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Inhibitory activity of 3′-fluoro-2′ deoxythymidine and related nucleoside analogues against adenoviruses in vitro[1]

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Abstract

Antiviral effects of nucleoside analogues against human adenoviruses (ADV) belonging to subgroup B (ADV3) and C (ADV2) were comparatively analysed using focus reduction assay on Fogh and Lund (FL) cells. 3′-Fluoro-2′-deoxythymidine (FTdR), 3′-fluoro-2′-deoxyuridine (FUdR), 2′,3′-dideoxycytidine (ddC) and 3′-fluoro-2′-deoxyguanosine (FGdR) emerged as potent and selective inhibitors. They were nontoxic for the FL cells at the tested doses. FTdR was proved to be the most effective inhibitor against both serotypes ADV2 and ADV3 (0.05 μM/0.02 μM). The inhibitory effect of FTdR was also analyzed on the level of viral proteins and viral DNA synthesis using radioimmunoprecipitation and PCR, respectively. Neither the main structural protein of ADV, the hexon, nor viral DNA could be detected in ADV-infected FL cells that had been exposed to FTdR. © 1997 Elsevier Science B.V.

Keywords: Adenovirus; Inhibition of virus replication; Modified nucleoside analogues; Focus reduction assay

1. Introduction

Adenoviruses (ADV) are ubiquitous agents and are associated with a wide range of illnesses.They can cause respiratory infections, gastroenteritis, hemorrhagic cystitis and necrotising pneumonia (Matsuse et al., 1994). Adenoviruses can be also etiologic agents of endemic and epidemic ocular infections, for instance keratoconjunctivitis (Jernigan et al., 1993). Despite the great advances in the knowledge of adenovirus since its discovery, currently there is no licenced vaccine or chemotherapy effective in preventing or interrupting this virus infection. Until now only some compounds

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[1] This paper is dedicated to Prof. Döhner on the occasion of his 65th birthday.

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were detected to be effective against adenovirus replication at cellular level. Between them are (*S*)-9-(3-hydroxy-2-phosphonyl-methoxypropyl) adenine ((S)-HPMPA) and (*S*)-1-(3-hydroxy-2-phosponylmethoxypropyl) cytosine ((S)-HPMPC), two acyclic nucleoside phosphonates, 2′-nor-cyclic GMP, Schiff bases of aminohydroxyguanidine and N-7 acyclic purine analog 2-amino-7-((1,3-dihydroxy-2-propoxy)methyl) purine (Baba et al., 1987; Gordon et al., 1991; Hui et al., 1994; Kodama et al., 1996). In vivo studies with HPMPC have demonstrated its efficiency against adenovirus type 5 infections in the New Zealand rabbit ocular model (De Oliveira et al., 1996).

The aim of our study was to evaluate several modified nucleoside analogues, which have been investigated earlier as inhibitors of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) replication (Meisel et al., 1990; Matthes et al., 1991) for their inhibitory effect on adenovirus. ADV2 of subgroup B, mainly responsible for respiratory infections and able to persistent infections as well as ADV3 belonging to subgroup C, particularly connected with ocular infections, were selected for the experiments.

2. Materials and methods

2.1. Virus and cells

Continuous passages of Fogh and Lund cells (FL cells), American Type Culture Collection (ATCC), CCL 62, were performed in Eagle's minimal essential medium (MEM) containing 7% heat inactivated fetal calf serum, 100 U/ml of penicillin G and 100 μg/ml of streptomycin. The prototype strains ADV2 (Ad 6: ATCC) and ADV3 (GB: ATCC) were propagated in FL cells using Eagle's MEM containing 2% fetal calf serum. Virus infected cells were frozen and thawed three times and after titration aliquots of viral stocks were stored at -80°C until use.

2.2. Compounds

The 2′,3′-dideoxy-3′-fluoro-β-D-ribofuranosyl nucleosides and the 3′-azido analogues were synthesized as described previously (Van Aerschot et al., 1989; Herdewijn et al., 1989; Kowollik et al., 1973). The 2′,3′-dideoxy-nucleosides were prepared by catalytic hydrogenation of the corresponding 2′,3′-unsaturated compounds (Chu et al., 1989). 2′,3′-Dideoxy-3′-thiacytidine was provided by R. Schinazi, Emory University, Atlanta, GA. The compounds were initially dissolved in sterile water and further diluted with maintenance medium immediately before use. All compounds were tested at concentrations of 0.01–100 μM.

2.3. Cellular toxicity

The cellular toxicity was determined by a dye uptake assay. Each concentration of the test compounds in a volume of 100 μl was mixed with 100μl of FL cell suspension (150 000 cells/ml) in 96-well culture plates and incubated for 48 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation the supernatant was removed and 200 μl neutral red solution in medium (0.005%) was added. After further incubation for 3 h at 37°C, the dye incorporated by viable cells was eluted by addition of 100 μl of ethanol/water/glacial acetic acid solution (50:50:1) and shaking for 15 min. Absorbance was measured at 540 nm. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced the absorbance of untreated cells by 50%.

2.4. Antiviral activity

The antiviral activity was evaluated by focus reduction assay (Mentel et al., 1996). Briefly, dilutions of the test compounds (100 μl) were mixed with 50 μl FL cells (300 000 cells/ml) in chamber slides for tissue culture (Lab Tek, Nunc). After incubation for 30 min at 37°C, 50 μl of adenovirus suspension were added. The virus doses were selected in such a way that the virus control was represented by approximately 40 fluorescent foci units (FFU). Cultures were incubated for 48 h at 37°C in a humidified 5% CO_2 atmosphere. After air drying and fixation by acetone, the cell sheet was stained with fluorescein isothiocyanate labeled antibodies against adenovirus ('in house' preparation in rabbits) for 30 min at 4°C. The

number of fluorescent foci were counted using a fluorescence microscope. The 50% antiviral inhibitory concentration (IC_{50}) was defined as the concentration that resulted in 50% reduction of the number of fluorescence foci in relation to virus-infected cells without test compounds and mock-infected cells, respectively.

2.5. Radio-immunoprecipitation

Adenovirus type 2-infected cells were incubated for 18 h in the presence of 1 μM FTdR. The cells were then labelled with [^{35}S]methionine for 5 h at 37°C, washed with PBS and harvested by scraping in 100 μl RIPA buffer. The cell extract was vortexed, centrifuged for 10 min at 4°C at 11 000 rpm and the supernatant was collected for analysis. Aliquots of cell lysate were added to specific anti-adenovirus polyclonal antibodies. The immune complex was adsorbed to protein-A sepharose and analyzed by polyacrylamide gel electrophoresis using 16.5% gel. [^{35}S]Methionine labelled proteins were visualized by autoradiography.

2.6. PCR assay

Adenovirus type 2-infected cells were incubated for 19 h in the presence of 10, 1 and 0.1 μM FTdR, respectively. Then DNA was extracted by phenol-chloroform. Samples were mixed with PCR reagents including Taq polymerase and incubated in a thermocycler by a three step profile: 94°C for 45 s, 55°C for 90 s and 72°C for 90 s for a total of 30 cycles. For PCR amplification oligonucleotide primers of the region 18 858–18 883 and 19 136–19 158 of the hexon gene were used. Nucleotide sequences were: 5′-GCCGCAGTGGTCTTACATGCACATC-3′ and 5′-CAGCACGCCGCGGATGTCAAAAGT-3′. A 300 bp sequence was amplified by PCR. After PCR amplification the results were analyzed on ethidiumbromide-stained 3% agarose gel. With serial dilutions of ADV2 genomic DNA the detection limit was 400 genome copies.

3. Results

The cytotoxicity of compounds was evaluated using a colorimetric assay. In our test system most compounds were tolerated by the FL cells at a concentration of 100 μM. Only FTdR showed a CC_{50} of 89 μM (Table 1).

The antiviral activity of 22 compounds were evaluated against ADV2 and ADV3. The 50% inhibitory doses are presented in Table 1. In the experiments with ADV2-infected cells, which were exposed to these inhibitors, FTdR was extremely effective and selective with an IC_{50} of 0.05 μM and a selectivity index of 1780. Furthermore, in this group of 3′-fluorosubstituted nucleosides antiviral activity was also found for FUdR, FGdR and FCdR. The inhibitory concentration ranged from 0.56 μM for FUdR to 67 μM for FCdR. The C-5 halogenated derivatives of FUdR and FCdR and also the methylated derivative of FCdR showed no increase of the antiviral effect in comparison to the parent compounds. No activity was observed for FAdR.

Within the 3-azido-2-deoxynucleosides, only N_3AdR exhibited a weak effect with an IC_{50} of 85 μM. N_3TdR and N_3GdR were not effective.

From the group of the 2′,3′-dideoxynucleosides ddC showed a remarkable inhibitory activity (IC_{50}: 0.83 μM). For the unsaturated derivative ddeC an IC_{50} of 20 μM was estimated. While 2,6-diaminopurine-2′,3′-dideoxy-3′-fluorodeoxyriboside was not active, a 50% inhibitory concentration of 79 μM was obtained for 2-aminopurine 2′,3′-dideoxy-3′-fluorodeoxyriboside.

In the experimental series with adenovirus serotype 3 the most effective inhibitor were also FTdR followed by ddC and FUdR (IC_{50}: 0.02, 0.05 and 0.7 μM). These data are consistent with the effect against ADV2. It was also observed that the substitution in position 5 by an halogen or methyl group did not increase the activity.

The replacement of the fluorine by an azido group displayed measurable effects for N_3TdR (18.8 μM) and N_3AdR (19.8 μM). N_3GdR showed no activity.

For ddC we calculated an IC_{50} of 0.05 μM, whereas the unsaturated derivative ddeC was 1000 fold less active. For the corresponding 2′,3′-

Table 1
Antiviral activity of modified nucleoside analogues against adenovirus type 2 and 3 in FL cells using a focus reduction assay

Compound	Abbreviation	IC_{50} (μM)		CC_{50} (μM)
		ADV2	ADV3	
3′-Fluoro-2′-deoxythymidine	FTdR	0.05	0.02	89.0
3′-Fluoro-2′-deoxyuridine	FUdR	0.56	0.7	>100
3′-Fluoro-5-chloro-2′deoxyuridine	FClUdR	34.5	>100	>100
3′-Fluoro-5-bromo-2′-deoxyuridine	FBrUdR	89.0	13.4	>100
3′-Fluoro-2′-deoxycytidine	FCdR	67.0	7.5	>100
3′-Fluoro-5-chloro-2′-deoxycytidine	FClCdR	>100	>100	>100
3′-Fluoro-5-methyl-2′-deoxycytidine	FMetCdR	69.5	55.9	>100
3′-Fluoro-2′-deoxyguanosine	FGdR	1.39	2.0	>100
3′-Fluoro-2′-deoxyadenosine	FAdR	>100	>100	>100
3′-Azido-2′-deoxythymidine	N_3TdR	>100	18.8	>100
3′-Azido-2′-deoxyguanosine	N_3GdR	>100	>100	>100
3′-Azido-2′-deoxyadenosine	N_3AdR	85.0	19.8	>100
2′,3′-Dideoxycytidine	ddC	0.83	0.05	>100
2′,3′-Dideoxyguanosine	ddG	47.0	>100	>100
2′,3′-Dideoxyadenosine	ddA	30.0	>100	>100
2′,3′-Dideoxythymidine	ddT	>100	>100	>100
2′,3′-Dideoxy-2′,3′-didehydrothymidine	ddeT	>100	13.1	>100
2′,3′-Dideoxy-2′,3′-didehydrocytidine	ddeC	20.0	52.5	>100
2′,3-Dideoxy-2′,3′-didehydroadenosine	ddeA	>100	>100	>100
2,6-Diaminopurine-2′,3′-dideoxy-3′-fluorodeoxyriboside	FDAPdR	>100	>100	>100
2-Aminopurine-2′,3′-dideoxy-3′-fluorodeoxyriboside	FAPdR	79.0	58.2	>100
2′,3′-Dideoxy-3′-thiacytidine	3TC	>100	50.0	>100

dideoxy-2′,3′-didehydrothymidine an IC_{50} of 13.1 μM was obtained. The IC_{50} of 2-aminopurine-2′,3′-dideoxy-3′-fluorodeoxyriboside and 2′,3′-dideoxy-3′-thiacytidine was equal to 58.2 and 50.0 μM, respectively.

It was also of interest to determine the inhibitory effect on the level of viral proteins and viral DNA synthesis. Viral polypeptides were analyzed by radioimmunoprecipitation. In the presence of 1 μM FTdR, the most effective substrate, there was a strong inhibitory effect on the production of the hexon, a 120 kDa protein, that represents the main structural protein of the viral capsid (Fig. 1). The effect of the compounds on viral DNA synthesis was determined by PCR. For DNAs extracted from adenovirus containing cell cultures a band of amplified DNA with the expected length of 300 bp was detected (Fig. 2; lane 1, 2, 3). No such band was seen in the presence of 10, 1 and 0.1 μM of FTdR (lanes 4, 5, 6) (Fig. 2).

4. Discussion

In the virus replicative cycle there are a number of events which are controlled by virus-specific proteins that could serve as specific targets for chemotherapeutic intervention (De Clercq, 1990, 1993). The research in the field of adenovirus has been directed toward learning details of the molecular mechanisms by which the responsible gene or genes produce the disease. Some of the E3 proteins of adenovirus have been demonstrated to be involved in determining the pathogenic properties of the virus (Wold and Gooding, 1991). The 19 kDa protein of the E3 region is a transmembrane glycoprotein that retains class 1 MHC antigens in the rough endoplasmic reticulum. As a consequence allogenic and antigenic specific T-cell recognition is drastically reduced. Meanwhile, Cotton and Weber (1995) have demonstrated that the adenovirus-encoded protease is required for virus entry into the host cells.

In addition new possibilities of the rapid diagnostic were developed using molecular methods and make a specific therapy against adenovirus very desirable (Jackson et al., 1996).

We could demonstrate that among a series of modified nucleosides several compounds have been proved as efficient inhibitors of adenovirus replication using the focus reduction assay. Of these modified nucleosides, FTdR, FUdR, ddC and FGdR emerged as most potent inhibitors of replication of adenovirus serotype 2 and 3. The IC_{50} estimated ranged between 0.02 and 2.0 μM. To our knowledge, they belong to the most efficient inhibitors against adenovirus replication, described so far.

The groups of evaluated compounds were tested earlier for their antiviral efficiency against HIV and HBV. FTdR, N_3TdR, FUdR, FClUdR, ddeT and ddC proved to be most effective against HIV-replication in T-lymphocytic cell lines and macrophages (Schinazi et al., 1992). FMetCdR, FGdR and ddG have been described as most potent inhibitors of HBV in Hep G2 2.2.15 cells (Matthes et al., 1990) and of duck hepatitis B virus in primary duck hepatocytes (Suzuki et al., 1988; Hafkemeyer et al., 1996).

Fig. 1. Effect of FTdR on viral protein synthesis in adenovirus type 2 infected FL cells. Adenovirus type 2-infected cells were labeled with [^{35}S]methionine. The cell lysate was immunoprecipitated and analyzed by SDS gel electrophoresis. Lanes: 1, mock-infected cells; 2, infected, untreated cells; 3–4, infected cells in presence of 1 μM FTdR; m, molecular weight marker 14.3–120 kDa.

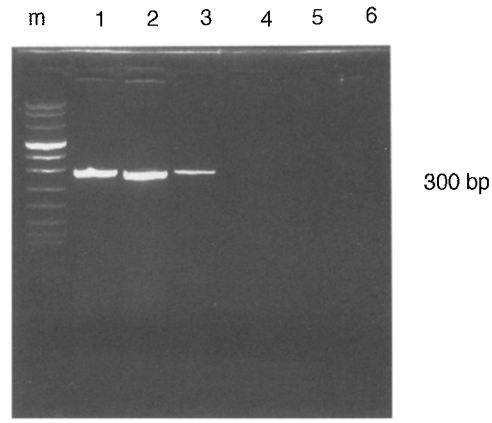

Fig. 2. Influence of FTdR on DNA synthesis of adenovirus type 2 in FL cells. Ethidium bromide-stained agarose gel analysis of PCR products (300 bp) generated from DNA extracted of FL cells infected with adenovirus type 2 in presence of different concentrations of FTdR. PCR was positive for AD DNA in the first three lanes. Lanes: 1, cells infected with 10^6 FFU; 2, 10^5 FFU; 3, 10^4 FFU. Lanes 4–6 were found to be associated with a negative PCR. Lane 4, cells infected with 10^6 FFU in presence of 10 μM FTdR; lane 5, in the presence of 1 μM FTdR; lane 6, in the presence of 0.1 μM FTdR. Lane m: DNA size marker 19–1114 bp.

Although the nucleosides tested have a high degree of similarity they display a quite different pattern of efficiency against the three viruses. Some of these differences could be explained by the varying ability of the different cell types to phosphorylate the nucleoside analogues. On the other hand the sensitivity of the viral targets might be different. For HIV-RT and HBV DNA polymerase the nucleoside triphosphates were found to act as inhibitors and/or competitive substrates of HIV RT and HBV DNA polymerase, respectively (Matthes et al., 1987, 1991; Meisel et al., 1990). Most probably some of them might act in the same way as inhibitors of adenoviral DNA polymerase.

Surprisingly, all nucleosides showed only modest antiproliferative effects against FL cells for up to 5 days of incubation. In contrast, MT-4 cells, a human T-lymphocytes cell line, proved to be very sensitive against FTdR ($CC_{50} = 1.1$ μM). In this system the CC_{50} values for FUdR, FGdR and ddC were 75, 197 and 41 μM, respectively. Moreover the effects of these compounds on the colony formation of mouse myeloid progenitor cells (CFU-GM) demonstrated the high cytotoxicity of ddC ($CC_{50} = 3$ μM) and FTdR ($CC_{50} = 15$ μM) in comparison to FUdR ($CC_{50} = 2200$ μM) and FGdR ($CC_{50} = 245$ μM) (Balzarini et al., 1988; Matthes et al., 1988 and unpublished data).

Further studies to better define the mode of action using a DNA polymerase assay and further investigations using the pneumonia model in hamsters (Mentel et al., 1993) of the most potent compounds are underway and will be reported in the future.

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