment with 5-FU for 24 h. Percentages of cell viability are indicated on the Y-axis according to drug concentrations on the X-axis. The drug concentration moderately affecting the cells was 700 µM: the ex, a non-cytotoxic dose.

CC: cell control, *p < 0.01, **p < 0.001; ***p < 0.0001

As seen in Figure 1, the reduction of cell viability depending on the different concentrations of mutagen was statistically significant compared with cell control ($p < 0.01$ and $p < 0.0001$).

50% tissue culture infective dose ($TCID_{50}$) assay

To evaluate whether 5-FU has an antiviral effect on HSV-1, we simultaneously performed 10 consecutive passages in Vero cells in the presence of 700 µM 5-FU (▲HSV-1) and absence of 5-FU (ΔHSV-1) (Figure 2).

At the end of each passage, we checked the viral titres using a standard microtitration test. Titration results indicated that there was selective pressure of 5-FU that inhibited virus infectivity as of the 1st passage, leading to a little more than 1 $\log_{10}$ reduction in the titre of ▲HSV-1 (Figure 3A). In this context, we calculated $TCID_{50}$ values of 1st passages for

▲HSV-1 and ΔHSV-1 as 4.64 x 10^4/mL and 1.0 x 10^6/mL, respectively. These values were also found at the end of the 10^{th} passage as 3.16 x 10^5/mL and 1.70 x 10^7/mL, respectively. When we compared the $TCID_{50}$/mL values of ▲HSV-1 and ΔHSV-1 statistically, there was a significant difference (p = 0.0497).

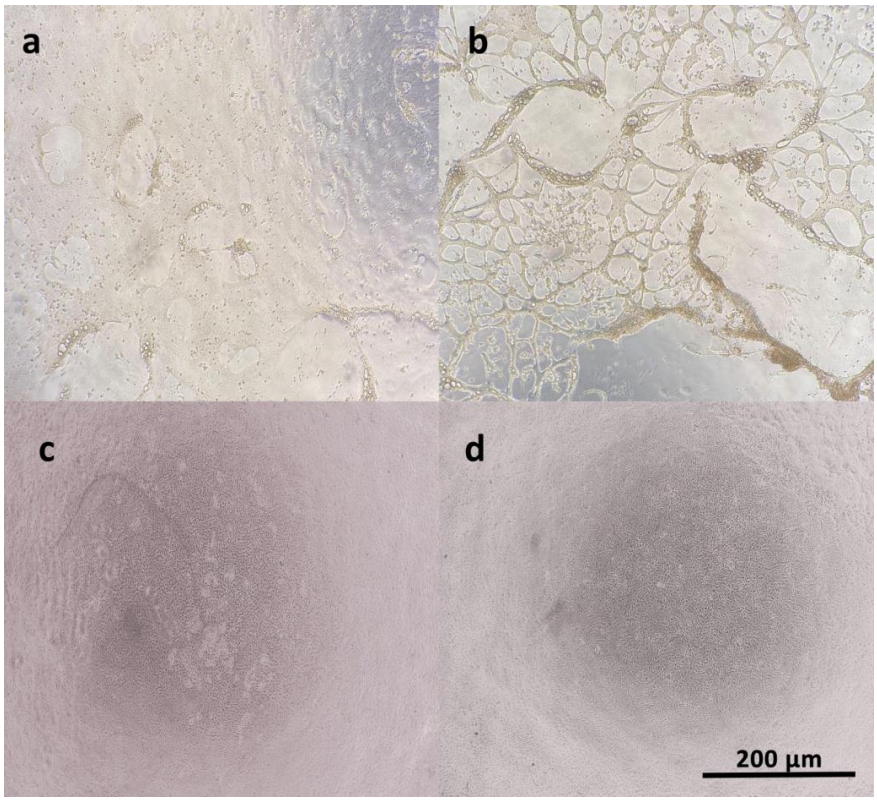

Figure 2.

Effects of 5-FU on HSV-1 growing in Vero cells. **a** and **b:** 10^{th} passage for ▲HSV-1 and ΔHSV-1, **c** and **d:** cell controls with and without 5-FU, respectively. ▲HSV-1 refers to virus under drug (5-FU) pressure, while ΔHSV-1 refers to virus without drug pressure.

*Phase contrast inverted microscopy was used for visualization

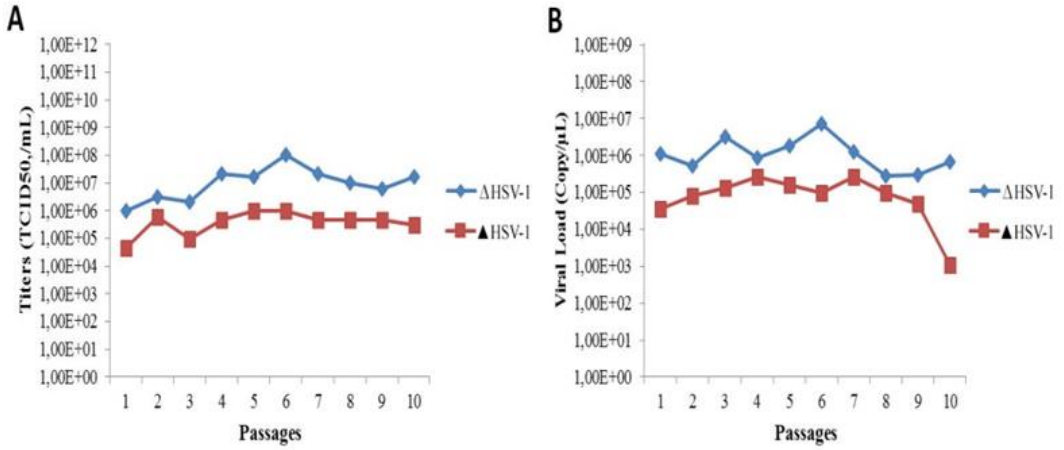

Figure 3.

Viral titres and loads with and without drug pressure. **A:** TCID50 values of ▲HSV-1 and ΔHSV-1. **B:** Viral loads *per* µL of ▲HSV-1 and ΔHSV-1. ▲HSV-1 refers to virus under drug (5-FU) pressure, while ΔHSV-1 refers to virus without drug pressure. The viral titres of ▲HSV-1 and ΔHSV-1, when statistically compared, were significantly different (p = 0.0497); also, viral load comparison for both ▲HSV-1 and ΔHSV-1 was statistically significant (p = 0.0292).

Determination of viral loads by qPCR

Real-time PCR (qPCR) was performed to calculate the viral loads from the cell lysate of each consecutive passages of ▲HSV-1 and ΔHSV-1. As seen in Figure 3B, the viral load for the 1^{st} passage of ΔHSV-1 drug was 1.13 x 10^6 copies/µL, whereas it was 3.66 x 10^4

copies/µL for ▲HSV-1. In the 10th passage, these values were 6.63 x 10^5 copies/µL for ΔHSV-1 and 1.06 x 10^3 copies/µL for ▲HSV-1. These results indicated that 5-FU suppressed HSV-1 by reducing the number of virus particles 625 times *per* micro-liter. The viral load comparison for both ▲HSV-1 and ΔHSV-1 was statistically significant (p = 0.0292). We also compared viral titre and viral loads of all passages for ▲HSV-1 and ΔHSV-1. There was also a significant marginal difference in the comparison of titre and load of ▲HSV-1 (p = 0.05), while comparing titre and load of ΔHSV-1 (p > 0.05) revealed a non-significant statistical difference.

UL-23 PCR assay and sequencing

We carried out conventional PCR to 1, 3, 5, 7 and 10 passages of ▲HSV-1 and ΔHSV-1, observing 1,335 base pair expected product in all selected passages. Following partial sequencing, we compared TK genes of 10 HSV-1 isolates recorded in GenBank and 10th passage isolates TK genes of the current study to investigate the natural polymorphism (Table I). There were > 97% nucleotide similarities among them. On the other hand, comparison of the 1st passage of both ▲HSV-1 and ΔHSV-1 indicated 100% nucleotide identity. This rate was found for a comparison of 10th passages of both as 99.80%.

Table I

Percentages of nucleotide and amino acid similarities between TK genes of our ▲HSV-1 and ΔHSV-1 10th passage isolates and TK genes of other HSV-1 strains obtained from Genbank

	GenBank Number	Strain Name	Amino Acid Similarities (%)											
			NC_001806	KT899744	KT425107	KM222725	KF498959	JN420342	HM585514	HM585508	GU734772	AB618031	OK274291	OK274290
Nucleotide Similarities (%)	NC_001806	17		99.40	99.10	99.40	99.10	99.40	99.40	99.10	99.10	99.40	99.40	98.79
	KT899744	KOS	99.30		99.10	100.00	99.70	100.00	100.00	99.70	99.70	99.40	100.00	99.40
	KT425107	Mckrae	99.20	99.30		99.10	99.40	99.10	99.10	98.80	99.40	99.10	99.10	98.49
	KM222725	F	99.20	99.70	99.20		99.70	100.00	100.00	99.70	99.70	99.40	100.00	99.40
	KF498959	RE	99.10	99.60	99.50	99.70		99.70	99.70	99.40	100.00	99.10	99.70	99.10
	JN420342	OD4	99.40	99.90	99.20	99.80	99.70		100.00	99.70	99.70	99.40	100.00	99.40
	HM585514	R11	99.40	99.90	99.20	99.80	99.70	100.00		99.70	99.70	99.40	100.00	99.40
	HM585508	CR38	99.30	99.80	99.10	99.70	99.60	99.90	99.90		99.40	99.10	99.70	99.10
	GU734772	H129	99.10	99.60	99.50	99.70	100.00	99.70	99.70	99.60		99.10	99.70	99.10
	AB618031	RH2	99.30	99.40	99.30	99.70	99.40	99.50	99.50	99.40	99.40		99.40	98.80
	OK274291	ΔHSV-1	99.40	99.90	99.20	99.80	99.70	100.00	100.00	99.90	99.70	99.50		99.40
	OK274290	▲HSV-1	99.20	99.70	99.00	99.60	99.50	99.80	99.80	99.70	99.50	99.30	99.80	

▲HSV-1 and ΔHSV-1 indicate the virus under 5-FU pressure and without 5-FU pressure, respectively

Table II

Comparison between TK genes of the HSV-1 reference strain (NC_001806) and our 10 HSV-1 isolates representing 1, 3, 5, 7 and 10th passages of ▲HSV-1 and ΔHSV-1

Passage no		GenBank no	Nt position[a]	Aa residue[b]	Nt position	Aa residue
			47257	**183**	**47305**	**167**
1	ΔHSV-1	OK180985	T	Thr	C	Ala
	▲HSV-1	OK180984	T	Thr	C	Ala
3	ΔHSV-1	OK274285	T	Thr	C	Ala
	▲HSV-1	OK274284	T	Thr	C	Ala
5	ΔHSV-1	OK274287	T	Thr	C	Ala
	▲HSV-1	OK274286	T	Thr	C	Ala
7	ΔHSV-1	OK274289	T	Thr	C	Ala
	▲HSV-1	OK274288	T	Thr	C	Ala
10	ΔHSV-1	OK274291	T	Thr	C	Ala
	▲HSV-1	OK274290	**G**	**Pro**	**T**	**Thr**

[a]positions refer to the nucleotide sequence of NCBI reference virus (Accession no. NC_001806); [b]residues refer to the amino acid position of TK encoded by the reverse complement of UL23 gene; ▲HSV-1 and ΔHSV-1 indicate virus under 5-FU pressure and without 5-FU pressure, respectively

Furthermore, selected passages of ▲HSV-1 and ΔHSV-1 were compared with the reference strain of HSV-1 with accession number NC_001806. We could not find any nucleotide changes between 1, 3, 5 and 7th passages of ▲HSV-1 and ΔHSV-1, but found two mutations in the 10th passage of ▲HSV-1. Substitutions in 10th passage UL-23 of ▲HSV-1 were identified at amino acid 167 and 183, changing alanine to threonine and threonine to proline, respectively, when compared with reference strain NC_001806 (Table II), while none of these two amino acid substitutions were observed in the 10th

passage UL-23 of ΔHSV-1. To the best of our knowledge, this sequence of ▲HSV-1 UL-23, was also the first sequence submitted to GenBank that included mutations. Because we screened 1,091 sequences of HSV-1 UL-23 gene recorded in GenBank, and none of them contained mutations.

Research to develop novel agents and drugs to treat viral infections continues, although it is costly. Arguments persist that developing drugs against emerging viruses is unfeasible because these pathogens can mutate over time, and antivirals and other treatments become less effective or even completely ineffective as resistance arises and evolves [10]. On the other hand, nucleoside and base analogues are thought to be an alternative antiviral strategy. Base analogues are molecules that can substitute for normal bases in nucleic acids and cause structural changes that affect DNA replication and RNA transcription. 5-FU is a thymine analogue that inhibits thymidylate synthase, an essential enzyme in thymine synthesis. It is used to treat some cancers by inhibiting DNA synthesis [11].

In addition to 5-FU, base analogues include ribavirin (guanosine analogue) and 5-azacytidine (cytidine analogue). These mutagenic compounds affect both RNA and DNA viruses [8, 17-20]. The current study investigates effects of 5-FU on the KOS strain of HSV-1, a clinically important DNA virus. We evaluated the growth of HSV-1 in Vero cells through sequential passage in the presence or absence of 700 µM 5-FU, designated as ▲HSV-1 and ΔHSV-1, respectively. CPE changes significantly decreased in the 10th passages of ▲HSV-1 and ΔHSV-1. More than 2-log lower titer values were determined for ▲HSV-1 in the 10^{th} passage compared with ΔHSV-1, and this titre difference was observed in most of the serial passages. Consistent with decreasing titre, the mutagen lowered viral load for ▲HSV-1 in the 10th passage approximately 625-fold. Reduction of titre and load could indicate that 5-FU has an antiviral effect on HSV-1, suppressing replication and viral activity, consistent with reports of antiviral activity of 5-FU on other viruses [17], through its known mechanisms of incorporating the mutations into viral RNA or DNA using its metabolized product 5-fluorouridine-triphosphate, which can be employed as a substrate for viral RNA-dependent RNA. or viral DNA-dependent DNA polymerases, leading to accumulation of viral mutations in a process known as lethal mutagenesis [17]. However, a limited number of studies concern antiviral effects on DNA viruses, including adeno-viruses [21], herpesviruses [22] and papillomaviruses [23]. All of these studies reported that 5-FU decreased titre as well as CPE.

The pursuit of drugs to treat herpesvirus infections has been ongoing for more than 60 years. ACV and its derivatives have been the first-line treatment, but resistance is a concern with long-term prophylactic and curative therapy. From 3.5% to 10% of immuno-compromised patients have ACV-resistant HSV strains [24, 25]. Up to 95% of ACV-resistant HSV-1 isolates from patients had mutations in the viral TK gene [6, 26], indicating these mutations may confer drug resistance. Approximately 63% of resistance mutations to ACV substitute amino acids in the TK gene [7].

The current study compares 1, 3, 5, 7 and 10 passages of ▲HSV-1 and ΔHSV-1 and identified nucleotide and amino acid changes in the TK gene. Comparisons of the TK genes of selected 10 HSV-1 isolates from Genbank, including reference strain NC_001806, with the TK genes of selected passages of ▲HSV-1 and ΔHSV-1, indicated no changes in the TK genes of selected 1, 3, 5 and 7^{th} passages of ▲HSV-1 and ΔHSV-1, as well as in reference strain NC_001806, except for the 10^{th} passage of ▲HSV-1 (OK274290). In terms of the TK nucleotide and amino acid identity comparisons, selected 1, 3, 5 and 7 passages of both ▲HSV-1 and ΔHSV-1 had 100% similarity to each other and 99.40% similarity with NC_001806. On the other hand, comparison of NC_001806 and ΔHSV-1 (OK274291) to the 10^{th} passage of ▲HSV-1 (OK274290) revealed 98.79% amino acid identity between NC_001806 and ▲HSV-1 (OK274290), stemming from two nucleotide changes that substituted two amino acids. Also the comparison of our selected 10^{th} passages of OK274291 and OK274290 with the KOS strain (accession No: KT899744) indicated 100% amino acid sequence identity between OK274291 and KT899744, with only one nucleotide change at position 47110 that substituted an alanine for a guanine. However, the situation differed for OK274290 and KT899744, because OK278290 compared to KT899744 had two nucleotide changes that were missense mutations, changing two amino acids.

We observed two nucleotide changes in OK274290 at positions 47257 and 47305, changing thymine to guanine and changing cytosine to thymine, respectively. These changes also led to two amino acid substitutions at position 183, replacing threonine with proline and turning alanine into threonine at position 167. These mutations were in the non-conserved region of the TK gene in ▲HSV-1, and it was important to determine whether the mutations caused the resistance. Previous studies reported that resistance substitutions in the non-conserved region in TK were condensed within amino acid regions 290 - 376, 117 - 215, 223 - 284, 89 - 161 and 64 - 82 [7]**.** This study demonstrated that two substitutions were within the resistance-mutation regions. The obtained results were interesting in terms of interpretation because if we had determined stability and/or increase in viral titre and viral load of ▲HSV-1(OK274290), these would have been evaluated as gaining of resistance against 5-FU, on the contrary, there were significant decreases in the 10^{th} passage of OK274290 when compared with the same passage of ΔHSV-1 (OK274291). Moreover,

we also observed these decreases in the majority of 10 serial passages between ▲HSV-1and ΔHSV-1. The current results indicate the antiviral effects of 5-FU on HSV-1, but the essential point is that drug have caused mutations in non-conserved regions associated with drug-resistance, meaning that HSV-1 could gain resistance against 5-FU and lead to the obtaining of drug-resistance mutants that may escape from the antiviral treatment if we extend our passages further.

Conclusions

The current feasibility study reinforced the observation that 5-FU can have an antiviral effect on HSV-1, using serial passages. Use of 5-FU, which causes mutations in the TK gene, can pave the way to suppress viral replication, reducing viral titre and load. However, viral drug resistance should not be underestimated. The number of consecutive passages under mutagen pressure was limited in our study, and these passages could not be enough for HSV-1 to induce mutagen resistance. We recommend increasing the number of passages under mutagen pressure beyond 15 and then sequencing the TK gene to identify HSV-resistant mutants.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E, The order *Herpesvirales*. *Arch Virol.*, 2009; 154(1): 171-177.
2. Mostafa HH, Thompson TW, Konen AJ, Haenchen SD, Hilliard JG, Macdonald SJ, Morrison LA, Davido DJ, Herpes simplex virus 1 mutant with point mutations in UL39 is impaired for acute viral replication in mice, establishment of latency, and explant-induced reactivation. *J Virol.*, 2018; 92(7): e01654-17: 1-12.
3. Jiang YC, Feng H, Lin YC, Guo XR, New strategies against drug resistance to herpes simplex virus. *Int J Oral Sci.*, 2016; 8(1): 1-6.
4. Brunnemann AK, Liermann K, Deinhardt-Emmer S, Maschkowitz G, Pohlmann A, Sodeik B, Fickenscher H, Sauerbrei A, Krumbholz A, Recombinant herpes simplex virus type 1 strains with targeted mutations relevant for aciclovir susceptibility. *Sci Rep.*, 2016; 6: 29903: 1-10.
5. Piret J, Boivin G, Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. *Antimicrob Agents Chemother.*, 2011; 55(2): 459-472.
6. Xie Y, Wu L, Wang M, Cheng A, Yang Q, Wu Y, Jia R, Zhu D, Zhao X, Chen S, Liu M, Zhang S, Wang Y, Xu Z, Chen Z, Zhu L, Luo Q, Liu Y, Yu Y, Zhang L, Chen X, Alpha-Herpesvirus Thymidine Kinase Genes Mediate Viral Virulence and Are Potential Therapeutic Targets. *Front Microbiol.*, 2019; 10: 941: 1-17.
7. Sauerbrei A, Bohn-Wippert K, Kaspar M, Krumbholz A, Karrasch M, Database on natural polymorphisms and resistance-related non-synonymous mutations in thymidine kinase and DNA polymerase genes of herpes simplex virus types 1 and 2. *J Antimicrob Chemother.*, 2016; 71(1): 6-16.
8. Geraghty RJ, Aliota MT, Bonnac LF, Broad-Spectrum Antiviral Strategies and Nucleoside Analogues. *Viruses*, 2021; 13(4): 667: 1-12.
9. Tataringa G, Spac A, Sathyamurthy B, Zbancioc AM, *In silico* studies on some dengue viral proteins with selected *Allium cepa* oil constituents from Romanian source. *Farmacia*, 2020; 68(1): 48-55.
10. Kataev VE, Garifullin BF, Antiviral nucleoside analogs. *Chem Heterocycl Compd (N Y)*, 2021; 57: 326-341.
11. Bhagavan NV, Ha CE, Chapter 21 - Structure and properties of DNA. in: Essentials of Medical Biochemistry (2nd Edition) – with Clinical Cases, Bhagavan NV, Chung-Eun Ha, Eds.; Academic Press: Elsevier, China, 2015; 381-400.
12. Kulu Y, Kawasaki H, Donahue JM, Kasuya H, Cusack JC, Choi EW, Kuruppu DK, Fuchs BC, Tanabe KK, Concurrent chemotherapy inhibits herpes simplex virus-1 replication and oncolysis. *Cancer Gene Ther.*, 2013; 20(2): 133-140.
13. Ghafouri-Fard S, Abak A, Tondro Anamag F, Shoorei H, Fattahi F, Javadinia SA, Basiri A, Taheri M, 5-Fluorouracil: A Narrative Review on the Role of Regulatory Mechanisms in Driving Resistance to This Chemotherapeutic Agent. *Front Oncol.*, 2021; 11: 658636: 1-21.
14. Reed LJ, Muench H, A simple method of estimating fifty percent endpoints. *Am J Epidemiol.*, 1938; 27(3): 493-497.
15. Namvar L, Olofsson S, Bergström T, Lindh M, Detection and typing of Herpes Simplex virus (HSV) in mucocutaneous samples by TaqMan PCR targeting a gB segment homologous for HSV types 1 and 2. *J Clin Microbiol.*, 2005; 43(5): 2058-2064.
16. van der Beek MT, Claas ECJ, van der Blij-de Brouwer CS, Morfin F, Rusman LG, Kroes ACM, Vossen ACTM, Rapid susceptibility testing for herpes simplex virus type 1 using real-time PCR. *J Clin Virol.*, 2013; 56(1): 19-24.
17. Agudo R, Arias A, Domingo E, 5-fluorouracil in lethal mutagenesis of foot-and-mouth disease virus. *Future Med Chem.*, 2009; 1(3): 529-539.
18. Beaucourt S, Bordería AV, Coffey LL, Gnädig NF, Sanz-Ramos M, Beeharry Y, Vignuzzi M, Isolation of fidelity variants of RNA viruses and characterization of virus mutation frequency. *J Vis Exp.*, 2011; 52: e2953: 1-11.
19. Khatun A, Shabir N, Yoon KJ, Kim WI, Effects of ribavirin on the replication and genetic stability of

porcine reproductive and respiratory syndrome virus. *BMC Vet Res.*, 2015; 11: 21: 1-11.
20. Pauly MD, Lauring AS, Effective lethal mutagenesis of influenza virus by three nucleoside analogs. *J Virol.*, 2015; 89(7): 3584-3597.
21. Wildner O, Hoffmann D, Jogler C, Uberla K, Comparison of HSV-1 thymidine kinase-dependent and -independent inhibition of replication-competent adenoviral vectors by a panel of drugs. *Cancer Gene Ther.*, 2003; 10(10): 791-802.
22. Dragún M, Rada B, Novotný L, Beránek J, Antiviral activities of pyrimidine nucleoside analogues: some structure--activity relationships. *Acta Virol.*, 1990; 34(4): 321-329.
23. Niwa K, Tagami K, Lian Z, Gao J, Mori H, Tamaya T, Topical vidarabine or 5-fluorouracil treatment against persistent HPV in genital (pre)cancerous lesions. *Oncol Rep.*, 2003; 10(5): 1437-1441.
24. James SH, Prichard MN, Current and future therapies for herpes simplex virus infections: mechanism of action and drug resistance. *Curr Opin Virol.*, 2014; 8: 54-61.
25. Skoreński M, Sieńczyk M, Anti-herpesvirus agents: a patent and literature review (2003 to present). *Expert Opin Ther Pat.*, 2014; 24(8): 925-941.
26. Burrel S, Deback C, Agut H, Boutolleau D, Genotypic characterization of UL23 thymidine kinase and UL30 DNA polymerase of clinical isolates of herpes simplex virus: natural polymorphism and mutations associated with resistance to antivirals. *Antimicrob Agents Chemother.*, 2010; 54(11): 4833-4842.