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# A luciferase-based screening method for inhibitors of alphavirus replication applied to nucleoside analogues

Leena Pohjala [a,b], Vladimir Barai [d], Alex Azhayev [c], Seppo Lapinjoki [c], Tero Ahola [a,*]

[a] *Program in Cellular Biotechnology, Institute of Biotechnology, P.O. Box 56 (Viikinkaari 9), University of Helsinki, 00014 Helsinki, Finland*
[b] *Drug Discovery and Development Technology Center, Faculty of Pharmacy, P.O. Box 56, University of Helsinki, 00014 Helsinki, Finland*
[c] *Department of Pharmaceutical Chemistry, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland*
[d] *Metkinen Chemistry Oy, Kuopio, Finland*

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**Abstract**

Several members of the widespread alphavirus group are pathogenic, but no therapy is available to treat these RNA virus infections. We report here a quantitative assay to screen for inhibitors of Semliki Forest virus (SFV) replication, and demonstrate the effects of 29 nucleosides on SFV and Sindbis virus replication. The anti-SFV assay developed is based on a SFV strain containing *Renilla* luciferase inserted after the nsP3 coding region, yielding a marker virus in which the luciferase is cleaved out during polyprotein processing. The reporter-gene assay was miniaturized, automated and validated, resulting in a Z′ value of 0.52. [$^3$H]uridine labeling for 1 h at the maximal viral RNA synthesis time point was used as a comparative method. Anti-SFV screening and counter-screening for cell viability led to the discovery of several new SFV inhibitors. 3′-Amino-3′-deoxyadenosine was the most potent inhibitor in this set, with an $IC_{50}$ value of 18 μM in the reporter-gene assay and 2 μM in RNA synthesis rate detection. Besides the 3′-substituted analogues, certain $N^6$-substituted nucleosides had similar $IC_{50}$ values for both SFV and Sindbis replication, suggesting the applicability of this methodology to alphaviruses in general.


*Keywords:* Semliki Forest virus; Sindbis virus; RNA replication; Luciferase; Antiviral screening

## 1. Introduction

*Semliki Forest virus* (SFV) is a positive-strand RNA virus belonging to the *Alphavirus* genus and *Togaviridae* family. Alphaviruses are relatively common in nature, and are usually spread by mosquitoes between mammalian or avian hosts. Some of these viruses are capable of causing epidemic diseases in humans and domestic animals (Griffin, 2001). The most recent example of a large alphavirus epidemic is the Chikungunya virus outbreak on islands in the Indian Ocean starting in 2005. Subsequently, an even larger Chikungunya outbreak occurred in India, with at least 1.4 million cases (Pialoux et al., 2007). There have been worries that the virus could establish itself in other regions, such as the Mediterranean (Charrel et al., 2007), and indeed the first locally transmitted cases have occurred in Italy (Angelini et al., 2007). Chikungunya and other 'Old World' alphaviruses usually cause fever, rash and arthritis, which can be very painful and persist for several months. Other representatives of these viruses include Ross River virus in Australia and the widespread Sindbis virus in Northern Europe and South Africa (Kurkela et al., 2005; Rulli et al., 2005). In Finland, strains of Sindbis virus cause a small epidemic every year in the early autumn, and a larger epidemic regularly every 7th year (Brummer-Korvenkontio et al., 2002). The 'New World' alphaviruses, such as Eastern, Western and Venezuelan equine encephalitis viruses commonly cause encephalitis in horses and humans. The most common of these

* Corresponding author. Tel.: +358 9 19159403; fax: +358 9 19159560.
*E-mail address:* tero.ahola@helsinki.fi (T. Ahola).

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viruses is Venezuelan equine encephalitis, which has caused recurrent epidemics (Griffin, 2001; Weaver and Barrett, 2004).

Despite their wide distribution and potential pathogenicity, neither chemotherapy nor vaccination is available for clinical alphavirus infections (Griffin, 2001; Sidwell and Smee, 2003). Furthermore, there are only a limited number of chemical agents described to inhibit alphavirus replication. Ribavirin (Virazole) was one of the first alphavirus inhibitors reported, showing a minimum inhibitory concentration (MIC) value of 32 μg/ml in reducing SFV-induced cytopathic effect (CPE) in chicken embryo fibroblasts (Huffman et al., 1973). Later, also a derivative of ribavirin, namely ribavirin-5′-sulphamate, has been described to inhibit SFV replication, with an $IC_{50}$ value of 10 μM in CPE reduction (Smee et al., 1988). Other studies on ribavirin's anti-alphaviral activity include the demonstration of its synergistic effects with the C-nucleoside analogues tiazofurin and selenazofurin against Venezuelan equine encephalitis virus (VEE) (Huggins et al., 1984) and with interferon α against Chikungunya and SFV (Briolant et al., 2004). However, probably the most effective alphavirus inhibitor found in the literature is 3′-fluoro-3′-deoxyadenosine ($Ado_{3'F}$), which was reported to have an $IC_{50}$ value of 4 μg/ml for SFV-induced CPE reduction (Van Aerschot et al., 1989) and of 10 μM against SFV and 5.3 μM against VEE in a plaque reduction assay (Smee et al., 1992). In fact, $Ado_{3'F}$ proved to be markedly inhibitory to the replication of a number of viruses including vaccinia virus, poliovirus-1, Coxsackie virus B4, SIN, reovirus-1 and SFV (Mikhailopulo et al., 1993). Other nucleosides that have been reported to have anti-alphaviral potential as tested with SFV include the carbocyclic nucleoside analogue, cyclopentenyl cytosine with $IC_{50}$ value of 0.4 μg/ml for CPE reduction (De Clercq et al., 1991), 6-azauridine ($IC_{50}$ 0.4 μg/ml for CPE reduction) (Briolant et al., 2004), and neplanocin A ($IC_{50}$ for CPE reduction 1 μg/ml) (De Clercq, 1985).

In addition to the modified nucleosides, also some non-nucleoside agents have been demonstrated to inhibit alphavirus replication. Arildone (4-[6-(2-chloro-4-methoxyohenoxy]hexyl-3,5-heptane dione) has been shown to significantly decrease SFV viral yield measured by a plaque assay at a concentration of 3 μg/ml (Kim et al., 1980). A more recent example of a non-nucleoside capable of inhibiting alphavirus replication is found by Kim et al. (2005), who demonstrated an $IC_{50}$ value of 1 μM for Sindbis virus production, using dioxan derivatives targeted against Sindbis capsid protein. The most widely applied endpoint in studies on alphavirus inhibitors is the reduction of virus-induced cytopathic effect measured by visual inspection, even though plaque reduction and RNA synthesis rate have also been used to some extent. Besides assay selection, other experimental parameters such as infection multiplicity and infection time course differ between different studies, making the comparison of quantitative results difficult.

The aim of the present study was to develop quantitative methods to detect potential antiviral agents against alphaviruses and to allow higher throughput screening for inhibitors of SFV replication. For this purpose, a luciferase reporter-gene assay based on recombinant SFV was developed. A set of 29 nucleoside analogues was screened for anti-SFV activity and counter-screened for effects on mammalian cell viability. Several nucleosides demonstrated activity against SFV and also against Sindbis virus, suggesting that the primary screening method was effective.

## 2. Materials and methods

### 2.1. Cells and viruses

Baby hamster kidney BHK-21 cell line was purchased from the American Type Culture Collection (ATCC code CCL-10). The cells were grown in Dulbecco's Modified Eagle's Medium (MEM) supplemented with 8% fetal calf serum (FCS), 2% tryptose-broth phosphate, 1% L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cultures were kept at 37 °C with 5% $CO_2$ atmosphere and 95% air humidity. Wild-type SFV and Sindbis virus stocks were derived from the infectious clones SFV4 and TOTO1101, respectively (Liljeström et al., 1991; Rice et al., 1987), by linearization, in vitro transcription and RNA transfection into BHK cells using electroporation. The initial virus stocks were titrated and amplified in fresh BHK cells at 0.01 PFU (plaque forming units)/cell for 24 h. These working stocks were titrated and used in all the experiments. Recombinant SFV containing *Renilla reniformis* luciferase insertion (SFV-Rluc) was produced from the infectious clone SFV-RlucH2, which was a kind gift of Dr. Andres Merits (University of Tartu, Estonia). Luciferase is inserted between the nsP3 and nsP4 coding regions in exactly the same way as EGFP in the clone SFV(3H)4-EGFP (Tamberg et al., 2007). The N-terminus of the inserted luciferase contains a preferred viral protease cleavage site amino acid sequence GIFSSDTGP and the C-terminus of luciferase contains 30 amino acids from the end of nsP3. Thus, it is efficiently released from the nonstructural polyprotein by the nsP2 protease. The virus stocks were obtained as described above. MEM containing 0.2% BSA and 20 mM Hepes (pH 7.2) was used as the medium for all dilutions and infections.

### 2.2. Nucleosides

Structures and chemical names are presented in Fig. 1 and the legend. Compounds **1** and **27** were purchased from Metkinen Chemistry (www.metkinenchemistry.com). Compounds **2–9** were prepared essentially as described (Barai et al., 2002a,b; Mikhailopulo et al., 1993; Zaitseva et al., 1994; Zinchenko et al., 1990; for a mini-review, see Mikhailopulo and Lapinjoki, 2006). Compounds **10–12**, **20–22** and **24** were prepared using recombinant enzymes as biocatalyst (Roivainen et al., 2007). Compounds **14–16** were prepared by a chemical method published earlier (Azhayev et al., 1979). Compounds **17–19**, **25** and **29** were prepared using whole *Escherichia coli* cells as a biocatalyst, essentially as published earlier (Zaitseva et al., 1999). Chemical synthesis of compounds **26** and **28** will be published elsewhere (A. Azhayev). Heterocyclic base **23** and a sample of nucleoside **22** for comparison were kindly supplied by Prof.