In vitro antiviral activity and kinetics of the inhibitory effect of some compounds against *Feline calicivirus* strain F9 – a surrogate model of human noroviruses

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**ABSTRACT**

In recent decades, searching for compounds inhibiting the caliciviruses (in particular the noroviruses) is of major scientific interest due to their substantial role in the infectious pathology. The development of effective anticalicivirus chemotherapy is important due to the lack of specific means for treatment and prevention of the calicivirus infections. Based on the previously obtained results from a screening for antiviral activity against *Feline calicivirus* (FCV) strain F9, four efficient antiviral compounds - oxoglaucine, ribavirin, PTU-23 and HBB were selected for further investigations. Cytotoxicity values and antiviral activity of the compounds were determined by neutral red uptake assay vs. three virus inoculation doses – 10, 100 and 1000 CCID₅₀ of FCV-F9 in Crandell-Reese feline kidney cell line (CRFK). A significant activity against FCV-F9 of oxoglaucine was detected; the compound ribavirin exhibited a moderate activity, while the compounds PTU-23 and HBB showed insignificant anticalicivirus effect. Kinetics of the antiviral activity of the tested compounds against FCV-F9 was determined by the one-step virus growth cycle experimental design in CRFK cells. All tested compounds showed activity against FCV-F9 when applied in the first hours after the virus infection (during the early stages of virus replication cycle).

**INTRODUCTION**

Family *Caliciviridae* includes a large number of important human and animal pathogens. It is now comprised of five genera, including *Norovirus*, *Sapovirus*, *Vesivirus*, *Lagovirus*, and *Nebovirus* (Robilotti et al., 2015; Alkan et al., 2015).

Genera *Norovirus* and *Sapovirus* include human enteric viruses. Members of these genera are responsible for sporadic cases and epidemic outbreaks of acute gastroenteritis in humans. Noroviruses are recognized as one of the leading etiological agents of acute viral gastroenteritis among the people of all ages in the industrialized countries. In recent two decades noroviruses are estimated to be responsible for over 90% of all outbreaks of acute viral gastroenteritis in Europe and the U.S.A., and for this reason they remain a serious problem of public health (Lopman et al., 2003; Zheng et al., 2010; Hall et al., 2012; Tumbarski et al., 2013; Vega et al., 2014).

Animal caliciviruses are known as causative agents of respiratory illness, conjunctivitis, stomatitis and hemorrhagic disease in animals (Bhella et al., 2008). Genus *Vesivirus* includes the *Feline calicivirus* (FCV) which causes acute upper respiratory disease, stomatitis, diarrhea and limping disease in cats (Radford et al., 2007); *Vesicular exanthema of swine virus* (VESV) associated with the vesicular exanthema in swine and *San-Miguel sea lion virus* (SMSV) which causes abortions and vesicular lesions in sea lions (Green et al., 2000). Genus *Lagovirus* includes the *Rabbit hemorrhagic disease virus* (RHDV) which causes the rabbit hemorrhagic disease and *European brown hare syndrome virus* (EBHSV) - the European brown hare syndrome in rabbits (McIntosh et al., 2007). Genus *Nebovirus* contains a single species – the bovine enteric calicivirus associated with enteric diseases in calves (*Newbury-I virus*) (Di Martino et al., 2011). A second nebo-like virus (*Kirklareli virus*) which may represent a second species within this genus has been described in cattle in Turkey (Alkan et al., 2015).

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Caliciviruses are small, nonenveloped, positive-sense RNA containing viruses. The viral genome is approximately 7.5 kb in length, encapsidated by an icosahedral capsid, which is 35 to 40 nm in diameter and consisting of 90 VP1 dimers. The genome contains up to four open reading frames (ORFs), the first of which (ORF1) encodes a polyprotein that is post-translationally cleaved to produce several nonstructural proteins critical to virus replication. ORF2 and ORF3 encode the major capsid protein VP1 and a putative minor structural protein VP2, respectively. ORF4 is still with unknown function (Bhella et al., 2008). In cell cultures, FCV strains grow fast and cause rapid cytopathic effects morphologically expressed in rounding and detachment of infected cells from the vessel surface followed by cell death (Ossiboff et al., 2007).

At present, there are not enough data published in the scientific literature for the specific treatment and prevention of the infections caused by caliciviruses, respectively noroviruses. Also little is known about the different antiviral compounds and their potential application as effective means for anticalicivirus chemotherapy. The inability of noroviruses to grow in cell cultures has greatly hampered the development of reliable methods for their detection, viability testing and finding of effective antiviral compounds. Therefore, knowledge of efficient inactivation by different methods or specific antivirals is limited and based on studies with cultivatable surrogate models of human noroviruses such as Feline calicivirus (FCV) (Bidawid et al., 2003; Duizer et al., 2004).

The aim of current study is to evaluate in vitro cytotoxicity, antiviral activity and kinetics of the effect of some highly efficient virus replication inhibitors with a different mode of action against the Feline calicivirus strain F9 - a surrogate model of human noroviruses.

MATERIALS AND METHODS

Cells

Monolayers of Crandell-Reese Feline Kidney cell line - CRFK (kindly supplied by Prof. S. Sattar, Centre for Research on Environmental Microbiology, Faculty of Medicine, the University of Ottawa, Canada) were used. Cells (2 × 10^5/mL) were cultured in Dulbecco’s modified Eagle’s medium - DMEM (Gibco, Grand Island, New York, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum - FCS (Gibco) and the antibiotics penicillin G (100 UI/mL), streptomycin (100 µg/mL) and gentamicin (30 µg/mL) at 37°C under humidified 5% CO₂ atmosphere for 24 hours. The maintenance DMEM for virus propagation was identical, but containing 2% of FCS.

Virus

Feline calicivirus (FCV) strain F9 (kindly supplied by Prof. S. Sattar, Faculty of Medicine, the University of Ottawa, Canada) was used.

Compounds

Four efficient calicivirus replication inhibitors with a different mode of action were used: oxoglucine (C_{20}H_{17}O_{3}N_{2}), synthesized by Stephan Philipov and provided by the Institute of Organic Chemistry, Bulgarian Academy of Sciences (BAS); ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), provided by Prof. Robert W. Sidwell, Logan, Utah, U.S.A.; PTU-23 (N-phenyl-N’-3-hydroxyphenylthiourea), synthesized by Prof. Georgi Vassilev, Institute of Plant Physiology, BAS, and HBB [2-(α-hydroxybenzyl)-benzimidazole], provided by Dr. Tatyana Dmitrieva, Moscow State University, Moscow, Russia. The molecular mass and the structural formula of the compounds are presented in Table 1.

All compounds with exception of ribavirin were preliminarily dissolved in dimethyl sulfoxide - DMSO (Fluka AG, Switzerland) and then in maintenance DMEM to the required molar concentrations.

Antiviral tests

Neutral red uptake assay

Neutral red uptake assay to determine the cytotoxicity and antiviral effect of all compounds was used. Confluent monolayers of CRFK cells in 96-well microplates (Cellstar, Greiner bio-one, Germany) after aspiration of the culture medium were infected with 100 µL per well of three viral inoculation doses - 10, 100 and 1000 CCID₅₀ (excluding the blanks and cell control wells). After 90 min of adsorption at 37°C and 5% CO₂, the excessive virus was discarded and 100 µL per well of serial dilutions of the tested compounds in DMEM were added. After 3 days of incubation at 37°C and 5% CO₂, the medium was removed and the cells were washed with 150 µL per well of prewarmed phosphate-buffered saline (PBS), which was aspirated and replaced with 100 µL per well of 0.4% neutral red (NR) (Fluka AG), preliminarily dissolved in DMSO. Cells were incubated for additional 3 hours at 37°C + 5% CO₂. Then NR was removed, monolayers were washed with PBS and 150 µL per well of NR-desorbt solution (acetic acid, ethanol and dd H₂O - 1:50:49) was placed. Plates were shaken for 10 min until NR has been extracted and formed a homogeneous solution. Measurement of the optical density (OD) was determined at λ = 540 nm in ELISA-reader 530 (Organon Teknika, Germany) according to the standard protocol.

One-step virus growth cycle experimental design

One-step virus growth cycle experimental design (timing-of-addition study) was carried out to determine the kinetics of the inhibitory activity of compounds against FCV-F9. Monolayers of CRFK cells were cultured for 24 hours in plastic tubes (Cellstar, Greiner bio-one, Germany). After removing the culture medium, viral inoculum (multiplicity of infection = 10) was placed for adsorption (90 min at 37°C and 5% CO₂). The excessive virus was discarded and the cells were washed with Hank’s solution. Then 900 µL per tube of maintenance DMEM were placed. The compound was added to DMEM (excluding the virus control tubes) in its maximal effective concentration at 0th, 1st, 2nd, 3rd, 4th and 5th hour after the virus adsorption. The samples were frozen subsequently at 2nd, 3rd, 4th, 6th and 8th hour for later titration. The samples were tested in monolayers of CRFK cells for presence/absence of cytopathic effect (CPE) and infectious virus titer was determined by Reed and Muench classical method (1938).
Table 1: Molecular mass and structural formula of the tested antiviral compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular mass</th>
<th>Structural formula*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxoglaucine (C_{20}H_{17}NO_{5})</td>
<td>351.3</td>
<td>![Oxoglaucine Structure]</td>
</tr>
<tr>
<td>Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide)</td>
<td>244</td>
<td>![Ribavirin Structure]</td>
</tr>
<tr>
<td>PTU-23 (N-phenyl-N'-3-hydroxyphenylthiourea)</td>
<td>244</td>
<td>![PTU-23 Structure]</td>
</tr>
<tr>
<td>HBB [2-(α-hydroxybenzyl)-benzimidazole]</td>
<td>225</td>
<td>![HBB Structure]</td>
</tr>
</tbody>
</table>


Cytotoxicity assay

Cytotoxicity tests of all compounds were done in the same plates simultaneously with antiviral tests. After formation of the cell monolayer, the growth medium was discarded and 100 μL of serial dilutions of the tested compounds in maintenance DMEM were added. During the incubation for 3 days, monitoring for microscopic cytotoxic effects at 24th, 48th and 72nd hour was carried out. The results were read at the 72nd hour by the neutral red uptake assay described above.

RESULTS AND DISCUSSION

As seen from the results presented in Table 2, the compounds ribavirin, HBB, and PTU-23 were relatively non-toxic for the CRFK cell line. In contrast, oxoglaucine was non-toxic only in the lower molar concentration and seemed to have a relatively low value of CC_{50}.

The tested antiviral compounds oxoglaucine, ribavirin, HBB, and PTU-23 showed inhibitory activity against FCV-F9 replication in varying degree. The inhibitory effect of compounds depended on their concentration in the culture medium as well as on the dosage of viral inoculum (Table 2).

The antiviral compounds demonstrated the inhibitory effect on FCV-F9 replication in low virus inoculation doses - 100 CCID_{50} and 10 CCID_{50}. At virus inoculation dose 100 CCID_{50}, only one compound - HBB did not show anticalicivirus activity.
At virus inoculation dose 10 CCID₅₀, all tested compounds showed activity against FCV-F9. These data are comparable to our previously published results from the primary screening obtained by the cytopathic effect (CPE) inhibition method (Tumbarski and Galabov, 2008).

Table 2: 50% cytotoxic concentrations (CC₅₀) and antiviral activity of the tested compounds against FCV-F9 expressed as 50% inhibitory concentration (IC₅₀) and selectivity index – SI (CC₅₀/IC₅₀) by neutral red uptake assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC₅₀ (µM/mL)*</th>
<th>IC₅₀ (µM/mL)*</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxoglaucine</td>
<td>7.97</td>
<td>0.88</td>
<td>9.06</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>180.11</td>
<td>48.53</td>
<td>3.71</td>
</tr>
<tr>
<td>PTU-23</td>
<td>607.97</td>
<td>108.75</td>
<td>5.59</td>
</tr>
<tr>
<td>HBB</td>
<td>409.9</td>
<td>-</td>
<td>73.63</td>
</tr>
</tbody>
</table>

*CC₅₀ and each IC₅₀ were calculated as mean value of two experiments/replicates.

Antiviral activity and kinetics of the inhibitory effect of oxoglaucine on the replication of FCV-F9

The recently explored and described aporphinoid alkaloid oxoglaucine isolated from the aerial parts of the plant Glaucum flavum Cranz, showed the highest anticaliciviral activity and selectivity ratio (CC₅₀/IC₅₀) - 39.85 (Table 2). Some authors reported remarkable inhibitory activity of oxoglaucine also against the replication of human rhinovirus 14 (HRV-14) and a large spectrum of enteroviruses (Georgieva and Galabov, 2008; Nikolaeva-Glomb et al., 2008).

Table 3: Antiviral effect of oxoglaucine on the replication of FCV-F9 in one-step virus growth cycle.

<table>
<thead>
<tr>
<th>Oxoglaucine</th>
<th>Infectious virus titer, log CCID₅₀/mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2⁰</td>
</tr>
<tr>
<td>0⁰</td>
<td>2.3</td>
</tr>
<tr>
<td>1⁰</td>
<td>2.3</td>
</tr>
<tr>
<td>2⁰</td>
<td>-</td>
</tr>
<tr>
<td>3⁰</td>
<td>-</td>
</tr>
<tr>
<td>4⁰</td>
<td>-</td>
</tr>
<tr>
<td>5⁰</td>
<td>-</td>
</tr>
<tr>
<td>KV**</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*The results were calculated as mean value of two experiments/replicates.

**KV – virus control.

Table 4: Antiviral effect of ribavirin on the replication of FCV-F9 in one-step virus growth cycle.

<table>
<thead>
<tr>
<th>Ribavirin</th>
<th>Infectious virus titer, log CCID₅₀/mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2⁰</td>
</tr>
<tr>
<td>0⁰</td>
<td>4.0</td>
</tr>
<tr>
<td>1⁰</td>
<td>4.0</td>
</tr>
<tr>
<td>2⁰</td>
<td>-</td>
</tr>
<tr>
<td>3⁰</td>
<td>-</td>
</tr>
<tr>
<td>4⁰</td>
<td>-</td>
</tr>
<tr>
<td>5⁰</td>
<td>-</td>
</tr>
<tr>
<td>KV**</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*The results were calculated as mean value of two experiments/replicates.

**KV – virus control.

In one-step virus growth cycle experiments, some characteristics of FCV replication cycle were taken into account - rapid growth, a lag phase of about 3.5 h, followed by exponential growth of about 3-4.5 h and maximal production of virus (peak yield) at 8-12 h post infection (p.i.) (Ossiboff et al., 2007).

Oxoglaucine revealed maximal inhibitory effect when added to the maintenance medium during the first four hours p.i. which coincided with the lag phase of FCV-F9 replication. This led to a significant reduction of the infectious virus titer with 2.6 log CCID₅₀/mL at the 4th h of virus replication compared to the virus control levels. Adding of oxoglaucine during the exponential phase of virus cycle did not affect the virus replication and it reached the peak titre at 8th h p.i. (Table 3).

Antiviral activity and kinetics of the inhibitory effect of ribavirin on the replication of FCV-F9

According to the antiviral activity and selectivity ratio, the broad-spectrum antiviral agent ribavirin occupied an intermediate position with selectivity ratio of 27.71 vs. virus inoculation dose 10 CCID₅₀ (Table 2). The antiviral compound ribavirin possesses a polycomponent mechanism of action. Its influence is based predominantly on various effects of the host cells by reduction of GTP pool; inhibition of 5’-cap formation on mRNAs, etc. (Galabov and Angelova, 2006). In vitro anticalicivirus efficacy of ribavirin was described by Povey (1978) who reported a marked effect on the FCV strain 255 by partial to complete suppression of the viral CPE in cell cultures. Antiviral activity of ribavirin was confirmed also by our previous study with FCV strain F9 (Tumbarski and Galabov, 2008).

As seen from the results in Table 4, ribavirin revealed a significant inhibitory activity when applied in the medium at the first four hours p.i. Adding of ribavirin during the late lag phase of the replication led to a marked and prolonged decrease of the infectious titer of FCV-F9 with 2.5 log CCID₅₀/mL at 4th h p.i. Application of ribavirin in exponential phase did not reduce the FCV-F9 titer and it reached the peak level at 8th h p.i. (compared to the relevant controls).

Antiviral activity and kinetics of the inhibitory effect of PTU-23 on the replication of FCV-F9

The antiviral compound PTU-23 showed a low cell protective effect against FCV-F9 at virus inoculation doses 100
CCID₅₀ and 10 CCID₅₀ and low selectivity index - 5.59 and 8.26 respectively (Table 2). PTU-23 is an antiviral compound that inhibits the synthesis of viral RNA, as a result of suppression of the synthesis of a viral protein with a regulatory function in the replication cycle (Galabov and Angelova, 2006).

**Table 5:** Antiviral effect of PTU-23 on the replication of FCV-F9 in one-step virus growth cycle.

<table>
<thead>
<tr>
<th>PTU-23</th>
<th>Infectious virus titer, log CCID₅₀/mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2⁰</td>
</tr>
<tr>
<td>0⁰</td>
<td>2.7</td>
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<td>4⁰</td>
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<td>5⁰</td>
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<tr>
<td>KV**</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*The results were calculated as mean value of two experiments/replicates.

**KV** – virus control.

PTU-23 demonstrated antiviral effect when added to the maintenance medium during the lag phase of the FCV-F9 replication. This led to a reduction of the infectious virus titer with 2 log CCID₅₀/mL at 3⁰ h p.i. compared to the virus control levels. PTU-23 did not affect the replication when added in the exponential phase and FCV-F9 rapidly reached the peak titre at 8⁰ h p.i. (Table 5).

**Antiviral activity and kinetics of the inhibitory effect of HBB on the replication of FCV-F9**

The antiviral compound HBB demonstrated the lowest anticalicivirus activity only vs. virus inoculation dose 10 CCID₅₀ and SI = 1.66 (Table 2). This substance is known as a selective picornavirus inhibitor which inhibits the replication of HRV-14 (Georgieva and Galabov, 2008). It does not affect processes like adsorption, uncoating or viral protein synthesis, however, it blocks viral RNA synthesis and inhibits the activity of virus-specific RNA-polymerase, resulting in a selective suppression of the ssRNA synthesis (dsRNA synthesis remaining intact) (Shimizu et al., 2000; Galabov and Angelova, 2006).

**Table 6:** Antiviral effect of HBB on the replication of FCV-F9 in one-step virus growth cycle.

<table>
<thead>
<tr>
<th>HBB</th>
<th>Infectious virus titer, log CCID₅₀/mL*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2⁰</td>
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<tr>
<td>0⁰</td>
<td>4.0</td>
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<td>1⁰</td>
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<td>4⁰</td>
<td>-</td>
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<tr>
<td>5⁰</td>
<td>-</td>
</tr>
<tr>
<td>KV**</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*The results were calculated as mean value of two experiments/replicates.

**KV** – virus control.

HBB exhibited weak inhibitory activity against FCV-F9. Similarly to the other of tested compounds, the maximal effect was observed when HBB was added to the maintenance medium during the lag phase of the FCV-F9 replication which led to a reduction of the infectious virus titer with 1.5 log CCID₅₀/mL at 4⁰ h p.i. compared to the virus controls. HBB did not show activity when added in the exponential phase of replication cycle and the FCV-F9 reached the peak titre at 8⁰ h p.i. (Table 6).

Searching for compounds suppressing the replication of caliciviruses is of great scientific and clinical importance due to their substantial role in the veterinary and human infectious pathology. The current study is the first report evaluating in details the inhibitory effect of some widely used in the chemotherapy practice antivirals and their potential application against the replication of FCV-F9 known as the best surrogate model of human noroviruses.

The second new and perspective approach of the present study was the investigation on the kinetics of inhibitory activity of these antiviral compounds on the replication of FCV-F9 by one-step virus growth cycle experimental design (timing-of-addition study). The data obtained from experimental analyses demonstrated that all tested compounds were effective against FCV-F9 when added to the maintenance medium in the first few hours post infection which coincides with the early stages of virus replication cycle (lag phase). These results can be taken into account in the development of more effective schemes for anticalicivirus chemotherapy.

**CONCLUSION**

All tested antivirals revealed inhibitory activity against the replication of FCV-F9 in varying degree. The current results obtained by neutral red uptake assay confirmed the previous data from the screening for anticalicivirus activity by CPE inhibition test. The effective compounds against FCV-F9 can be arranged as follow: oxoglaucine > ribavirin > PTU-23 > HBB. The results obtained from the investigations on the kinetics of the effect by one-step virus growth cycle showed that all antiviral compounds were most active against FCV-F9 when applied in the first hours of the virus infection. These data could be useful for the medical practice and could find application in the development of further strategies for treatment and prevention of calicivirus infections, respectively human norovirus infections.

**ACKNOWLEDGEMENTS**

All experiments were carried out at the Department of Virology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria. The author is thankful to Luchia Mukova (MSc) for assisting with some of the laboratory analyses as well as to Prof. Lilia Vassileva and Prof. Angel Galabov for the kind support during the work at the Institute.

**CONFLICT OF INTERESTS**

There is no conflict of interests.

**REFERENCES**


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