Effect of fluorinated $N$-alkylthioamides on HSV-1 multiplicity

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The number of promising viral targets and classes of compounds with substantial antitherpetic properties considerably increased during the last decade. However, no new effective and low-toxicity clinical drugs against both wild-type viruses and drug-resistant strains have appeared. This situation makes the search for new antitherpetic drugs and their new targets a high priority task. **Aim.** To study the effect of fluorinated $N$-alkylthioamides on the herpes simplex virus-1 (HSV-1). **Methods.** The influence of these compounds on the multiplicity and infectivity of HSV-1 was determined by the MTT-assay, virucidal assay, adsorption and penetration assays, PCR and infectious virus yield reduction assay. **Results.** The 10S-23 and 10S-24 compounds prevented the adsorption and penetration of HSV-1 into cells by up to 26%. HSV-1 DNA replication was moderately inhibited by the 10S-24 compound (up to 39 %). The 10S-23 and 10S-24 compounds in concentrations of 100 – 33 μg/ml reduced HSV-1 virus production by 70–99% and >99%, respectively. **Conclusion.** The 10S-24 compound may be used as a therapeutic agent to reduce the penetration, replication and translation of HSV-1.

**Key words:** Herpes simplex virus, Fluorinated N-alkylthioamides, Antiviral activity

Introduction

The character of diseases caused by *Alphaherpesviruses* has changed over the last decade. The severity of disease and the frequency of acyclovir resistance increased with an increase in the number of immunocompromised patients. Most modern drugs for the treatment of herpetic infections are based on the use of modified nucleosides or their prodrugs [1]. Noteworthy, the drugs do not save the patient from the recurrent character of the disease, and their prolonged administration can cause the emergence of resistant virus strains. Moreover, to date, none of the currently approved antiviral drugs has been able to eliminate an established latent infection [2, 3]. Many of the newer experimental agents target the essential processes unique to herpesvirus replication and, therefore, are potentially highly selective [4].
Due to synthetic accessibility and high chemical and biological stability, the fluorine containing compounds are popular building-blocks from the standpoint of medicinal chemistry. Because of similarity in size to hydrogen, it has been shown that microorganisms or enzymes often do not recognize the difference between a natural substrate and its analogue wherein a C – H bond of the substrate is replaced with a C – F bond. This observation is a basis of what is regarded as the “mimic effect” of fluorine for hydrogen. This strongly suggests that not only single fluorine displacement but also various fluorine-substitution patterns in the substrate analogues or inhibitors could be adapted to biological systems in a molecular – recognition mode similar to that of the natural substrates [5]. The presence of a fluorine atom in the molecule influences the chemical, physical and biological properties of compounds. A fluorine atom is involved in many biochemical reactions as activator and inhibitor of enzymes, of the metabolism and thyroid hormone, the synthesis of nucleic acids, proteins and lipids. Thus, fluorinated analogs of biological molecules are useful tools to study and modify the function of biological systems. It is known that the insertion of fluorine atoms in the molecule of biologically active compounds can affect not only their pharmacokinetic properties, but also the allocation in the tissues, path and metabolic rate of the fluorine analogue and the pharmacodynamics and toxicology [6, 7]. All these factors play a key role in the creation of the drugs based on nucleoside and non-nucleoside analogues and cause a considerable interest to the medicinal chemistry of fluorine compounds [8, 9].

In the present study, the effect of new fluorinated derivatives of amino acids on the herpes simplex virus-1 (HSV-1) multiplicity was studied.

Materials and Methods

Virus and cells

The Syrian hamster kidney fibroblasts (BHK-21) and the strain US of herpes simplex virus type 1 (HSV-1/US) were used in this study. The cells and virus were cultured according to standard methods.

Tested substances

The general characteristics of the fluorinated derivatives of amino acids (fluorinated N-alkylthioamides) were shown by Pikun et al [10]. The compounds are classified according to the type of amino acid, its configurations, the number of fluorine atoms and the position of the fluorine substitution (2’, 3’, or both) (Fig. 1).

Cellular toxicity

Cellular toxicity of compounds was tested in vitro according to a cell viability assay previously reported [11]. Monolayers of BHK 21 cells in 96-multiwell plates were incubated with the compounds in concentration of 1000 – 15.6 μg/ml for 48 h, then 20 μl of a 5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) were added to the medium. The plates were read using an automatic plate reader Multiskan FC (Thermo Scientific, USA) at 538 nm test wavelength.

Antiviral Assay

We used a modification of an MTT-assay developed for screening anti-HSV compo-
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und [12]. 50 µl of virus suspension (a multiplicity of infection (MOI) of 0.5 and 0.05) were added to BHK 21 cells. In 2 h, an unabsorbed virus was aspirated and 200 µl of serial three-fold drug-containing medium were added to each well, and incubated at 37°C and 5% CO₂ for 2 days. The percent of protection was calculated by the following formula:

\[
\frac{(OD \text{ exp.}) - (OD \text{ virus control})}{(OD \text{ cell control}) - (OD \text{ virus control})} \times 100\%
\]

where (OD exp.), (OD virus control), (OD cell control) indicate the absorbencies of the tested sample, the virus control and the cell control, respectively.

**Plaque reduction assay**

The BHK 21 cells monolayer was infected with 0.02 MOI of HSV-1 in the absence or presence of the substances. After 2 h of adsorption, the cell monolayer was covered with overlay medium. The infected cell monolayer was fixed and stained with 1% Crystal Violet in 20% ethanol. The antiviral activity of substances was determined by the following formula [13]:

\[
\text{Percentage inhibition} = \left[1 - \frac{\text{number of plaque (tested)}}{\text{number of plaque (control)}}\right] \times 100%
\]

**Virucidal assay**

100 µL of \(2^X\) compounds in a supportive medium (66, 22 and 7.4 µg/ml) were mixed with 100 µL of \(2^X\) HSV-1 in microfuge tubes and incubated at 37°C for 30 min and 2 h. Then, 50 µL of each mixture were added to a separate well on a 96-well plate containing BHK 21 cells. The plates were incubated at 37°C and 5% CO₂ for 2 h. The cells were washed

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Fig. 1. Structure of compounds and scheme of their synthesis
with PBS (pH=7.4) and 200 µl of overlay medium were added [14, 15]. The MOI of HSV-1 after dilution was 0.03. Plaques were counted as described above.

**Attachment assay**
The attachment assay described by De Logu et al. [16, 17] was used in this study with a minor modification. Briefly, BHK 21 cell monolayer was pre-chilled at 4°C for 1 h and then infected with 0.03 MOI HSV-1 in the absence or presence of serial dilution compounds. After 3 h of incubation at 4°C, the cell monolayer was washed with PBS three times and overlaid with medium containing 0.06% agarose. Plaques were counted as described above.

**Viral Penetration Assay**
The assay of HSV-1 penetration into BHK 21 cells was performed according to the published procedures, with minor modifications [18]. A BHK 21 cell monolayer was pre-chilled at 4°C for 1 h, infected with 0.3 MOI HSV-1 and incubated at 4°C for further 3 h to allow the attachment of HSV-1. After incubation, 25 µL of 2x concentration of compounds were added. Infected cell monolayer was then incubated at 37°C to maximize the penetration of viruses. In 10 min, the infected cell monolayer was treated with PBS at pH 3 for 1 min to inactivate non-penetrated virus. PBS at pH 11 was then immediately added to neutralize acidic PBS. The neutral PBS was removed and the cell monolayer was covered with overlay medium. After further 48 h incubation, the cell monolayer was fixed and stained. Plaques were counted as described above.

**Real-time PCR analyses**
Real-time PCR was utilized to derive the total number of viral genomes within the lysate. Lysates were collected at 48 h p.i., and 200 µL of each suspension were used for the extraction of viral DNA. Viral DNA was extracted using innuPREPVirus DNA Kit (Analityk Jena AC, Germany) according to the manufacturer’s instructions. PCR tests were performed with “AmpliSens®HSV-screen-FL» (AmpliSens, Russia) according to the manufacturer’s recommendations. The Thermocycler qTOWER 2.2 (Analytic Jena, Germany) was used.

**Infectious virus yield reduction assay**
Monolayer of BHK 21 cells was infected by adsorption of HSV-1 at a MOI of 0.02 and 0.2 for 2 h at 37°C and 5% CO₂. The compounds in concentrations ranging between 100 and 3.7 µg/ml were added immediately after adsorption. In 48h after virus inoculation, the cells in culture medium were lysed by freezing and thawing (three times). Ten-fold serial dilutions of HSV-1 and virus-treated extracts of HSV-1 were prepared prior to the infection. The number of plaque-forming units was determined and a decrease of virus titer was calculated [19]:

\[
\text{Percentage inhibition of virus reproduction} = (1 - \frac{T}{C}) \times 100\%
\]

where T is the compounds-treated viral titers and C is the control viral titers.

**Results and Discussion**
The first stage of the antiviral assay is necessary to determine the concentrations of the compounds that are not toxic to the cells. According to the MTT test results, the CC₅₀
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values of 10S-20, 10S-23 and 10S-24 for BHK 21 cells were 1731.8, 1000.7 and 1004.2 µg/ml, respectively (Table 1). The compounds 10S-20 in concentration of 1500 µg/ml, 10S-23 and 10S-24 in concentration of 500 µg/ml exhibited a weak cytotoxic effect, and 96% of cells survived (data not shown). The compounds 10S-21 and 10S-22 demonstrated CC$_{50}$ values equal to 510.3 and 304.5 µg/ml, respectively.

The most perspective compounds in the concentration range used to assess their activity did not induce any visible changes in the BHK 21 cell morphology or density. The derivatives of amino acids exhibited in vitro anti-HSV-1 activities at different magnitudes of potency; the EC$_{50}$ for 5 tested compounds was in the range of 15 – 247 µg/ml, their inhibitory effect on HSV-1 multiplication was MOI dependent (Table 1). ACV was used as a reference compound. The 10S-23 and 10S-24 compounds showed potent anti-HSV-1 activity, their EC$_{50}$ increased from 47.9 to 70.9 µg/ml and 15.4 to 35.0 µg/ml, respectively, whereas MOI increased from 0.05 to 0.5. Other 3 compounds were either mild or weakly active to suppress the HSV-1 infection, their EC$_{50}$ 102 – 247 µg/ml.

The selectivity index (SI) for the anti-HSV-1 assay was in the range of 2 - 67. SIs of 10S-23 and 10S-24 for MOI 0.05 were 14 and 29, for 0.5 were 21 and 67, respectively. Based on these results, 10S-23 and 10S-24 were selected for further research on their antiviral activity and targets of action.

The antiviral effects of compounds against HSV-1 infection were evaluated by the plaque reduction assay. The results revealed that both 10S-23 and 10S-24 inhibit HSV-1 and their plaque formation in a dose-dependent manner (Fig. 2).

10S-24 at concentrations of 33 – 150 µg/ml inhibited the HSV-1 plaque formation by 70 – 95 %, whereas 10S-23 significantly inhibited the plaque formation at higher concentrations (100 and 150 µg/ml).

Herpes virus reproduction is characterized by a complex sequence of different steps at which antiviral agents might interfere. The need for additional anti-herpetic drugs, the

Table 1. Anti-HSV-1 activity, cell cytotoxic effect and selectivity index of fluorine-containing derivatives of amino acids for BHK 21 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral activity, EC$_{50}$ b (µg/ml)</th>
<th>Cell cytotoxic effect, CC$_{50}$ b, (µg/ml)</th>
<th>Selectivity index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>moi 0.05</td>
<td>moi 0.5</td>
<td>moi 0.05</td>
</tr>
<tr>
<td>10S-20</td>
<td>--*</td>
<td>247.2±5.2</td>
<td>1731.8±36.1</td>
</tr>
<tr>
<td>10S-21</td>
<td>102.9±3.4</td>
<td>125.4±3.7</td>
<td>510.3±7.5</td>
</tr>
<tr>
<td>10S-22</td>
<td>131.3±3.8</td>
<td>175.1±4.4</td>
<td>304.5±5.8</td>
</tr>
<tr>
<td>10S-23</td>
<td>47.9±2.3</td>
<td>70.9±2.8</td>
<td>1000.7±20.5</td>
</tr>
<tr>
<td>10S-24</td>
<td>15.4±1.3</td>
<td>35.0±2.0</td>
<td>1004.2±10.8</td>
</tr>
<tr>
<td>Aciclovir</td>
<td>5.6±0.8</td>
<td>7.7±0.9</td>
<td>1006.0±14.3</td>
</tr>
</tbody>
</table>

a – Antiviral activity and cell cytotoxic effect were determined by MTT assay. b – Values represent the mean ± S.D. for three independent experiments. SI is the ratio of CC$_{50}$ to EC$_{50}$. EC$_{50}$ the concentration, which corresponded to 50% inhibition of HSV-1 multiplication. CC$_{50}$ was the concentration that showed 50% cellular cytotoxic effect. --* – no anti-HSV-1 activity.
mechanisms of action of which differ from that of ACV, remains a high priority in the antiviral drug research today. For example, polyamino-acid-based derivatives, such as poly-L-lysine and poly-L-arginine, appear to act primarily by inhibiting early stages of virus replication, prevent HSV-1 binding to cellular receptors and consequently internalization of the virus into the cells [2, 20].

To determine a stage of the HSV-1 infection inhibited by the derivatives of L-phenylalanine, the virus and cell were treated with the compounds at various times before and after the HSV-1 infections. The fluorinated derivatives of amino acids have been reported to possess a variety of biological activities, including antiviral, antimicrobial, antitumor, antioxidant, enzyme-inhibiting, etc. [5, 9]. For antiviral activity, it is believed that the inhibitory effect is caused by binding of the derivatives of amino acids to the protein coat of the virus and/or the host cell membrane. The virus adsorption

Table 2. Dependence of anti-HSV-1 activity of fluorine-containing derivatives of L-phenylalanine on treatment schedule

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Compounds present:</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min. before adsorption</td>
<td>2 h before adsorption</td>
</tr>
<tr>
<td>10S-23</td>
<td>33 2.91±0.57</td>
<td>24.02±1.63</td>
</tr>
<tr>
<td></td>
<td>11 2.94±0.57</td>
<td>2.01±0.47</td>
</tr>
<tr>
<td></td>
<td>3.7 2.96±0.58</td>
<td>0</td>
</tr>
<tr>
<td>10S-24</td>
<td>33 0</td>
<td>8.84±1.03</td>
</tr>
<tr>
<td></td>
<td>11 0</td>
<td>6.95±0.92</td>
</tr>
<tr>
<td></td>
<td>3.7 0</td>
<td>4.91±0.77</td>
</tr>
</tbody>
</table>

Each point represents the mean ± S.D. for three independent experiments. Significant difference between tested sample and control (P < 0.05).
and eventual virus penetration are arrested. The compounds affected the virus penetration process possibly through detaching the virus already bound to the cell, perhaps by the disturbance of viral glycoproteins [1].

Table 2 shows that different inhibitory effects were observed when the compounds were added 30 min and 2 h before adsorption, during virus adsorption and penetration.

The 10S-24 compound had no significant direct virucidal effect on HSV-1 even at the concentration of 33 µg/ml or lower, whereas 10S-23 reduced the HSV-1 infectivity by 24% when added to virus 2 h before adsorption.

The results indicate that the 10S-23 and 10S-24 compounds at higher concentrations showed a reducing effect on the viral adsorption and penetration (Table 2). It was found that 10S-23 in concentrations of 33 µg/ml reduced the HSV-1 attachment to cells by 21%.

Our studies revealed that 10S-23 and 10S-24 in concentration of 33 µg/ml were able to prevent the penetration of HSV-1 into cells by 18.5 and 25.9 %, respectively.

Based on previously reported findings, as well as on our data, and in view of the chemical nature of compounds, we hypothesized that 10S-23 and 10S-24 might prevent the formation of a full and infectivity virus progeny. Phenylalanine residues are aromatic and hydrophobic amino acids known to be involved in protein-protein interactions and to play an important role in the virus assembly and a critical regulatory function in the infectious virus production [21]. The antiviral activity assessed via real-time PCR and infectious virus yield reduction assay demonstrated the inhibitory effect of these compounds at the late stage of the virus reproduction.

As shown in Fig. 3, in the presence of 10S-24 in high concentrations, the HSV-1 DNA replication was moderately inhibited and the viral DNA copy number reduced to 73 and 61% of the number of viral control, for MOI 0.02 and 0.2, respectively. 10S-23 irrespective of the multiplicity of infection of the virus could not fundamentally suppress DNA synthesis of HSV-1 in the BHK-21 cells, DNA replication of virus decreased by 5 – 18% only.

The compounds were added to a cell at the end of the virus adsorption period (2 h after infection), a significant delay in the growth of HSV-1 was observed, and a much lower yield of infectious virus was also obtained (Table 3).

It was found that at the concentration of 100 – 33µg/ml, the compound 10S-24 regardless of multiplicity of infection considerably reduces (by >99%) the titer of virus obtained de novo, whereas for all analyzed concentrations of the compound 10S-23 a decrease of virus titer by 83 – 98% and 68 – 99% was observed for MOI 0.2 and 0.02, respectively.

Interestingly, the anti-HSV activity of 10S-21, 10S-23 and 10S-24, containing L-phenylalanine, was greater in comparison to 10S-20 and 10S-22, which contain other amino acids in the molecules. However, only sodium salts of L-phenylalanine demonstrated potent anti-viral activity. Noteworthy, the low virus-inhibitory action of 10S-23 compared with 10S-24 can be attributed to the absence of a tetrafluoropropanethioyl group in the structure. This suggests that the anti-HSV activity of this group of the compounds depends not only on amino acid but also on the number of the fluorine atoms in the molecule.
Conclusion

Taken together, our results showed that 10S-23 and 10S-24 in non-toxic concentrations possess the anti-HSV activity, which is realized via multiple mechanisms. The compound 10S-23 reduces the viral adsorption, penetration and yield of virus particles. The compound 10S-24 blocks the viral penetration into cells, inhibits the viral DNA replication and infectivity of viral progeny, which is probably caused by decreasing protein synthesis or capsid assembly. Taking into account the promising antitherpetic activity of sodium salts of L-phenylalanine herein reported, further investigation is needed to explore the antiviral mechanism of these compounds in detail.

Funding

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Table 3. Effect of fluorinated sodium salts of L-phenylalanine on HSV-1 infectivity

<table>
<thead>
<tr>
<th>Concentration of compound, (µg/ml)</th>
<th>HSV-1 at moi 0.02</th>
<th>HSV-1 at moi 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus titer PFU/ml</td>
<td>% of inhibition</td>
</tr>
<tr>
<td>10S-23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>4,0 x10^5</td>
<td>70,87</td>
</tr>
<tr>
<td>50</td>
<td>2,7 x10^4</td>
<td>98,06</td>
</tr>
<tr>
<td>33</td>
<td>1,4 x10^4</td>
<td>99,00</td>
</tr>
<tr>
<td>11</td>
<td>1,6 x10^5</td>
<td>88,35</td>
</tr>
<tr>
<td>3,4</td>
<td>4,4 x10^5</td>
<td>67,96</td>
</tr>
<tr>
<td>10S-24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1,3 x10^3</td>
<td>99,90</td>
</tr>
<tr>
<td>50</td>
<td>1,2 x10^4</td>
<td>99,13</td>
</tr>
<tr>
<td>33</td>
<td>6,7 x10^3</td>
<td>99,51</td>
</tr>
<tr>
<td>11</td>
<td>1,1 x10^4</td>
<td>99,22</td>
</tr>
<tr>
<td>3,4</td>
<td>1,2 x10^5</td>
<td>91,46</td>
</tr>
<tr>
<td>Control of virus</td>
<td>1,4x10^6</td>
<td>–</td>
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</table>
REFERENCES


Влияние фторированных N-алкилтиоамидов на репродукцию ВПГ-1

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О. Ю. Повница, Н. В. Пикун, Ю. Г. Шермолович,
С. Д. Загородняя

В течение последнего десятилетия увеличилось количество перспективных вирусных мишеней и классов соединений со значительными противогерпетическими свойствами. Однако еще не появились новые эффективные и низкотоксичные препараты, которые активны как против вирусов дикого типа, так и против резистентных штаммов. Цель. Исследовать влияние фторсодержащих N-алкилтиоамидов на репродукцию вируса простого герпеса первого типа (ВПГ-1).

Методы. С использованием МТТ-анализа, ПЦР и метода редукции количества бляшек проводили исследование вирулицидности и действия соединений на адсорбцию, проникновение, репликацию и инфекционные титры вируса. Результаты. Показано, что соединения 10S-23 и 10S-24 на 26 % предотвращали адсорбцию и проникновение ВПГ-1 в клетки. Соединение 10S-24 способно до 39 % подавлять репликацию ВПГ-1. Установлено, что при концентрации 33–100 мкг/мл 10S-23 и 10S-24 снижали титр вируса, синтезированного de novo, на 70–99 % и > 99 %, соответственно. Вывод. Полученные данные свидетельствуют о перспективности использования соединения 10S-24 в качестве терапевтического средства для снижения проникновения ВПГ-1 в клетки, его репликации и трансляции.

Ключевые слова: вирус простого герпеса, фторированные N-алкилтиоамиды, противовирусная активность.