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In vitro effect of some nucleoside reverse transcriptase inhibitors against HSV-1 replication

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Abstract. – OBJECTIVE: The current study was designed to investigate the effects of some nucleoside reverse transcriptase inhibitors (NRTIs) on HSV-1 infection.

MATERIALS AND METHODS: Initially, the SwissTargetPrediction server was used to predict the interactions between HSV-1 thymidine kinase and acyclovir, stavudine, zidovudine, didanosine, and entecavir. The effect of each component on Vero cell viability was assessed by the MTT assay. After treatment, the cell supernatants were collected, and HSV-1 replication was analyzed by quantitative real-time PCR.

RESULTS: The qPCR results revealed that viral titers were reduced 41, 40, 19, 44, and 31-fold in the presence of acyclovir, zidovudine, stavudine, didanosine, and entecavir, respectively.

CONCLUSIONS: Our findings indicate that NRTIs significantly reduce HSV-1 replication in cell culture.

Key Words:
Herpes Simplex Virus (HSV), NRTIs, Acyclovir (ACV), Stavudine (STV), Zidovudine (ZDV), Didanosine (ddI), Entecavir (ETV).

Introduction

Herpes simplex virus type 1 (HSV-1) is one of the most prevalent, incurable virus infections worldwide, belonging to the Herpesviridae family[1]. The ability to establish and maintain latent and recurrent infections are characteristic of all herpes viruses. Following the primary infection in epithelial cells, the latent infection leads to life-long virus persistence in sensory nerve ganglia[2]. HSV-1 is frequently associated with oral and perioral infections. It leads to severe infections, such as keratitis, corneal blindness, and encephalitis. The advent of potent anti-inflammatory drugs and their extensive use in medical wards have led to increased HSV-1-related diseases, especially in developing countries. Despite the remarkable advances in anti-HSV therapies, recurrent infections are frequently found among immunocompromised patients, and there is not yet a cure for latent infections[3,4].

Nucleoside analogs have had impressive impacts on health development and care as the cornerstone of anticancer and antiviral medications. For example, antiviral therapy of neonatal HSV infections using nucleoside analogs, including high-dose acyclovir (ACV), has led to a significant reduction in infant mortality (from 50% to 4%). Acyclovir has been used for more than three decades to treat herpes simplex virus infections. Despite the widespread application of acyclovir worldwide, the prevalence of acyclovir resistance in immunocompetent patients remains stable at approximately 0.3%. In immunocompromised patients, the prevalence of resistant virus has similarly remained stable but at a higher level, typically 4% to 7%[5]. Therefore, new antiviral drugs alone or in combination with acyclovir should be developed to control resistance. Nucleoside analogue reverse transcriptase inhibitors (NRTIs), for example, stavudine (STV)/d4T, zidovudine (ZDV)/AZT, didanosine (ddI), and entecavir (ETV), are commonly used in the treatment of human immunodeficiency virus (HIV) infections and other reverse transcriptase coding viruses such as hepatitis B virus (HBV)[6-8]. Given the severe consequences of HSV recurrence in patients with a suppressed immune system, including cancer, organ transplantation, and HIV-infected individuals, it is essential and inevitable to search for novel and combination therapies that can target both HSV and HIV with minimum side effects. Alongside this, the current study was aimed to evaluate the effect of some NRTIs against HSV-1 replication in Vero cell line.

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Materials and Methods

Bioinformatics Analysis

All NRTIs were analyzed regarding their mechanism of action using http://www.expasy.org (as well as the SwissTargetPrediction database (STP). The NRTIs with thymidine kinase (TK) interactions such as stavudine (STV), zidovudine (ZDV), didanosine (ddI), entecavir (ETV), and acyclovir (ACV, as a control group) were selected.

Cell Lines and Drugs

The African green monkey kidney (Vero) cell line was obtained from the Pasteur Institute of Iran. The cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), IU/ml penicillin, and 1 µg/ml streptomycin (Gibco, Grand Island, NY, USA). All cells were incubated at 37°C in 5% CO_2 and 90% humidity. Didanosine (D0162) and stavudine (D1413) drugs were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), and acyclovir was obtained from Combino Pharm (Combino Pharm S.L., Barcelona, Spain) as sodium salts. Entecavir and zidovudine were obtained from Bakhtar Bioshimi (Bakhtar BioShimi Co., Kermanshah, Iran).

Plaque Assay

Pre-cultured vero cells were seeded 5×10^5 cells into six well plates to reach a confluent monolayer. Then, 10-fold dilutions of HSV-1 stock (obtained from the Pasteur Institute of Iran) were prepared in DMEM, and a 500 µl volume of each dilution was added to each well. The plates were placed at 37°C with 5% CO_2 for 1 h. The viral inoculum was replaced with a 1:1 mixture of 2x DMEM and agarose (1.5% in Milli-Q® water) with 2% FBS. The plates were incubated at 37°C with 5% CO_2 for 48 h. The cells were fixed with 10% formaldehyde and stained with 1% crystal violet in ethanol.

Cytotoxicity Assay

Cell toxicity of each nucleoside analog and optimum concentrations for cell viability were measured using MTT assay[9]. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich; St. Louis, MO, USA) powder was dissolved in PBS at the concentration of 5 mg/ml. Vero cells were plated in 96-well plates at an initial density of 1×10^4 cells/well to reach 70-80% confluency. On the next day, the cells were treated with different concentrations of each nucleoside analogs. Approximately 48 h after drug treatments, the medium was replaced with a fresh medium containing 0.5 mg/ml MTT solution and incubated for 4 h at 37°C. After incubation, culture media were discarded. Afterward, 100 µL of dimethylsulfoxide (DMSO) was added to each well. The blue formazan crystals were dissolved in DMSO, and the optical density (OD) was measured at 570 nm wavelength via ELx800 microplate reader (BioTeck, Winooski, VT, USA).

Cell Treatment with HSV-1 and NRTIs

The cells were cultured in 24-well plates to reach 70-80% confluency. The cells were cultured in 24-well plates to reach 70-80% confluency and were infected with HSV-1 at MOI 0.01 and appropriate concentration of each drug (ACV: 13 µM, ddI: 5 µM, STV: 10 µM, ZDV: 1 µM, ETV: 1.7 µM). About 72 h after infection, the supernatant of cell cultures was centrifuged (8000 g, 5 min, 4°C) and stored at -20ºC until DNA extraction. HSV-1 DNA was extracted using a High Pure Viral Nucleic Acid kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.

Primers and Probe

In the present study, the HSV-1 primers and probe set (Metabion, GmbH, Plannegg, Germany) were designed in the NCBI primer BLAST to specifically detect the *UL27* region, which encodes the viral glycoprotein B (*gB*). The sequence and characteristics of the oligonucleotides used in this study are shown in Table I.

Table I. The sequences of primers and probe used in the study.

Oligonucleotide	Sequence	Region in gB gene	Amplicon size
Probe	5′-6-FAM-ATCACCACCGTCAGCACCTTCATCG-BHQ-1-3′	702-726	
Sense primer	5′-TCCAGCATGGTGATGTTGAG-3′	680-996	96 bp
Antisense primer	5′-CGTGTACTTCGAGGAGTA CG-3′	756-775	

Real-time PCR

Real-time PCR was performed using the QuantiNova Probe PCR kit (Qiagen, GmbH, Hilden, Germany). Each reaction contained 2 µl template DNA, 1 µl Rox, 0.2 µM probe, 0.4 µM of each primer, 10 µl Qiagen Taqman Master Mix (2x), and up to 20 µl water. The amplification was carried out as follows: initial incubation at 95°C for 2 min, 35 cycles of 95°C for 10 s, and 60°C for 15 sec. The standard curve was drawn using 10-fold serial dilutions of HSV-1 sample to determine the reaction's efficiency.

Statistical Analysis

The statistical analysis was performed with Prism V 5.01 (GraphPad Software Inc., San Diego, CA, USA). To compare the variables, we carried out the ANOVA test, followed by the Tukey post hoc test. Values of $p < 0.05$ were considered significant at a 95% confidence interval (CI).

Results

Drug Screening

Nucleoside analogs are commonly used in HIV-1 infection therapy. To date, nucleoside reverse transcriptase analogs have not been used against HSV-1. Activation of nucleoside analogs requires initial phosphorylation, which is catalyzed by nucleoside kinases. ACV can be phosphorylated both by viral and cellular kinase, whereas STV, ddI, ZDV, and ETV are phosphorylated by cellular kinases.

NRTIs and Cytotoxicity

The MTT assays were performed for each drug to assess the most suitable dose of NRTIs. The NCBI primer BLAST was used with a minimum toxicity effect on the vero cells. Measurements were performed, and the required concentration was determined for a 50% inhibition of viability (IC50). The highest doses of ACV, ETV, ZDV, ddI, and STV with minimum toxicity were as follows: 13 µM, 1.7 µM, 1 µM, 5 µM, and 10 µM, respectively. The dose-response curves of each drug are shown in Figure 1.

Real-Time PCR

Real-time relative quantification PCR was conducted on the supernatants of HSV-1-infected cells treated with the highest non-toxic dose of each NNRTIs. ACV was considered as the control group. A standard curve of the Ct values, which have been plotted against the serial dilutions of virus concentrations, was drawn to evaluate primers and qPCR reactions. Reaction efficacy was about 100%, which is acceptable for comparative qPCR between treated and non-treated sample groups (Figure 2).

NRTIs treatment significantly reduced the viral titers in comparison with the non-treatment control. Viral titers were reduced 40, 39.7, 43.3, 29.7, and 17.7-fold in the presence of acyclovir, zidovudine, didanosine, entecavir, and stavudine, respectively. Our findings indicate that there is a significant difference in viral titers between NRTIs treatment groups and the non-treatment control ($p < 0.05$, ANOVA). The Tukey's test showed that didanosine ($p < 0.06$) and zidovudine ($p < 0.99$) act similarly to acyclovir, while entecavir and stavudine had no advantage over acyclovir (Figure 3). As compared to zidovudine, didanosine significantly reduced viral titer ($p < 0.03$). In this study, acyclovir was used as a standard drug that has been intended to compare the efficacy of other nucleoside analogs.

Discussion

Although there have been advances in the management of HSV infections, due to the presence of drug resistance mutations, new or improved therapeutic agents are required[10]. Currently, ACV and its analogs, such as valacyclovir and penciclovir, are the only approved and the most common treatments for HSV infections[11]. Since long-term acyclovir therapies might be required for infection management in organ transplant recipients and AIDS patients, drug-resistant viruses emerge in nearly 7% of these immunocompromised transplant patients[11-13]. In the current study, we used several NRTIs against the HSV-1, assuming that there is a possibility of resistance to ACV among the virus strains. We selected nucleoside analog drugs that have not been used for HSV-1 treatment and might be effective on acyclovir. The cellular kinases are necessary to convert the nucleoside analogs into an active and usable form. The interaction of the nucleoside analogs and TK was evaluated using the STP database. STP has been primarily developed for the identification of molecular targets that have been known as bioactive. However, it is able to upload any small molecule, even without prior knowledge of its potential effects[14]. ACV is considered as an internal control, and its interaction with the HSV TK has been

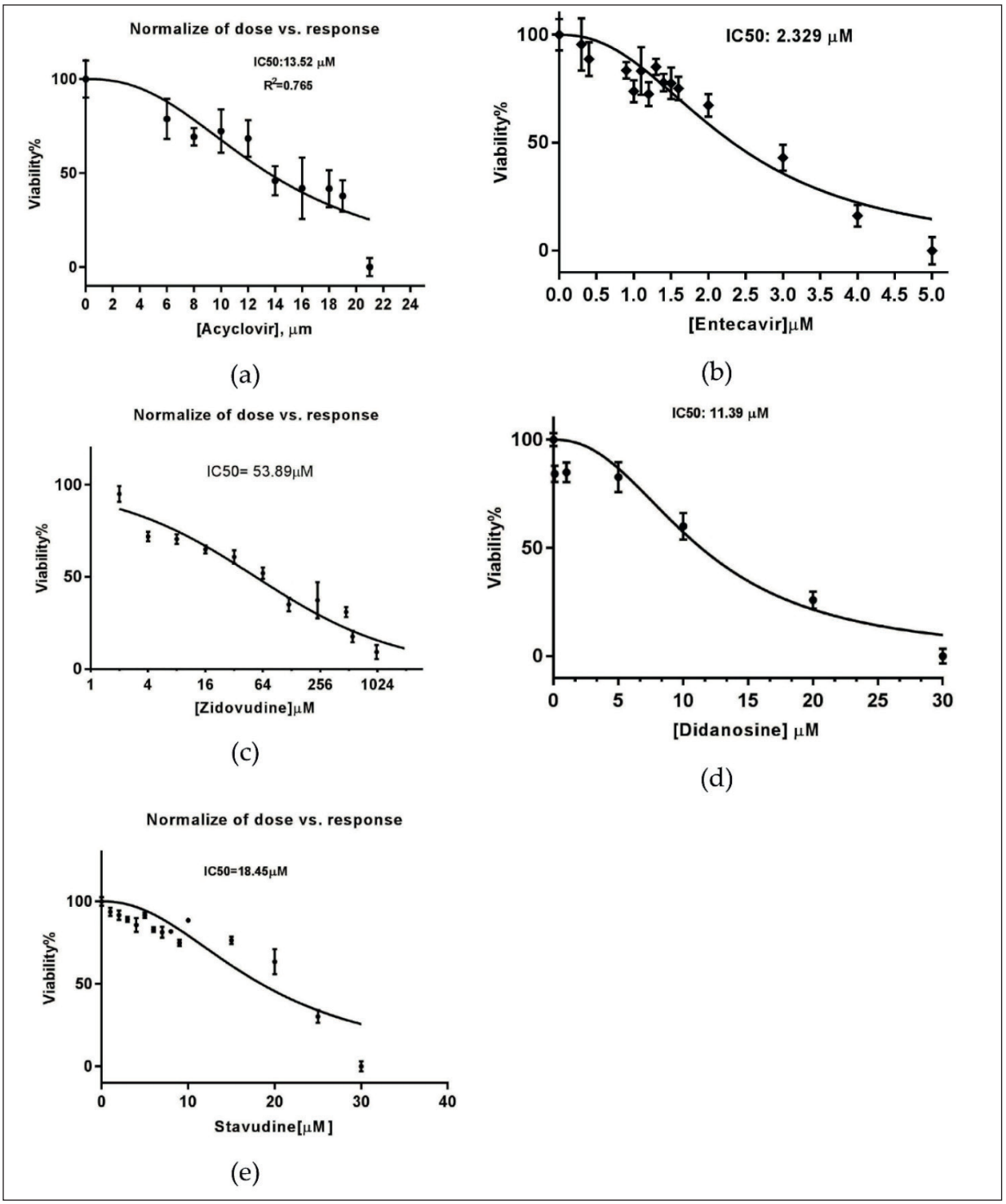

Figure 1. The dose-response curves for MTT assay of ACV (**A**), ETV (**B**), ZDV (**C**), ddI (**D**), and STV (**E**) on Vero cells.

confirmed. In the current study, the results of STP target prediction for the selected nucleoside analogs with TK compared to ACV were as follows: ddI ≥ACV≥ZDV>ETV>STV. Although there are differences between the reverse transcriptase and DNA polymerase, remarkable similarities prevail between the two enzymes when their active sites are considered[15]. Therefore, NRTIs might be used as a DNA polymerase inhibitor, and consequently, it can be employed to suppress HSV-1.

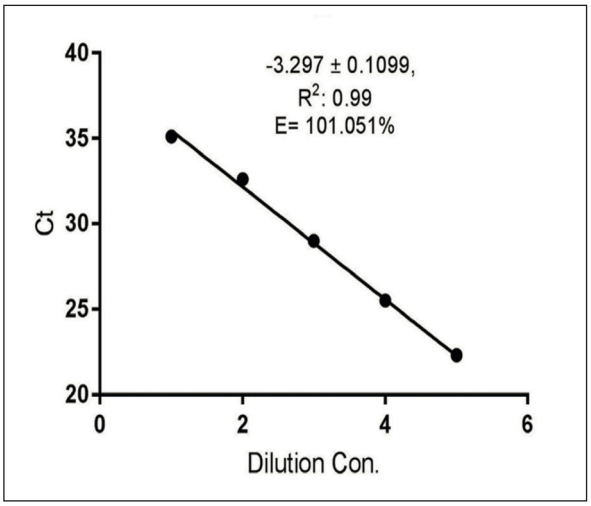

Figure 2. qPCR standard curve of gB primers and probe.

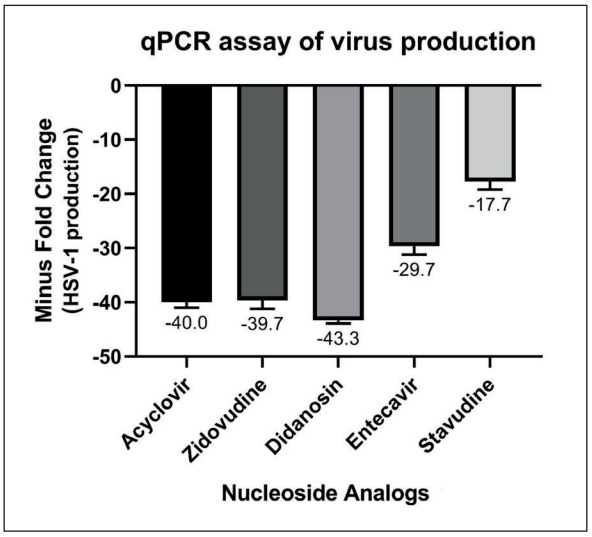

Figure 3. The effects of NRTIs on HSV-1 production in Vero cells. The values represent the mean of 3 independent assays.

The MTT assay was performed to determine the lowest toxic dose of the nucleoside analogs for treating Vero cells. MTT is a simple, rapid, cost-effective, and convenient approach compared to other toxicity-measuring methods such as the lactate dehydrogenase (LDH) assay. For each NRTI, a different concentration range was used, none of which had toxicity under 50%. Therefore, we applied the standard concentrations of all the NRTIs, which had been reported elsewhere. As one of the most accurate techniques for measuring the viral quantity, the plaque assay was used to determine the virus titer.

Therefore, we measured the viral replication in the presence of some NRTIs. For virus propagation, infections are commonly performed at low MOI to avoid the appearance of defective virus. Different dilutions of the viral DNA were applied for PCR standardization. To specify and discriminate between HSV-1 and HSV-2, we designed the probe and primers to target the *gB* gene. The *gB* gene is a type-specific gene that can be used to distinguish between two types of HSVs[16]. Although real-time PCR is a sensitive and precise method, it is better to use a probe-based approach that is specific to the viral agent[16,17]. In the current study, the quantitative real-time PCR was used to compare two cell groups (NRTIs-treated groups *vs.* the control group) using relative quantification. The relative quantification was applied according to 2(-ΔCt). In the presence of ACV (as the standard control group), ZDV, ddI, ETV, and STV, the replication of the HSV-1 was decreased 40, 39.7, 43.3, 29.7 and 17.7-fold, respectively. These findings indicate that NRTIs presumably have therapeutic effects against HSV-1 infections and might be considered as a therapy for severe infections.

Conclusions

The results showed that some NRTIs, especially didanosine, have surprisingly remarkable effects against HSV-1 in cell culture; therefore, it is recommended to investigate their activity against acyclovir-resistant isolates.

Conflict of Interests

The authors declare that they have no conflict of interests.

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