Antiviral Activity of Nucleoside Reverse Transcriptase Inhibitors in Adenovirus In Vitro

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Abstract：Background：Adenoviral keratoconjunctivitis is recognized as one of the major pathogens of ophthalmological nosocomial infection worldwide. No specific anti－adenoviral agent has yet been established for the treatment of adenoviral infection. We evaluated the anti–adenoviral effect of nucleoside reverse transcriptase inhibitors in this study. Methods：Two anti－HIV agents, zalcitabine and stavudine were subjected to in vitro evaluations. A549 cells were used for the viral cell culture, and adenovirus serotypes 3, 4 and 8 were used. After calculating CC_{50} of each agent by MTS method, adenovirus was cultured with the agents for seven days, and the extracted adenoviral DNA was then quantitatively measured by real－time PCR. Results：Both zalcitabine and stavudine showed significant anti－adenoviral activity in a dose－dependent manner in all serotypes. Conclusions：These results indicate that zalcitabine and stavudine are possible candidates for the local and systemic treatment of adenoviral infection. The chemical properties regarding the clinical safety for systemic and local application need to be determined in order to for these drugs to be accepted for the treatment of adenovirus in clinical settings.

Key words：**Adenovirus, Conjunctivitis, Zalcitabine, Stavudine**

have yielded conflicting results.[3)4)] Recently, cidofovir was found to be beneficial in several small-scale studies involving patients with life-threatening AdV infections.[3)5)] In the present study, we combined the indirect parameters of AdV replication (cytopathic effect and viability of infected cells) with a real-time PCR analysis to directly quantify AdV progeny in virus-infected cells. Epidemic keratoconjunctivitis (EKC) is mainly caused by subgroup D adenoviruses of serotypes 8, 19 and 37.[6)] Serotypes 3, 4 and 7, which belong to subgroup B, also cause conjunctivitis and pharyngoconjunctival fever.[7)] It is therefore is necessary to include various serotypes of AdV to evaluate the antiviral effect of chemical compounds. We therefore selected AdV3, AdV4 and AdV8 for evaluation in this study. Using these methodologies, we demonstrated that several nucleoside reverse transcriptase inhibitors display a potent and selective anti-AdV activity in cell culture.

Materials and methods

Chemical compounds

Zalcitabine and stavudine were subjected to an in vitro evaluation. They were obtained from commercial sources.

Cells

A549 cells (alveolar epithelial; ATCC #CCL-185) were obtained from the American Type Culture Collection (ATCC, Rockville, GA) and cultured in Eagle's Minimum Essential Medium (MEM) containing 2 mM L-glutamine, 0.1 mM non-essential amino acids and 7% fetal calf serum (FCS).

Viruses

The viruses used were AdV type 3 (AdV3), type 4 (AdV4) and type 8 (AdV8). These were prototype strains of each serotypes. These strains were propagated in A549 cells and stored at −80℃ until use. ^{35}S-methionine labeled AdV was prepared in A549 cells. Briefly, AdV-infected A549 cells were radiolabeled with 100 μCi ^{35}S-methionine for 12 h until a massive cytopathic effect (CPE) was observed. The radiolabeled virus particles were purified on a CsCl gradient by ultracentrifugation,[8)] and 100-μl aliquots were then tested to determine the virus infectivity titer and radioactivity.

Cytotoxicity assay

The cytotoxicity of the test compounds was evaluated in A549 cells. All assays were performed on confluent cell layers seeded in 96-well microplates (Falcon 3072, Becton Dickinson, Lincoln Park, NJ). Dilutions of drugs were prepared in Eagle's MEM supplemented with 2% FCS. Five concentrations of the test compound were used. After 7 days' incubation at 37℃ with 5% CO_2, the cells in the plates were then subjected to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based colorimetric assay for cell viability according to the manufacturer's instructions (Promega, Leiden, The Netherlands). The A490 values, corrected for the cytotoxicity exerted by GRGDSP peptide (as determined in mock-infected cultures), were used to calculate the percent cell viability. The 50% cytotoxic concentration (CC_{50}) was determined as the value causing a destruction of 50% of the monolayer cells, based on a regression analysis.

Antiviral experiments in A549 cells using real-time PCR

First, A549 cells were seeded in wells of 96-well plates at 10,000 cells per well and incubated for 4 or 5 days until confluency was reached. Fifty microliters of AdV, diluted in medium to obtain a virus input of 5 PFU per well, was added to each well. After 2 h at 37℃, the virus was aspirated and replaced by serial dilutions of the test compounds (200 μl per well). The concentrations of chemical compounds were determined according to 1/100 CC_{50} of each agent. Mock-treated cultures receiving only test compounds were included in each plate. After 7 days of incubation at 37℃, microscopy was performed to score the virus-induced CPE. After the removal of the culture supernatant, cells and virus particles were lysed by the addition of 70 μl lysis buffer (10 mM Tris-HCl [pH 7.8], 0.5% sodium dodecyl sulfate [SDS], 5 mM Na_2EDTA, 80 μg proteinase K per ml) and incubated at 50℃ for 1 h and then at 65℃ for 20 min to inactivate proteinase K. After clarification (23,000 ×g, 10 min), the cell extracts were stored at −20℃ until real-time polymerase chain reaction (PCR)

was performed. The extracts were diluted 100－fold in water. The 2 μl diluted extract was added to each well on optical plates containing 23 μl of SYBR green PCR master mix (Applied Biosystems, Foster City, Calif.), and the forward and reverse primers (300 μM) were then added to the wells. The primers, derived from GenBank sequences, were chosen to amplify a 137－bp fragment in the conserved AdV hexon DNA sequence, thus allowing an analysis of all known AdV types (forward primer, 5′–CGCTGGACATGACTTTTGAG–3′ ; reverse primer, 5′–GAACGGTGTGCGCAGGTA–3′). A real–time PCR analysis was performed in an ABI Prism 7,000 apparatus (Applied Biosystems) and consisted of 10－min activation at 95℃, followed by 40 thermal cycles, with 1 cycle consisting of 15 s at 95℃ and 90 s at 60℃. The dissociation profile was obtained at the end of each analysis to confirm the specificity of the PCR amplification. In each individual experiment, a standard curve ($R^2 > 0.98$ within the range of 103 to 108 copies per reaction mixture) was obtained by the amplification of known amounts of a pGEM T–vector in which a 691 –bp fragment of AdV hexon DNA was inserted using common cloning procedures. These standard curves were used to convert the cycle threshold values for the A549 extracts into the absolute number of AdV hexon DNA copies. If a dose－dependent antiviral activity was observed, then similar experiments in more detail and with lower drug concentrations were carried out using candidate agents. EC_{50} was calculated by extrapolation as the compound concentration at which the number of viral DNA copies at 7 days post－infection was 50% compared to the value obtained for the virus control.

Results

Cytotoxity of compounds

CC_{50} of each agent is shown in Table 1.

Screening for inhibitory effect of antiviral agents

The results of screening for anti－adenoviral activity are shown in Figures 1 and 2. A dose－dependent decrease in adenoviral DNA copies was found for both zalcitabine and stavudine, thus indicating a possible anti–adenoviral activity. The antiviral activity against serotypes were similar for both compounds based on a screening test.

Table 1. CC_{50} of each chemical compound in A549 cells

Zalcitabine	4,282 μg/ml (20.3 mM)
Stavudine	547 μg/ml (2.5 mM)

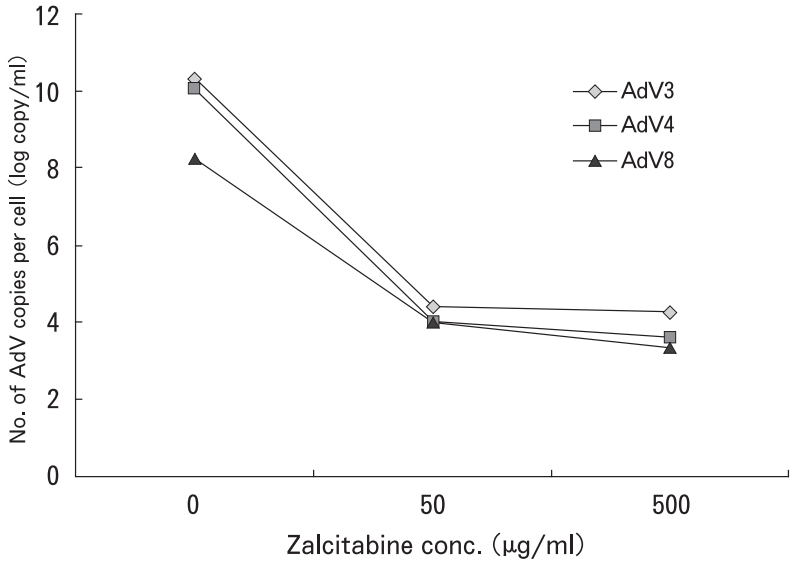

Figure 1. Antiviral activity of zalcitabine against AdV serotypes in A549 cells
A Dose dependent decrease of AdV copies is observed in all serotypes. Error bar indicates standard deviation.

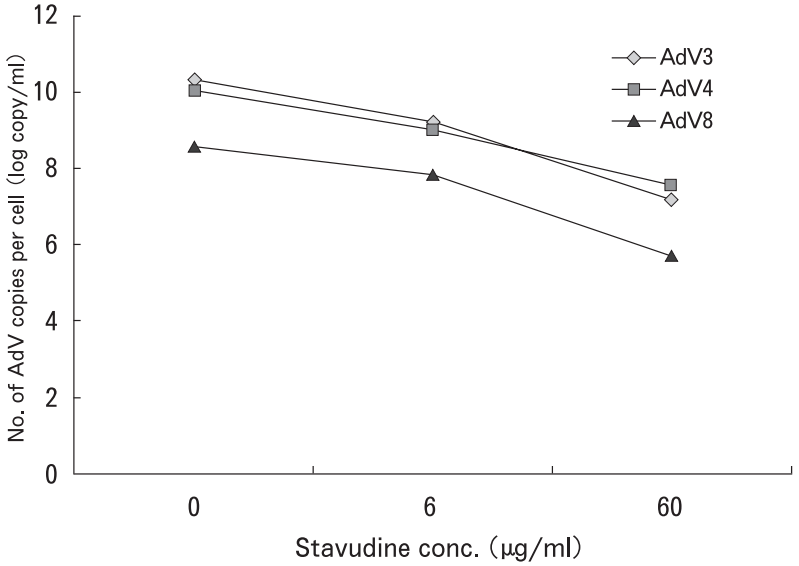

Figure 2. Antiviral activity of stavudine against AdV serotypes in A549 cells A dose dependent decrease of AdV copies is observed in all serotypes. Error bar indicates standard deviation.

Table 2. Antiviral activity of zalcitabine against AdV in A549 cells and EC_{50}

Zalcitabine conc. (μg/ml)	No. of AdV copies per cell (log copy/ml)		
	AdV3	AdV4	AdV8
10	5.69±0.10	5.52±0.22	4.24±0.01
2	8.48±0.28	7.36±0.03	5.63±0.08
0.4	10.03±0.08	9.40±0.14	6.90±0.04
0.08	10.26±0.30	10.08±0.14	8.11±0.40
0.016	10.39±0.15	10.38±0.17	8.17±0.23
0	10.34±0.12	10.44±0.23	8.21±0.19
EC_{50} (ng/ml)	384 (1.82 μM)	66 (0.31 μM)	117 (0.55 μM)

Table 3. Antiviral activity of stavudine against AdV in A549 cells and EC_{50}

Stavudine conc. (μg/ml)	No. of AdV copies per cell (log copy/ml)		
	AdV3	AdV4	AdV8
60	7.07±0.16	7.35±0.23	6.17±0.15
12	9.68±0.24	8.92±0.21	6.95±0.20
2.4	10.04±0.12	9.27±0.37	7.94±0.28
0.48	10.29±0.05	9.68±0.17	8.67±0.02
0.096	10.37±0.04	10.11±0.16	8.16±0.34
0	10.57±0.12	10.44±0.23	8.73±0.39
EC_{50} (ng/ml)	0.50 (2.29 μM)	0.091 (0.42 μM)	0.99 (4.52 μM)

Antiviral activity of zalcitabine and stavudine and EC_{50}

The relationship of the concentration of each agent and decrease of virus copy is shown in Tables 3 and 4. Zalcitabine at 10 μg/ml inhibited replication of all serotypes of AdV to approximately to 100,000− to 10,000−fold lower than control, and to 1000− to 100−fold lower at 2 μg/ml (Table 4). EC_{50} obtained by real−time PCR of zalcitabine ranged between 0.31 and 1.82 μM. Stavudine also inhibited AdV proliferation 100− to 1000−fold lower at 60 μg/ml compared to control, and 5− to 50−fold

at 12 μg/ml, thus showing a similar effect in each serotype, but stavudine appeared to be particularly effective against AdV3 and AdV4 (Table 5). EC_{50} of stavudine ranged between 0.42 and 4.52 μM, thus indicating approximately a 10−fold lower anti−adenoviral activity than zalcitabine.

Discussion

Several nucleoside analogues have been discovered to have antiviral activity against AdV by inhibiting viral DNA polymerase－mediated DNA replication in AdV－infected cells.[9)10)] Nucleoside analogues require activation by cellular enzymes to exert their antiviral activity.[11)] The active intracellular diphosphate form of the agent exerts its mechanism of action as both a competitive inhibitor and an alternative substrate for 2−deoxycytidine 5′−triphosphate in the viral DNA polymerase reaction. Cidofovir is a nucleotide analogue which contains a phosphate group that does not require cellular phosphorylation. Both ribavirin and cidofovir exhibit inhibitory activity in vitro against AdV.[11)12)] It has been reported that trifluridine (5−trifuoromethyl−2′−deoxyuridine) is effective against AdV8, AdV19 and AdV37 in vitro.[13)] The importance in the design of novel nucleoside analogues for anti－AdV therapy is their ability to discriminate between viral DNA polymerase and cellular DNA polymerases. Several experimental anti－AdV agents are associated with significant side effects. The local toxicity of cidofovir in the skin of the eyelids and conjunctiva is clinically similar to the signs of primary adenoviral keratoconjunctivitis.[14)] Therefore, there is an urgent need to develop selective anti－AdV drugs with more favorable safety profiles than the currently available nucleoside and nucleotide analogues.

None of the currently available anti−HIV agents has a potent anti−AdV activity. We demonstrated a marked anti－AdV activity of zalcitabine, a nucleoside re4verse transcriptase inhibitor (NRTI),[15)] and it exhibits antiviral activity against AdV both in vitro and in vivo.[16)17)] However, the biochemical basis for its anti−AdV effect (related to inhibition of AdV DNA polymerase) has not yet been studied in sufficient detail. The triphosphates of zalcitabine are typical chain－terminating inhibitors of HIV reverse transcriptase, but their mode of interaction with the AdV DNA polymerase remains to be investigated. The finding of significant inhibitory activity of zalcitabine in the present study was similar to that reported by Kaneko et al using an MTS－based cell viability staining method.[18)] We conducted a direct quantitation of virus progeny by real－time PCR in this study. Naesens et al. reported that zalcitabine and alovudine showed the most active and selective anti－AdV activity among several classes of nucleoside and nucleotide analogues by real−time PCR quantitation of adenoviral DNA.[11)] However, they selected only AdV type 2 for their evaluation, in contrast AdV3, AdV4 and AdV8 were included in our study. Considering the serious systemic side effects of zalcitabine as an NRTI, its local toxicity cannot be excluded. Therefore, its therapeutic efficacy along with the degree of local ocular adverse effects should be evaluated in an animal model in a future study.

Stavudine (STV ; 2′,3′−didehydro−3′−deoxythymidine/d4T) is a pyrimidine nucleoside analogue used in the treatment of HIV infection. It inhibits viral reverse transcriptase as does zalcitabine, and it is also member of NRTI. There has been no report on the effectiveness of stavudine for the inhibition of viral replication in AdV ; however, considering the common pharmacological characteristics of stavudine with the NRTIs, anti−adenoviral activity could be expected. Our study showed that stavudine exerted a similar but relatively weaker antiviral activity against AdV than zalcitabine. Uckun et al. recently reported that stampidine, a phenyl phosphoramidate derivative of stavudine, is the least cytotoxic and most effective of the stavudine derivatives against AdV.[19)] Further studies focusing on the inhibitory activity of stampidine in various AdV serotypes including those causing oculo−genital infections in both in vitro and in vivo experiments are thus called for.

In conclusion, zalcitabine and stavudine were shown to be potent and selective agents not only against HIV, but also against AdV. If these drugs are non−toxic and non−irritating to mucosal epithelial cells, including the ocular surface, then they could be potentially applied to the eye as either eye drops or in ointment form.

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