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

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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

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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.

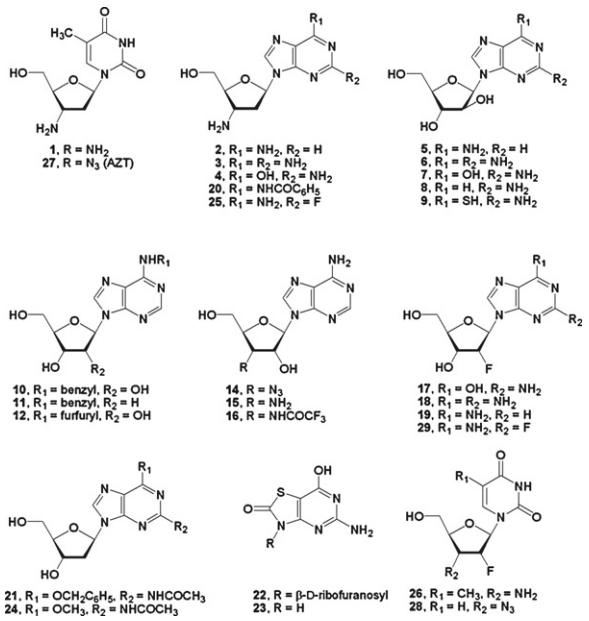

Fig. 1. The chemical structures of the modified nucleosides tested. (**1**) 3′-Amino-3′-deoxythymidine, (**2**) 3′-amino-2′,3′-dideoxyadenosine, (**3**) 2,3′-diamino-2′,3′-dideoxyadenosine, (**4**) 3′-amino-2′,3′-dideoxyguanosine, (**5**) 9-(β-D-arabinofuranosyl) adenine, (**6**) 2-amino-9-(β-D-arabinofurano syl)adenine, (**7**) 9-(β-D-arabinofuranosyl)guanine, (**8**) 2-amino-9-(β-D-arabinofuranosyl)purine, (**9**) 2-amino-9-(β-D-arabinofuranosyl)-6-mercaptopurine, (**10**) N^6-benzyladenosine, (**11**) N^6-benzyl-2′-deoxyadenosine, (**12**) N^6-furfuryladenosine, (**14**) 3′-azido-3′-deoxyadenosine, (**15**) 3′-amino-3′-deoxyadenosine, (**16**) 3′-trifluoroacetamido-3′-deoxyadenosine, (**17**) 2′-deoxy-2′-fluoroguanosine, (**18**) 2-amino-2′-deoxy-2′-fluoroadenosine, (**19**) 2′deoxy-2′-fluoroadenosine, (**20**) 3′-amino-2′,3′-dideoxy-N^6-benzoyladenosine, (**21**) N^2-acetyl-O^6-benzoyl-2′-deoxyguanosine, (**22**) 8-oxo-7-thiaguanosine, (**23**) 8-oxo-7-thiaguanine, (**24**) N^2-acetyl-O^6-methyl-9-(2-deoxy-β-D-ribofuranosyl)purine, (**25**) 3′-amino-2′,3′-dideoxy-2-fluoroadenosine, (**26**) 3′-amino-2′-fluoro-2′,3′-dideoxythymidine, (**27**) 3′-azido-3′-deoxythymidine, (**28**) 3′-azido-2′,3′-dideoxy-2′-fluorouridine and (**29**) 2′-deoxy-2′-fluoro-2-fluoroadenosine. Note that compound **13** (structure not shown) was excluded from the study due to its poor stability.

Frank Seela (University of Osnabrück, Germany). Nucleoside **22** was prepared by an enzymatic transglycosylation of base **23** as described for compounds **10**–**12** (Roivainen et al., 2007). Structures of nucleosides were supported by physico-chemical methods (UV, ^{1}H and ^{13}C NMR) and their purity was ≥97% as verified by TLC and RP HPLC. Ribavirin used as a reference compound was purchased from Sigma Aldrich.

2.3. Characterization of SFV-Rluc

The genetic stability of SFV-Rluc was analyzed by plaque purification followed by detection of luciferase positivity for each clone. Virus from 25 individual plaques was extracted after infection with the working stock of SFV-Rluc. Each extracted sample was then used to infect fresh cells, and the luciferase activities in the cultures were assayed 14 h post infection. In this experiment, 91% (21 out of 23) of the clones were luciferase positive. In addition to plaque extraction, Western blot analysis was used to verify the correct processing of nsP3 in SFV-Rluc. After lysis with 1% SDS, the samples for immunoblotting were boiled for 2 min and resolved by SDS-PAGE in a 10% gel before transfer to nitrocellulose membranes. Anti-nsP3 rabbit antiserum was used at 1:10,000 and swine anti-rabbit IgG used as secondary antibody at 1:3000.

2.4. RNA labeling

Viral RNA synthesis rate was determined by labeling BHK cell cultures on 35 mm dishes with 15 μCi of [5-^{3}H]uridine (GE Healthcare) for 1 h in 1 ml of medium, ending at the time points indicated in each experiment. To prevent host cell RNA synthesis, the cultures were treated with 2 μg/ml actinomycin D starting 1 h prior to labeling. To determine the time points of maximal RNA synthesis, 1 h pulses for MOI 0.001 were made hourly from 10 h to 17 h post infection, for MOI 0.01 from 8 h to 14 h, for MOI 0.1 from 6 h to 12 h, for MOI 1 from 4 h to 10 h, and for MOI 10 and 100 from 2 h to 8 h, respectively. For each pulse, the label was added directly to the actinomycin D-containing medium and the culture was incubated at 37 °C. Afterwards, the cultures were washed three times with PBS, the cells lysed with 1% SDS and the lysates heated to 70 °C for 1 min. The amount of incorporated label was determined by precipitation with 10% trichloro acetic acid followed by collection onto glass fiber filters and scintillation counting.

2.5. Luciferase detection

The *Renilla* luciferase activity indicating the production of nonstructural proteins and thus replication of SFV-Rluc was determined with Renilla Luciferase Assay System (Promega) according to the Provider's instructions. At the selected time, the cultures were lysed and 20 μl of the lysate was mixed with luciferase substrate solution followed by the luminometric measurement.

2.6. Plaque reduction assay

For viral yield analysis, a plaque assay was used, in which BHK cells were infected with serial dilutions of the SFV working stock (4.5×10^9 PFU/ml). After 1 h virus adsorption, the cultures were washed and incubated for 48 h in MEM supplemented with FCS, glutamine, Hepes, penicillin and streptomycin and containing 0.45% carboxymethyl cellulose. Afterwards, the cultures were checked under a microscope to exclude toxic effects by the nucleosides. Then, the cells were stained with crystal violet and the viral titer was calculated based on the number of plaques. For efficacy evaluation, 10-fold virus dilutions from 10^{-3} to 10^{-9} were used, with 100 μM nucleoside present throughout the experiment. In dose–response studies, virus dilutions 10^{-7}, 5×10^{-8}, 10^{-8}, 5×10^{-9} and 10^{-9} were used with each nucleoside concentration (1 μM, 5 μM, 10 μM, 25 μM, 50 μM and 100 μM).

2.7. Cytotoxicity assays

For host cell toxicity assessment, BHK cells were exposed in 96-well plates to the compounds ($V = 100$ μl) at various concentrations. After exposure, WST-1 reduction assay (Ishiyama

et al., 1995) and ATP level determination (Crouch et al., 1993) were performed. In the WST-1 assay, the cultures were washed twice with Hank's balanced salt solution, and 10 μl of WST-1 cell proliferation assay reagent (Roche Diagnostics) was added. After 1 h incubation, the absorbance at 440 nm was measured to detect the presence of the reduced formazan product, indicating the metabolic activity of the cell culture. In the ATP assay, the intracellular ATP content of the cells was determined by adding 100 μl Cell Titer GLO luminescent cell proliferation assay (Promega) into the wells and detecting the luminometric signal within 1 h.

2.8. Assay miniaturization and automation

The antiviral assays based on SFV-Rluc, as well as the assays used for mammalian cell viability assessment, were miniaturized to 96-well plate format. In this format of the antiviral assay, BHK cells were seeded into opaque white 96-well plates and the confluent cultures (approximately 10^5 cells/well) were infected with 100 PFU of SFV-Rluc. To automate the methods, pipetting protocols for Biomek FX workstation were created. The data quality in the miniaturized assays was controlled by exploiting the assay quality parameters signal-to-background $S/B = \mu_{signal}/\mu_{background}$, signal-to-noise $S/N = (\mu_{signal} - \mu_{background})/\sqrt{(\sigma_{singal}{}^2 + \sigma_{background}{}^2)}$ and signal window coefficient $Z' = 1 - ((3\sigma_{singal} + 3\sigma_{background})/(\mu_{signal} - \mu_{background}))$; μ = mean, σ = standard deviation (Zhang et al., 1999) throughout the experiments. Threshold values $S/B > 5$, $S/N > 10$ and $Z' > 0.5$ were set to assay robustness. The validation and automation of the cell viability assays was performed as recently described (Pohjala et al., 2007).

2.9. Data analysis

In primary assays, the inhibitory potency was expressed as a percentage of RNA synthesis or luciferase activity remaining, compared to the untreated control. For the compounds active in the primary screen, antiviral IC_{50} values were determined by fitting the assay points from dose–response studies into sigmoidal dose–response curves with GraphPad Prism 3.0 software.

3. Results

3.1. Antiviral assay based on RNA labeling

To set up a quantitative antiviral assay for determining the SFV RNA synthesis rate, a series of pilot experiments was performed. Based on consecutive 1 h uridine labeling pulses, the time course of SFV infections in BHK cells was investigated using multiplicities from 0.001 PFU/cell to 100 PFU/cell. The results indicate that each 10-fold decrease in the infection multiplicity causes an approximately 2 h delay in the maximal RNA synthesis time point (Table 1). A similar maximal uridine incorporation rate (about 30,000 cpm under these conditions) was always reached. This should represent the point when all the cells in the culture were infected, and maximal numbers of RNA replication complexes were formed. Based on these results, the infection multiplicity of 0.001 PFU/cell and sample collection time of 14 h post infection were selected for the antiviral assays.

Table 1
Maximal RNA synthesis time point during SFV-wt and SFV-Rluc infections

Multiplicity	Time point (h)[a]	
	SFV-wt	SFV-Rluc
0.001	14	14
0.01	11	
0.1	9	
1	7	
10	4.5	4.5
100	4	

[a] The time point when maximal viral RNA synthesis rate was reached after infection of BHK cells with different SFV-wt and SFV-Rluc multiplicities. The RNA synthesis rates were determined by uridine labeling using consecutive 1 h labeling pulses for each MOI (see Section 2). The time given is the end of the pulse.

3.2. Reporter-gene based antiviral assay

To allow a simplified assay protocol and higher throughput, a recombinant SFV containing *Renilla reniformis* luciferase reporter-gene was propagated. The luciferase gene, together with an additional cleavage site for the nsP2 protease of the virus, was inserted in the end of the nsP3 region of SFV genome, leading to release of the luciferase during the viral polyprotein processing. After propagation, the marker virus was characterized to have a replication profile similar to the wild-type virus, indicated by the infection time scale (Table 1) and polyprotein processing (nsP3 immunoblotting, data not shown), as well as by correlation of RNA synthesis rate to luciferase expression, and demonstration of reasonable genetic stability (luciferase positivity of isolated plaques >90%).

The SFV-Rluc was utilized to develop an antiviral assay more suitable for larger screens, as the avoidance of the filtration step required in radiometric detection enables a more miniaturization and automation-friendly assay protocol. In this assay, the viral replication is detected by determining the luciferase activity from the lysed cultures. Starting from 35 mm dishes, the assay was miniaturized into 96-well format, and the miniaturized assay was also successfully automated. After optimization and validation, reasonable assay robustness was achieved, as indicated by validation parameters shown in Table 2.

3.3. Effects of the modified nucleosides on SFV replication

The RNA labeling assay and the reporter-gene assay were utilized to investigate the effects of a set of modified nucleosides (Fig. 1) on SFV replication. The efficacy of each nucleoside was assayed in the primary screen for antiviral activity using a concentration of 200 μM, and the potency of the nucleosides active in the primary screen was estimated by dose–response experiments. Fig. 2 represents the primary screen activities of each nucleoside, and IC_{50} values are shown in Table 3 together with the results from further experiments (see below). In these studies, several new inhibitors of SFV replication were discovered.

Table 2
Validation parameters for the optimized reporter-gene assay

Parameter	Value
CV	17.1
S/B	152
S/N	6.4
Z'	0.52
Plate-to-plate	8.9%
Day-to-day	11.6%
Hit limit	<70%
Control *c*	25 μM
Control limits	$8 < x < 27\%$

CV = coefficient of variation, S/B = signal-to-background ratio, S/N = signal-to-noise ratio, Z' = signal window coefficient, plate-to-plate = signal deviation between the plates, day-to-day = signal deviation between the days, control c = concentration of 3′-amino-3′-deoxyadenosine used as a positive control.

Particularly nucleosides **10–16** seem to exhibit inhibitory capacity at micromolar concentrations. The results also demonstrate a good correlation between the IC_{50} values yielded by the two methods, even though the values determined by the luciferase assay are slightly higher. Thus, 3′-amino-3′-deoxyadenosine was selected as a positive control for the screening assay, and assay validation was completed by setting the control thresholds (Table 2).

3.4. SFV plaque reduction

To further study the effects of these selected nucleosides, plaque reduction assay was performed in which BHK cells infected with SFV were incubated under CMC medium for 48 h, with the nucleosides present at 100 μM concentration. Data from plaque assays, shown in Table 3, indicates that 3′-amino-3′-deoxyadenosine (**15**) is the most efficacious analogue of this set in reducing the number of plaques, causing an 820-fold decrease in virus titer in these conditions. IC_{50} values in the plaque reduction assay were similar to those observed by luciferase and RNA synthesis assays (Table 3). In liquid culture, it was also observed that nucleoside **15** gave the largest reduction in virus yield, decreasing the virus titer by three orders of magnitude.

3.5. Inhibition of Sindbis virus replication

To estimate the wider anti-alphaviral potential of the nucleosides inhibiting SFV replication, antiviral IC_{50} values for these compounds were determined in Sindbis virus infections using uridine labeling. Similar infection time course experiments were performed for Sindbis virus infection (MOI 0.001 PFU/cell) as described above for SFV, and as the maximal replication rate was found to be approximately 2 h delayed compared to SFV, the 16 h post infection time point was selected for sample collection. As demonstrated by the IC_{50} values in Table 3, the inhibitory potency of these nucleosides against Sindbis virus is quite similar to that against SFV.

3.6. Effects of the nucleosides on cell viability

To estimate the toxic potential of the nucleosides in mammalian cells, two cell viability assays were utilized after BHK cell exposure to the compounds. A 14 h exposure time was used to assess the impact of cell viability on antiviral activity (data not shown). Due to the rapid proliferation rate of BHK cells, as well as the sensitivity of the cytotoxicity assays with endpoints in metabolic activity (Mueller et al., 2004; Slater, 2001), 24 h exposure time was selected for general toxicity evaluation (Fig. 3). All the derivatives, except for ara-A (**5**), which showed toxicity only after 24 h exposure, showed decreased BHK cell viability in the 14 h experiment. Even though certain nucleosides had cytocidal effects, the antiviral effect did not generally correlate with toxicity. Comparison of the graphs in Figs. 2 and 3 indicates that of the 13 significantly effective antiviral compounds, six had some toxic properties whereas seven did not, and the

Table 3
Anti-alphaviral activity of selected nucleoside analogues

Compound	IC_{50} (μM)[a]			SFV plaque reduction[b]	
	SFV RNA labeling	SFV luc activity	SIN RNA labeling	Efficacy (fold)	Potency (IC_{50} μM)
5	45 ± 6	58 ± 7	42 ± 5	–	
6	>200	>200		–	
10	5 ± 1	12 ± 3	16 ± 3	87	18
11	2 ± 1	18 ± 4	10 ± 5	71	19
12	15 ± 3	8 ± 2	11 ± 3	16	23
14	13 ± 2	21 ± 3	25 ± 5	–	
15	2 ± 2	18 ± 4	12 ± 4	820	6
16	25 ± 4	31 ± 5	36 ± 7	–	
17	157 ± 14	187 ± 23		–	
18	111 ± 10	121 ± 15		–	
24		75 ± 6			
25		119 ± 9			
29		107 ± 11			

[a] IC_{50} values represent the nucleoside concentration causing a 50% decrease in the antiviral assay endpoint (uridine incorporation or luciferase expression) at 14 h post infection. The results (mean ± S.D.) represent data from at least four replicates, using a concentration range of 0.01–200 μM for each dose–response curve.

[b] In plaque reduction assays, the fold reduction of the number of plaques compared to the untreated control was determined using a 100 μM concentration of each nucleoside. See Section 2 for detailed experimental information. "–" indicates "no effect". IC_{50} values for plaque reduction were determined using two replicates.

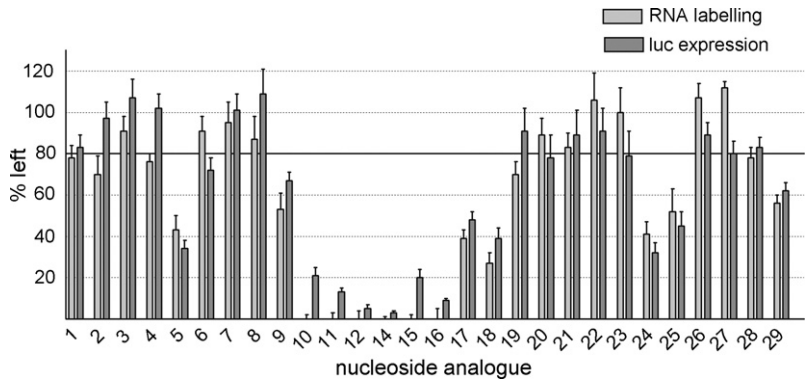

Fig. 2. Primary screen results from the antiviral assays using the two alternative assay methods. Nucleoside analogues were present throughout the 14 h experiment at a concentration of 200 μM. The compounds were added to the cells at the same time with the virus (infection multiplicity was 0.001 PFU/cell). The data represents results (mean ± S.D.) from a minimum of three experiments.

toxic compound **4** did not have an antiviral effect. The cytocidal effects occurred at significantly higher concentrations than the antiviral effects, since the antiviral IC_{50} values attained 2 μM in the best cases (**15** and the completely nontoxic **11**; Table 2), but the decrease in cell viability only reached ≥50% for a few compounds (Fig. 3).

4. Discussion

To screen for novel antiviral agents against alphaviruses, a non-radiometric reporter-gene anti-SFV assay was set up, using conventional RNA labeling as a reference method and to control the presence of possible interfering aspects (e.g. inhibition of luciferase activity as such). The reporter-gene approach proved to be successful in setting up a more automation-friendly assay for antiviral testing.

In pilot experiments for infection time scale, the observation was made that independent of the infection multiplicity, the same maximal RNA synthesis rate was eventually achieved. In the infected cells, the alphaviruses appear to make only a certain number of replication complexes, and afterwards the synthesis of further minus strands (required in the replication complexes as templates for plus strand synthesis) and also the synthesis of new nonstuctural proteins is turned off (Kääriäinen and Ahola, 2002). The interplay between viral and host cell factors in this self-limiting regulation is yet to be understood (Sawicki et al., 2006). The selection of infection multiplicity 0.001 PFU/cell and detection time 14 h allows assaying the compounds' effects during more than one complete infection cycle. As by this time all the cells have become infected, the screen should also detect inhibitors of virus egress and entry. At the same time, the assay time has been kept relatively short to avoid the influence of complicating factors such as compound half-life.

The antiviral effects of 29 nucleosides were evaluated against two alphaviruses, SFV and Sindbis virus. Correlation of IC_{50} values obtained for RNA labeling and reporter-gene assay was achieved, even though the values determined with the reporter-gene assay are often slightly higher (Table 2), although the difference is not statistically significant in most cases. This phenomenon may result from the extremely high sensitivity of the luciferase detection system, whereas the higher background level limits the sensitivity in radiometric detection. The miniaturization and automation of the reporter-gene assay further increased the throughput capacity, and the assay performance was also indicated by its successful use in screening of a library of natural compounds (unpublished results, L. Pohjala). Despite the presence of two biological variants, i.e. virus and cell, the criteria set to the assay robustness as assay quality parameter thresholds were successfully achieved.

In this study, several new inhibitors of alphavirus replication were discovered. The most potent antiviral agents in this set of tested nucleosides were the adenosine analogues 3′-azido-3′-

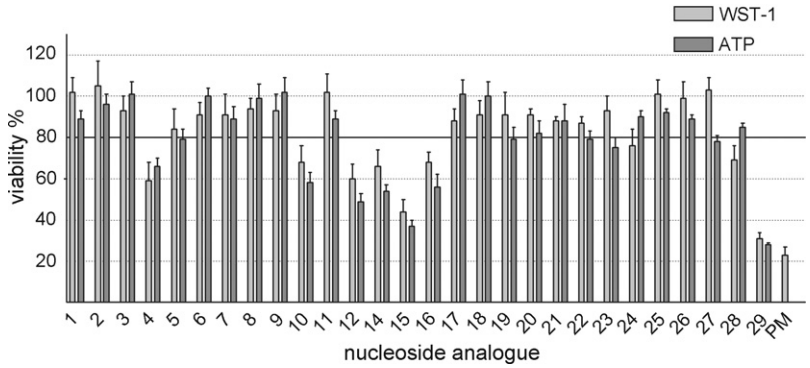

Fig. 3. Effects of the nucleosides on BHK cell viability after 24 h exposure to 200 μM of compound. The data represents results (mean ± S.D.) from a minimum of three experiments. PM = polymyxin B (at 7500 IU/ml) was used as a control compound for its well-known toxic effects.

deoxyadenosine (**14**) and 3′-amino-3′-deoxyadenosine (**15**). In addition to RNA labeling and luciferase activity detection, the antiviral activity of 3′-amino-3′-deoxyadenosine (**15**) was also observed in a plaque reduction assay. Interestingly, 3′-fluoro-3′-deoxyadenosine, which is one of the most potent SFV inhibitors published previously, contains a substitution at the same position (Mikhailopulo et al., 1991; Smee et al., 1992). However, similar to the earlier report, also these more recent 3′-substituted adenosines may have harmful effects on mammalian cell viability at higher concentrations. The other structural subset of anti-alphaviral agents identified in this study was the adenosine analogues having a large, cyclic substituent at N^6 (compounds **10**, **11** and **12**). Even though similar structures are currently under intense investigation as potential cancer chemotherapeutics (Ishii et al., 2003; and references therein), to our knowledge this is the first time when their antiviral activities were studied. These agents might also have some toxic potential (for example, cell viability after 24 h exposure to 200 μM compound **10** is 62% by ATP assay), but their harmful effects on mammalian cells is less obvious than for the 3′-substituted compounds. In general, antiviral IC_{50} values determined in these studies are, at best, at low micromolar level, which compares favorably with most, if not all published alphavirus inhibitors. Under these conditions, ribavirin showed an IC_{50} value of 95 μM for SFV (reporter-gene assay) and 63 μM for Sindbis virus (uridine labeling).

In addition to the studies on SFV, the effects of the nucleosides on Sindbis virus were investigated. The antiviral IC_{50} values for the nucleosides tested were rather similar between these two viruses. As SFV and Sindbis represent members of distant branches of the alphavirus family (Griffin, 2001), this suggests that the nucleosides may have inhibitory potential against a wider range of alphaviruses.

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