New effective chemically synthesized anti-smallpox compound NIOCH-14

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Antiviral activity of the new chemically synthesized compound NIOCH-14 (a derivative of tricyclodicarboxylic acid) in comparison with ST-246 (the condensed derivative of pyrroledione) was observed in experiments in vitro and in vivo using orthopoxviruses including highly pathogenic ones. After oral administration of NIOCH-14 to outbred ICR mice infected intranasally with 100 % lethal dose of ectromelia virus, it was shown that 50 % effective doses of NIOCH-14 and ST-246 did not significantly differ. The ‘therapeutic window’ varied from 1 day before infection to 6 days post-infection (p.i.) to achieve 100–60 % survival rate. The administration of NIOCH-14 and ST-246 to mice resulted in a significant reduction of ectromelia virus titres in organs examined as compared with the control and also reduced pathological changes in the lungs 6 days p.i. Oral administration of NIOCH-14 and ST-246 to ICR mice and marmots challenged with monkeypox virus as compared with the control resulted in a significant reduction of virus production in the lungs and the proportion of infected mice 7 days p.i. as well as the absence of disease in marmots. Significantly lower proportions of infected mice and virus production levels in the lungs as compared with the control were demonstrated in experiments after oral administration of NIOCH-14 and ST-246 to ICR mice and immunodeficient SCID mice challenged with variola virus 3 and 4 days p.i., respectively. The results obtained suggest good prospects for further study of the chemical compound NIOCH-14 to create a new smallpox drug on its basis.

INTRODUCTION

After completion of the Global Smallpox Eradication Program and cessation of smallpox immunization in 1980, the world is facing a dangerous situation when more than half of the world population has no immunity against orthopoxvirus infections. Nowadays there exists the threat of smallpox emergence as a result of intentional or accidental spread of the pathogen from such potential sources as probably existing illegal depositaries (Henderson et al., 1999; Jahrling, Fritz & Hensley, 2005; Anderson & Bokor, 2012), the remains of people who died from smallpox found during archaeological excavations in permafrost soils where the virus can persist for a long time (Herrlich, 1960; Biagini et al., 2012; McCollum et al., 2014), or virus created artificially on the basis of data on its DNA primary structure (Parker et al., 2012). In addition, the magnitude and frequency of epidemic outbreaks of other orthopoxvirus infections such as monkeypox, cowpox and buffalopox have increased in the past decades (Baxby et al., 1994; Damaso et al., 2000; Wienecke et al., 2000; Reed et al., 2004; Favier et al., 2011). At present, the two most effective and orally bioavailable antiviral drugs for emergency prophylaxis and treatment of orthopoxvirus infections are at the completion stages of clinical trials and production: CMX001 (ether-lipid analogue of cidofovir with inhibitory activity at the stage of DNA replication) (Magee et al., 2005, 2008) and ST-246 (an inhibitor of formation of the enveloped virus form) (Yang et al., 2005;
Jordan et al., 2010; Grosenbach et al., 2011). However, due to the increased urgency of finding measures to prevent orthopoxvirus infections in humans and the ability of the causative agents to mutate and acquire drug resistance (Smee et al., 2002, 2005; Yang et al., 2005) it is necessary not only to search for new chemical compounds with different mechanisms of action, but also to develop new generations of antiviral drugs.

Since 2001, the State Research Center of Virology and Biotechnology (SRC VB) Vector has been searching for new low-molecular-mass inhibitors of orthopoxviruses in collaboration with a number of research institutes and universities. A total of more than 7000 compounds have been tested. In particular, we have synthesized a series of compounds that have structural fragments similar to ST-246 and tested their antiviral activities in order to develop new highly efficient antiviral preparations (Selivanov et al., 2011). As a result of the research conducted, a number of compounds possessing high antiviral activity against orthopoxviruses were identified in tests on cell cultures. In particular, these compounds include derivatives of fluorinated benzotriazines, fluorinated derivatives of benzimidazoles, derivatives of polyhedran compounds, derivatives of hydrazine and thiosemicarbazide, derivatives of tricyclodicarboxylic acid and condensed derivatives of pyrroledione.

Screening and extensive testing of compounds produced in vitro revealed that a large number of compounds among derivatives of tricyclodicarboxylic acid and condensed derivatives of pyrroleonide possessed high antiviral activity against orthopoxviruses (Selivanov et al., 2011). 7-((4-trifluoromethyl)phenyl)formohydrazido)carbonyl)tricyclo(3.2.2.0²,4)non-8-en-6-carboxylic acid (NIOCH-14) demonstrated the highest antiviral activity as compared with other compounds tested.

In this regard, the goal of the present work was to study the antiviral activity of the chemically synthesized compound NIOCH-14 in experiments in vitro and in vivo using ectromelia virus (ECTV) and orthopoxviruses that are highly pathogenic for humans (monkeypox virus, MPXV, and variola virus, VARV).

RESULTS

Antiviral activity of NIOCH-14 in a cell culture infected with ECTV and orthopoxviruses highly pathogenic for humans

Several chemical compounds (Fig. 1), derivatives of tricyclodicarboxylic acid (NIOCH-14) and condensed derivatives of pyrroledione (NIOCH-32 and ST-246) exhibiting high activity against orthopoxviruses were identified in testing in vitro (in Vero cell culture). All chemical compounds studied had TC₅₀ (50 % toxicity concentration) > 100 μg ml⁻¹. In experiments using ECTV (strain K-1), more pronounced antiviral activity of ST-246 (IC₅₀ = 0.003 μg ml⁻¹) and NIOCH-14 (IC₅₀ = 0.011 μg ml⁻¹) compared with NIOCH-32 (IC₅₀ = 0.54 μg ml⁻¹) was observed. Despite the fact that the indicator of antiviral efficacy of NIOCH-14 against MPXV (strain V79-1-005) was lower (IC₅₀ = 0.013 μg ml⁻¹) than that of ST-246 (IC₅₀ = 0.003 μg ml⁻¹), no differences were observed between the values of this indicator for the given drugs in experiments involving four VARV strains: India-3a, 6-58, Congo-9 and Butler (IC₅₀ = 0.001–0.004 μg ml⁻¹). At the same time, NIOCH-32 demonstrated the lowest antiviral activity in all these studies (IC₅₀ = 0.153 μg ml⁻¹ for MPXV and IC₅₀ = 0.032–0.078 μg ml⁻¹ for four VARV strains). The therapeutic indices (TI) of the substances against orthopoxviruses, which we studied, had the following maximal values: TI of NIOCH-32 was > 3100, TI of NIOCH-14 and ST-246 were > 100 000.

Antiviral activity of NIOCH-14 in ICR mice infected with ECTV

A comparative evaluation of the 50 % effective dose (ED₅₀) values of NIOCH-14 and ST-246 administered orally in different doses to ICR mice infected intranasally (i.n.) with 2.57 log₁₀ p.f.u. (10 LD₅₀) of ECTV was preliminarly performed before conducting in vivo studies involving orthopoxviruses highly pathogenic for humans. It was noted that ED₅₀ values of NIOCH-14 [3.59 (2.29–5.63)]
Chemically synthesized anti-smallpox compound NIOCH-14

µg (g mouse weight)^{-1} and ST-246 [5.08 (3.04–8.48) µg (g mouse weight)^{-1}] did not differ significantly. When estimating the survival rate (SR) and the average life expectancy (ALE) of mice, it was shown that at doses of both agents equal to 12.5 µg g^{-1} and larger these indicators were significantly higher (P≤0.003) than in the control. Using NIOCH-14 at the dose 6.25 µg g^{-1} resulted in an increase in both SR and ALE of infected mice, whereas ST-246 induced an increase only in ALE, but not SR, relative to the control. At the same time, when NIOCH-14 was administered at a dose of 3.12 µg g^{-1}, the value of ALE (16.8 ± 5.6 days) significantly differed (P≤0.001) from that of the control (10.2 ± 2.8 days) but did not differ from the ALE observed when using ST-246 at the same dose (13.6 ± 5.3 days, not different from the control). Based on the analysis of the results obtained, it can be concluded that NIOCH-14 is comparable to ST-246 in antiviral efficacy against ECTV in vivo.

The ‘therapeutic window’ (the period during which the administration of a drug p.i. is effective) was determined for NIOCH-14 in experiments where ICR mice were infected i.n. with ECTV at a dose of 2.57 log₁₀ p.f.u. (10 LD₅₀) (Table 1).

It was shown that if NIOCH-14 was first administered to mice 1 day or 1 h before infection (b.i.) with ECTV or 1 or 2 days p.i. followed by daily administration until 9 days p.i., the survival rate in these groups of mice was 100%. The initial administration of NIOCH-14 within 3 or 4 days after infecting mice with ECTV also provided 90 and 100% survival of animals, respectively. Further delay in the start of treatment until 5 or 6 days p.i. reduced their survival rate to 60%, although this indicator still remained significantly higher than in the control group. We found that without the use of anti-poxvirus agents the peak of death (up to 50%) of mice was observed 7 days p.i.; therefore, it was appropriate for this research to start the administration of NIOCH-14 within 7 days after infecting mice with ECTV.

When conducting studies to assess ECTV concentration in organs and tissues of mice 6 days p.i., its absence was recorded in sera of both control and experimental animals to which drugs were administered at a dose of 50 µg g^{-1} 1 h p.i. and then daily for 5 days p.i. At the same time, the highest virus concentrations were revealed in the nose, lungs, spleen and liver of the control mice (Fig. 2). In other organs (trachea, brain, liver, kidney and pancreas) ECTV titres were significantly lower than in the lungs. The administration of NIOCH-14 and ST-246 resulted in a reduction of ECTV titres in the lungs as compared with the control by 1.33 and 1.50 log₁₀, respectively. In other organs examined using both antiviral compounds, the virus concentrations also became significantly lower than those in the control. As a result, the use of both NIOCH-14 and ST-246 resulted in a significant reduction of ECTV production in the lungs, nose and brain as well as essentially complete absence in trachea, liver, spleen, kidneys and pancreas of infected mice.

Pathomorphological investigation of the lungs of infected mice in the control and experimental groups showed an observable effect of anti-poxvirus agents. The lungs of animals in the control group had significant haemodynamic disorders expressed as sharp hyperaemia of capillaries with a massive release of plasma and red blood cells in alveoli, which often spread to the entire lung (Fig. 3a). Medium-size and small bronchi were necrotic with abundant infiltration of the walls and the surrounding parenchyma by neutrophils. In animals treated with NIOCH-14 and ST-246, haemodynamic and inflammatory-necrotic effects in the lungs were similar and significantly smaller than in the control group. Hyperaemia of capillaries of interalveolar walls and airines violations like distelectasis and atelectasis were mainly observed, areas of reduced aeration of parenchyma in some cases were accompanied by moderate inflammatory cellular reaction with rare haemorrhages. Only in one animal that received NIOCH-14 and two others treated with ST-246, were necrotic lesions of individual bronchi detected (Fig. 3b, c).

Table 1. Determination of the therapeutic window of NIOCH-14 administered orally at a dose of 50 µg g^{-1} to ICR mice infected i.n. with ECTV (strain K-1) at a dose of 2.57 log₁₀ p.f.u. (10 LD₅₀)

<table>
<thead>
<tr>
<th>Time of NIOCH-14 administration to mice</th>
<th>Number (%) of survivors</th>
<th>ALE (days; mean ± SD)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day before infection (b.i.)</td>
<td>10§ (100)</td>
<td>21.0 ± 0.0§</td>
</tr>
<tr>
<td>1 h b.i.</td>
<td>10§ (100)</td>
<td>21.0 ± 0.0§</td>
</tr>
<tr>
<td>1 day p.i.</td>
<td>10§ (100)</td>
<td>21.0 ± 0.0§</td>
</tr>
<tr>
<td>2 days p.i.</td>
<td>10§ (100)</td>
<td>21.0 ± 0.0§</td>
</tr>
<tr>
<td>3 days p.i.</td>
<td>9§ (90)</td>
<td>19.8 ± 3.8§</td>
</tr>
<tr>
<td>4 days p.i.</td>
<td>10§ (100)</td>
<td>21.0 ± 0.0§</td>
</tr>
<tr>
<td>5 days p.i.</td>
<td>6§ (60)</td>
<td>17.5 ± 4.9§</td>
</tr>
<tr>
<td>6 days p.i.</td>
<td>6§ (60)</td>
<td>16.1 ± 6.4</td>
</tr>
<tr>
<td>Placebo (control) 1 day b.i.∥</td>
<td>0 (0)</td>
<td>10.2 ± 2.8</td>
</tr>
</tbody>
</table>

*NIOCH-14 was administered to mice according to the following schemes: 1 day b.i., 1 h b.i., 1, 2, 3, 5 and 6 days p.i. and then daily for 9 days after the first administration of the agent.
†ALE of mice was calculated taking into account the maximum animal observation period – 21 days p.i. (the guaranteed time of termination or specific death of infected mice was accepted as the maximum life expectancy for surviving mice).
‡Difference from the control by the χ² test at P<0.003.
§Difference from the control by Mann–Whitney U-test, P<0.001.  
∥Solution containing 0.75 % methylcellulose and 1 % Tween 80 was administered to mice at a dose of 0.2 ml 1 day b.i. and then daily for 9 days after the first administration of the agent.
Antiviral activity of NIOCH-14 in ICR mice and marmots infected with MPXV

The preventive efficacies of the drugs were estimated in ICR mice infected i.n. with a dose of 3.4 log_{10} p.f.u. (10 ID_{50}) of MPXV by recording the presence and titres of the virus in the lungs of these animals 7 days p.i. The results of these investigations are presented in Table 2. According to Table 2, it was noted that the amount of MPXV in the lungs of mice treated with NIOCH-14 and ST-246 and the percentage of infected animals 7 days p.i. were significantly lower than those of the control (at P≤0.05). It was found that NIOCH-32 did not significantly suppress these parameters as compared with the control. The use of NIOCH-14 and ST-246 at the same doses did not reveal any significant differences between the efficacies of these drugs. Histological examination of the lungs of infected mice treated with NIOCH-14 and ST-246 showed either complete absence of inflammatory-necrotic changes or their rare occurrence and mild degree as compared with the control-infected animals (not shown in Fig. 3).

The next series of experiments on the comparative assessment of therapeutic and preventive efficacies of NIOCH-14 and ST-246 was carried out using MPXV at a dose of 3.7 log_{10} p.f.u. (30 ID_{50}) by infecting marmots i.n. and recording clinical signs of the disease (including death). Before the experiment, the whole group of animals dedicated for research demonstrated the absence of anti-MPXV antibodies in the neutralization reaction. The study results are presented in Table 3.

The data in Table 3 show that all marmots in the control group became ill and showed clinical symptoms (pyrexia, single- or double-sided submandibular lymphadenitis, discrete pox-like rash on visible skin and mucosa areas, serous-purulent rhinitis, conjunctivitis, blepharitis, violation of coordination, tremors of extremities, increased aggressiveness, ruffled fur) 9–12 days p.i. At the same time, in marmots of experimental groups treated with NIOCH-14 and ST-246 no signs of the disease were observed throughout the whole observation period after intranasal infection with MPXV as compared with the control animals (Fig. 3d, e). This showed that both tested agents had equally significant therapeutic and preventive effects.

Relatively high titres of anti-MPXV antibodies were detected in the neutralization test 28 days p.i. in all marmots of both the surviving animals and the experimental group (Table 3).

Antiviral activity of NIOCH-14 in ICR and SCID mice infected with VARV

In the next series of experiments, a comparative assessment of the prophylactic efficacies of ST-246 and NIOCH-14 was...
performed in ICR and SCID mice infected i.n. with VARV at doses of 3.7 \( \log_{10} \) p.f.u. (10 LD_{50}) and 4.5 \( \log_{10} \) p.f.u. (10 LD_{50}), respectively. The presence and titres of the virus were recorded in the lungs of ICR mice 3 days p.i. and in SCID mice 4 days p.i. The results of these studies are presented in Tables 4 and 5.
Table 2. Therapeutic-prophylactic activities of substances administered orally to ICR mice infected i.n. with MPXV V79-1-005 strain at a dose of 3.4 log10 p.f.u. (10 ID50)

<table>
<thead>
<tr>
<th>Indicator</th>
<th>NIOCH-14</th>
<th>NIOCH-32</th>
<th>ST-246</th>
<th>Placebo*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily dose of administered substances (mg kg−1)</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>*</td>
</tr>
<tr>
<td>Number of animals in the experiment</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Virus amount in the lungs (log10 p.f.u. per lungs) of each mouse</td>
<td>&lt;1.7</td>
<td>4.9</td>
<td>&lt;1.7</td>
<td>4.9</td>
</tr>
<tr>
<td>7 days p.i.</td>
<td>1.7</td>
<td>4.9</td>
<td>&lt;1.7</td>
<td>4.9</td>
</tr>
<tr>
<td>Mean ±4√(log10 virus amount) (n=6)</td>
<td>1.7 ±0.05</td>
<td>4.5±0.7</td>
<td>1.9±0.6</td>
<td>4.9±0.1</td>
</tr>
<tr>
<td>VPII (log10) in mouse lungs§</td>
<td>3.2</td>
<td>0.4</td>
<td>3.0</td>
<td>ND</td>
</tr>
</tbody>
</table>
| Number (%) of infected mice IPC of mice¶ | 0 || (0) | 0 (100) | 1 || (17) | 6 (100)
| DPC of marmots§ | 50 | 50 | ND |
| Anti-MPXV antibody titres in marmots || From 1 : 25 to 1 : 625 to 1 : 125 and 1 : 3125 |

Table 3. Therapeutic-prophylactic activities of substances administered orally to marmots infected i.n. with MPXV V79-1-005 strain at a dose of 3.7 log10 p.f.u. (30 ID50)

<table>
<thead>
<tr>
<th>Indicator</th>
<th>NIOCH-14</th>
<th>ST-246</th>
<th>Placebo*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily dose of administered substances (mg kg−1)</td>
<td>40</td>
<td>40</td>
<td>*</td>
</tr>
<tr>
<td>Number of animals in the experiment</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Number (%) of sick marmots</td>
<td>0</td>
<td></td>
<td>(0)</td>
</tr>
<tr>
<td>SPC of marmots§</td>
<td>100</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>Number (%) of dead marmots</td>
<td>0</td>
<td></td>
<td>(0)</td>
</tr>
<tr>
<td>DPC of marmots§</td>
<td>50</td>
<td>50</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-MPXV antibody titres in marmots</td>
<td></td>
<td>From 1 : 25 to 1 : 625 to 1 : 125 and 1 : 3125</td>
<td></td>
</tr>
</tbody>
</table>

ND, Value was not determined.

Substances were administered 1 day b.i., 2 h p.i. and then daily for 6 days p.i.

*Solution containing 0.75 % methylcellulose and 1 % Tween 80 was administered to mice of the control group at a dose of 0.2 ml per mouse in the same scheme.

†<1.7, a value below the sensitivity threshold (1.7 log10 p.f.u. per lungs) of the titration method (the limit of detection was used to calculate the mean).

§Difference from the control by Mann–Whitney U-test and Student’s t-test at P≤0.05.

§VPI, virus production inhibition index= log10 virus concentration in the mouse lungs in the control−log10 virus concentration in the mouse lungs in the experiment.

||Difference from the control by the χ2 test and Fisher exact test at P≤0.05.

†|IPC, infection protection coefficient=percentage of infected mice in the control—percentage of infected mice in the experiment.

It was noted (Table 4) that the number of infected ICR mice treated with ST-246 and NIOCH-14 and infected with VARV 3 days p.i. was significantly (P≤0.05) lower than that in the control group. Moreover, the drugs used significantly reduced VARV production in mouse lungs 3 days p.i. as compared with the control.

The data in Table 5 show that 4 days p.i. the number of SCID mice containing VARV in the lungs among those treated with NIOCH-14 was significantly lower than that among mice in the control group (at P≤0.05), which was not the case for ST-246. At the same time, 4 days p.i., both agents caused a significant reduction of the mean virus concentrations in mouse lungs as compared with the control. NIOCH-14 and ST-246 administration at equal doses showed no significant differences in the anti-poxvirus efficacies of these agents.

Histological examination of lungs of the control SCID (Fig. 3f) and ICR mice (not shown in Fig. 3) infected with VARV revealed similar pathological processes as observed in ICR mice infected with ECTV. The only difference was that in all animals infected with VARV, haemorrhagic events were less pronounced and oedema was stronger. The effect of using anti-smallpox agents was also clearly seen in SCID mice (Fig. 3g, h) and in ICR mice (not shown in Fig. 3). It manifested itself as a less pronounced swelling of interalveolar walls than in the control groups with preserved aeration of the lung tissue and the absence of inflammatory-necrotic events in bronchi.
Table 4. Therapeutic-prophylactic activities of substances administered orally to ICR mice infected i.n. with VARV Ind-3a strain at a dose of 4.5 log₁₀ p.f.u. (10 ID₅₀)

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Indicators for mice treated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIOCH-14</td>
</tr>
<tr>
<td>Daily dose of administered substances (μg g⁻¹)</td>
<td>60</td>
</tr>
<tr>
<td>Number of animals in the experiment</td>
<td>7</td>
</tr>
<tr>
<td>Virus amount in the lungs (log₁₀ p.f.u. per lungs) of each mouse 3 days p.i.†</td>
<td>2.4</td>
</tr>
<tr>
<td>Mean ± I₉₅ virus amount (log₁₀ p.f.u. per lungs) in mouse lungs</td>
<td>1.6 ± 0.6‡</td>
</tr>
<tr>
<td>VPII (log₁₀) in mouse lungs§</td>
<td>2.0</td>
</tr>
<tr>
<td>Number (% of infected mice)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>IPC of mice¶</td>
<td>57</td>
</tr>
</tbody>
</table>

ND, Value was not determined.
Substances were administered 1 day b.i., 2 h p.i. and then daily for 2 days p.i.
*Solution containing 0.75 % methylcellulose and 1 % Tween 80 was administered to marmots of the control group at the dose of 5 ml per marmot in the same scheme.
†<1.1, a value below the sensitivity threshold (1.1 log₁₀ p.f.u. per lungs) of the titration method (the limit of detection was used to calculate the mean).
‡Difference from the control by Mann–Whitney U-test and Student’s t-test at P≤0.05.
§VPII, virus production inhibition index= log₁₀ virus concentration in the mouse lungs in the control – log₁₀ virus concentration in the mouse lungs in the experiment.
¶Difference from the control by χ² test and Fisher exact test at P≤0.05.

DISCUSSION
Our chemical compound NIOCH-14 is a predecessor in synthesis stages and the closest analogue of ST-246 (Shishkina et al., 2015). The assessment of antiviral activity of NIOCH-14 against human adenovirus serotype 5, herpes simplex virus type 2, coxsackievirus A7 and West Nile virus showed that this compound did not inhibit their replication in vitro at concentrations up to 80 μg ml⁻¹. The lack of activity against two types of DNA-containing viruses and two types of RNA-containing viruses by these agents suggests the specificity of NIOCH-14 action against orthopoxviruses (Selivanov et al., 2011). It is known that ST-246 was not active against respiratory syncytial virus (Paramyxoviridae, single-stranded negative RNA), rotaviruses (Reoviridae, double-stranded RNA), bovine diarrhea virus (Flaviviridae, positive single-stranded RNA), Rift Valley fever virus (Bunyaviridae, single-stranded negative RNA) and some other RNA- and DNA-containing viruses except orthopoxviruses (Yang et al., 2005).

In experiments using 10 ID₅₀ of ECTV and ICR mice (Fig. 2), NIOCH-14 as well as ST-246 reduced the virus amount in the lungs in the control—percentage of infected mice in the experiment.

Table 5. Therapeutic-prophylactic activities of substances administered orally to SCID mice infected i.n. with VARV Ind-3a strain at a dose of 4.5 log₁₀ p.f.u. (10 ID₅₀)

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Indicators for mice treated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIOCH-14</td>
</tr>
<tr>
<td>Daily dose of administered substances (μg g⁻¹)</td>
<td>50</td>
</tr>
<tr>
<td>Number of animals in the experiment</td>
<td>10</td>
</tr>
<tr>
<td>Virus amount in the lungs (log₁₀ p.f.u. per lungs) of each mouse 4 days p.i.†</td>
<td>2.1 ± 2.2</td>
</tr>
<tr>
<td>Mean ± I₉₅ of each mouse</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>
| Difference from the control by Mann–Whitney U-test and Student’s t-test at P≤0.05.
§VPII, virus production inhibition index= log₁₀ virus concentration in the mouse lungs in the control – log₁₀ virus concentration in the mouse lungs in the experiment.
¶Difference from the control by χ² test and Fisher exact test at P≤0.05.

ND, Value was not determined.
Substances were administered 1 day b.i., 2 h p.i. and then daily for 3 days p.i.
*Solution containing 0.75 % methylcellulose and 1 % Tween 80 was administered to marmots of the control group at the dose of 5 ml per marmot in the same scheme.
†<1.1, a value below the sensitivity threshold (1.1 log₁₀ p.f.u. per lungs) of the titration method (the limit of detection was used to calculate the mean).
‡Difference from the control by Mann–Whitney U-test and Student’s t-test at P≤0.05.
§VPII, virus production inhibition index= log₁₀ virus concentration in the mouse lungs in the control – log₁₀ virus concentration in the mouse lungs in the experiment.
¶Difference from the control by χ² test and Fisher exact test at P≤0.05.

ND, Value was not determined.
Substances were administered 1 day b.i., 2 h p.i. and then daily for 2 days p.i.
*Solution containing 0.75 % methylcellulose and 1 % Tween 80 was administered to marmots of the control group at the dose of 5 ml per marmot in the same scheme.
†<1.1, a value below the sensitivity threshold (1.1 log₁₀ p.f.u. per lungs) of the titration method (the limit of detection was used to calculate the mean).
‡Difference from the control by Mann–Whitney U-test and Student’s t-test at P≤0.05.
§VPII, virus production inhibition index= log₁₀ virus concentration in the mouse lungs in the control – log₁₀ virus concentration in the mouse lungs in the experiment.
¶Difference from the control by χ² test and Fisher exact test at P≤0.05.
concentration in the primary target organs (lungs, trachea and nose) and particularly in the secondary target organs (brain, liver, kidney, spleen, pancreas) 6 days p.i. As a result, both agents inhibited systemic dissemination of the virus and protected mice from lethal ECTV infection (Table 1). Data about reduction of ECTV titres in mouse organs 4, 6 and 8 days after intranasal infection that are close to ours were obtained in studies on the protective action of ST-246 at doses of 50 and 100 μg (g mouse body weight)^−1 after lethal challenge with ECTV (Moscow strain, 10 and 100 LD₉₀) (Yang et al., 2005; Quenelle et al., 2007). The authors showed that in mice treated with ST-246, the virus titres in liver, spleen and lungs were below the detection limit.

Anti-MPXV antibody titres in surviving marmots after intranasal infection with MPXV and the therapeutic administered and preventive measures using NIOCH-14 and ST-246 were sufficiently high. This can be explained by the fact that the mechanism of antiviral action of these compounds is focused on the inhibition of formation of different enveloped forms of the virus (intracellular, cell-associated and extracellular). Another form of the virus (mature intracellular) located in infected target cells can also infect neighbouring cells (although to a limited extent) (McIntosh & Smith, 1996; Stern et al., 1997; Zhang et al., 2000), which, most likely, provided the formation of a relatively high level of antigenic viral load in these animals resulting in substantially increased humoral immunity approaching that of the control group of surviving marmots and after intranasal infection with MPXV. Similar results showing the accumulation of high titres of anti-orthopoxvirus antibodies in experiments involving ST-246 in different animals were also obtained by other researchers (Huggins et al., 2009; Stabenow et al., 2010; Smith et al., 2011).

NIOCH-14 and ST-246 did not significantly differ in ED₉₀ values defined in experiments using ECTV. However, in studies involving ST-246 and cowpox virus performed under similar conditions by other scientists (Quenelle et al., 2007; Quenelle & Kern, 2010) the value of this indicator for mice was significantly higher – about 30 μg g⁻¹.

When assessing the ‘therapeutic window’ of NIOCH-14 (50 μg g⁻¹) for mice infected with ECTV (10 LD₉₀), it was noted that its use was effective when started 1 h b.i. or within 1–6 days p.i. The same period during which it was appropriate to start using this drug after intranasal challenge with ECTV (8 LD₉₀) was noted in mice in similar research using ST-246 (100 μg g⁻¹) (Parker et al., 2012).

Despite some similarity of structures of NIOCH-14 and ST-246, it should be noted that the process of producing NIOCH-14 has a number of advantages as compared with the methods described for ST-246 synthesis. For example, ST-246 synthesis is performed using catalysts and an absolute solvent (waterless ethanol) by heating the reaction mixture (87 % product yield) (Bailey et al., 2007). In the patents published, absolute solvents and catalysts are not used; however, ST-246 is produced by heating (boiling in ethanol overnight), followed by purification with column chromatography (51 % product yield) (Jordan et al., 2006, 2008, 2012).

NIOCH-14 production does not require catalysts, absolute solvents and heating the reaction mixture (Shishkina et al., 2015) as used in ST-246 production (Bailey et al., 2007). In addition, column chromatography is not used in NIOCH-14 separation and purification in contrast to ST-246 production according to USA patents (Jordan et al., 2006, 2008, 2012). NIOCH-14 synthesis occurs at the temperature of the reaction mixture plus 2–10 °C, the precipitate is filtered and washed on a filter with cold ethanol (yield 96 %) (Shishkina et al., 2015). Therefore, the process of NIOCH-14 synthesis is simpler and more profitable than ST-246 production.

In most of our studies involving three orthopoxviruses, the chemicals NIOCH-14 and ST-246, which are close analogues, were administered to experimental animals at doses of 40–60 μg g⁻¹. This dose range was chosen based on literature data demonstrating that ST-246 at doses from 30 to 100 μg g⁻¹ was effective against many orthopoxvirus infections in experiments on animals other than primates (Nalca et al., 2008; Quenelle & Kern, 2010; Smith et al., 2011).

Thus, in the studies presented, the chemical compound NIOCH-14 possessed high antiviral efficacy against ECTV, MPXV and VARV in vitro and in vivo, which was comparable to the similar compound ST-246 in all parameters tested. NIOCH-14 has advantages over ST-246 relating to its production method. This allows us to draw a conclusion about the prospects of further investigation of the mechanisms of NIOCH-14 antiviral action to develop a new anti-poxvirus drug on this basis.

**METHODS**

All experiments with live VARV and MPXV were conducted at SRC VB Vector in a maximum containment facility (BSL-4) using insulating pneumatic suits.

**Animals.** The studies involved the use of outbred male and female 10- to 14-day-old and 16- to 20-day-old ICR mice (weighing 8–10 g and 12–14 g, respectively) obtained from the nursery of SRC VB Vector. These animals that are similar to HA (ICR) mice from the Jackson laboratory originated from Swiss Webster mice purchased previously by the former Soviet Union. The studies also involved male and female 18- to 21-day-old immunodeficient SCID Hairless Outbred SHO-Pkrdc⁻/⁻Hrd⁻/⁻ mice (weighing 12–14 g) obtained from the SPF vivarium of ICG (RMEM61914 x 0005 & RMEM612214 x 0010) where this lineage came from the Charles River Laboratories in 2012 as well as 1- to 2-year-old male and female Baibak marmots (Marmota bobak) weighing 3–4 kg and obtained from the Pushkin nursery, Moscow region.

Experimental animals were kept on a standard diet with enough water in accordance with the veterinary legislation and requirements for humane animal care and use in experimental studies (National Research Council of the National Academies, 2011). Research and
Cell culture. A continuous Vero cell culture obtained from the Cell Culture Collection of FBRIC VC Vector was used to produce a virus-containing suspension and to titrate various samples. Vero cell monolayer was grown in DMEM medium (OJSC BioloT) in the presence of 10 % FBS (HyClone) supplemented with penicillin (100 IU ml⁻¹) and streptomycin (100 μg ml⁻¹). The same medium supplemented with 2 % FBS, penicillin (100 IU ml⁻¹) and streptomycin (100 μg ml⁻¹) was used for supporting virus cultivation.

Viruses. ECTV (strain K-1), MPXV (Central African strain V79-1-005) and VARV (four strains : 6-58, Ind-3a, Congo-9 and Butler) obtained from the State Collection of Viral Infections and Rickettsioses Agents of SRC VC Vector were used for in vitro and in vivo studies involving NIOCH-14 and ST-246. Virus-containing suspensions with concentrations from 5.6 to 6.7 log₁₀ p.f.u. ml⁻¹ were prepared in Vero cell culture medium using these strains. Virus-containing material was packaged in individual tubes and stored at a temperature of −70 °C.

Chemical compounds under study. The study involved the use of the chemical compound NIOCH-14 [7-[[{[(4-trifluoromethyl)phenyl]formohydrazido[carbonyl]}tricyclo(3.2.2.0²,6)non-8-en-6-carboxylic acid] synthesized at NIOCH according to the technique described previously (Shishkina et al., 2015). The chemical compound with an established activity against smallpox: ST-246. NIOCH-32 and ST-246 [N-[3,5-dioxo-4-azatricyclo(5.3.2.0²,6)dodec-11-en-4-yl]-4-(trifluoromethyl)benzamide] synthesized for research purposes at NIOCH according to the technique described by Bailey et al. (2007) was used as a positive control. In some cases the chemical compound NIOCH-32 [N-[3,5-dioxo-4-azatricyclo(5.3.2.0²,6)dodec-11-en-4-yl]-2-hydroxybenzamide hydroxyhydrate] possessing a weaker anti-orthopoxviral effect than NIOCH-14 and ST-246 in vitro was also used (Kabanov et al., 2013). The chemical names of the compounds are given under the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC).

Determination of cytotoxicity and anti-orthopoxviral activities of compounds. To determine the cytotoxicity and antiviral activities of agents against ECTV, MPXV and VARV, we adapted a method using 96-well plates (Baker et al., 2003; Kabanov et al., 2013). The Emax plate spectrophotometer (Molecular Devices) and the SoftMax 4.0 Software (Molecular Devices), which automatically calculated the TC₅₀ and IC₅₀ concentrations of the agents, were used to evaluate the results: TC₅₀ is the concentration at which 50 % of cells in uninfected monolayer are destroyed; IC₅₀ is the substance concentration at which 50 % of cells in infected monolayer are preserved. The therapeutic index (TI) was determined by the ratio of TC₅₀ and IC₅₀ values.

Methods used to infect the animals and virological analysis of samples. In all cases mice were challenged i.n. after isoflurane inhalation anaesthesia by administering a total of 0.03 ml of virus-containing liquid in both nostrils. The animals were euthanized by cervical dislocation.

In experiments with ECTV to study the efficacy of drugs, ICR mice (weighing 12–14 g) were infected i.n. with a dose of 2.57 log₁₀ p.f.u. (10 LD₅₀) and observed for 21 days p.i. until their deaths. Six days p.i., blood was taken from retro-orbital venous sinuses of four mice in some groups before euthanasia, and after euthanasia lungs, nasal septum with mucosa, trachea, brain, liver, spleen, kidneys and pancreas were collected to prepare 10 % homogenates from each animal separately and for the subsequent determination of ECTV titres. In addition, a portion of the lungs was used for histological examination. ICR mice (weighing 8–10 g) were used as model animals to study the drug efficacy in experiments with MPXV (Sergeev et al., 2015a). The animals were challenged with a dose of 3.4 log₁₀ p.f.u. (10 ID₅₀ determined by the presence of an infectious process in the animals’ lungs 7 days p.i.), and 7 days p.i. their whole lungs were collected after euthanasia to prepare 5 % homogenates from each animal separately.

Similar experiments were conducted using VARV and ICR mice (weighing 8–10 g) and SCID mice (weighing 12–14 g) as model animals (Titova et al., 2015). The animals were challenged with doses of 3.7 log₁₀ p.f.u. (10 ID₅₀ determined by the presence of an infectious process in the lungs of the animals’ lungs 3 days p.i.) and 4.5 log₁₀ p.f.u. (10 ID₅₀ determined by the presence of an infectious process in the lungs of ICR mice 4 days p.i.). Three and four days p.i., respectively, their whole lungs were collected after euthanasia to prepare 4 % homogenates from each animal separately.

The concentration of viable virus in the samples was determined by the plaque assay using Vero cell monolayer (Leparc-Goffart et al., 2005). The minimum virus amount that could be detected with the titration method used (the method threshold) was 0.3 log₁₀ p.f.u. ml⁻¹ (0.6 log₁₀ p.f.u. per lung) for ECTV and 1.1 log₁₀ p.f.u. per lung for VARV and 1.7 log₁₀ p.f.u. per lung for MPXV.

Marmots were also used as model animals to study the drug efficacy in experiments with MPXV (Sergeev et al., 2015b). The animals were challenged with the intranasal dose of 3.7 log₁₀ p.f.u. (30 ID₅₀ determined by the presence of a clinical picture of a pox-like disease) by administering 0.5 ml of virus-containing material in each nostril (a total of 1 ml). Before this procedure, the animals were anaesthetized intramuscularly with Zoletyl (at a dose of 25 μg g⁻¹) consisting of a mixture of active ingredients (tiletamire hydrochloride and zolazepam hydrochloride) in a 1 : 1 ratio. After the experiment, surviving marmots were euthanized by injecting lethal intravenous doses (60 μg kg⁻¹) of Zoletyl anaesthetic agent.

Schemes to administer chemicals to experimental animals. ICR mice infected with VARV received orally 0.2 ml of suspension of compounds under study prepared on the basis of methylcellulose with Tween 80, at a dose of 50 μg (g mouse weight)⁻¹ 1 h p.i. and then daily for 5 or 9 days p.i. (depending on the purpose of the experiment). To determine the therapeutic window, NIOCH-14 was first given once daily at a dose of 50 μg g⁻¹ at different times before and after challenge with ECTV: 1 day b.i., 1 h b.i., 1, 2, 3, 4, 5 and 6 days p.i. The same medium was then given for 9 days after the first administration of the drug. To determine ED₅₀ the drugs were given in doses of 0.78, 1.56, 3.12, 6.25, 12.50, 25.00, 50.00 μg (g mouse weight)⁻¹ 2 h p.i. and then daily for 9 days p.i.

ICR mice infected with MPXV were administered orally with 0.2 ml of suspensions of compounds under study at a dose of 60 μg g⁻¹ 1 day b.i., 2 h p.i. and then daily for 6 days p.i. Marmots infected with MPXV were administered orally with the drugs at a dose of 40 mg (kg weight)⁻¹ 1 day b.i., 2 h p.i. and then daily for 6 days p.i. After manual fixing without anaesthesia the animals received 5 ml of suspension of either compound in the oral cavity through the corner of the mouth behind large molars using an automatic pipette.

In experiments with VARV, ICR mice were administered orally with 0.2 ml of suspensions of compounds under study at a dose of 60 μg (g mouse weight)⁻¹ 1 day b.i., 2 h p.i. and then daily for 2 days p.i., and SCID mice received orally the compound at a dose of 50 μg g⁻¹ 1 day b.i., 2 h p.i. and daily for 3 days p.i.

Experimental animals in the control were administered with the solution containing 0.75 % methylcellulose and 1 % Tween 80 used to prepare the suspension of NIOCH-14, NIOCH-32 and ST-246 as a placebo according to the same schemes.
Determination of anti-MPXV antibody titres in marmots. The titres of anti-MPXV antibodies were determined in the plaque reduction neutralization test (Leparc-Goffart et al., 2005) using Vero cell monolayer and MPXV (Central African strain V79-1-005) for all marmots b.i. and for surviving ones p.i. in an experiment to study the drugs' efficacies. Marmots' blood samples were collected from the superficial vein of the lower leg after anaesthesia by intramuscular administration of Zoletyl at a dose of 25 μg kg⁻¹.

Histological examination. Organ samples were fixed in 4 % paraformaldehyde solution for light optical examinations. Further processing of material was conducted according to a generally accepted technique: successive dehydration in alcohol solutions with increasing concentrations, impregnation with xylene/paraffin mixture and embedding into paraffin blocks. Four–five micrometre thick paraffin sections were prepared on an HM 360 automatic rotary microtome (Microm). The sections were stained with haematoxylin and eosin. Light-optical examination and microphotography were performed on an Imager Z1 microscope (Zeiss) equipped with a high resolution camera (HRc). The software package AxioVision Rel.4.8.2 (Carl Zeiss MicroImaging) was used to analyse the images.

Statistical treatment of results. Statistical treatment of results was carried out with standard methods (Zaks, 1976) using the software package Statistica 6.0 (StatSoft) with assessment of significant differences (P ≤ 0.05) for 95 % confidence level (I95). ED₅₀ was calculated on the basis of animal survival indices according to the Spearman–Karber method. In experiments to evaluate the therapeutic and prophylactic effects of the agents, the comparison of the proportions of live or infected animals in groups was conducted by the χ² test and the exact Fisher test. ALE values are presented as mean ± se, and the virus amount in organs as mean ± I95. In the cases when the virus concentration in organ homogenates was below the sensitivity threshold of the titration method, the minimum detectable value, i.e. the sensitivity threshold of the titration method, was used to calculate the mean. The Mann–Whitney U-test was used to compare the mouse ALE, and the Mann–Whitney U-test and Student’s t-test were used to compare the virus titres in the animals’ organs.

REFERENCES


Chemically synthesized anti-smallpox compound NIOCH-14


