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ScienceDirect
Antiviral Research 72 (2006) 34–41
Antiviral Research
www.elsevier.com/locate/antiviral

A rapid quantitative PCR-based assay for testing antiviral agents against human adenoviruses demonstrates type specific differences in ribavirin activity

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Received 20 January 2006; accepted 20 March 2006

Abstract

Human adenovirus (HAdV) infections are increasingly frequent and potentially fatal as a disseminated disease in highly immunocompromised patients. Determining the in vitro sensitivity of HAdV to antiviral agents is not an easy task because HAdV CPE reduction assays are difficult to interpret and may take more than 1 week. We developed a phenotypic assay for testing the antiviral activity during the first round of replication using HAdV DNA concentration as an objective readout within 30 h. After evaluating the assay with cidofovir, we focused on determining the antiviral of ribavirin against different HAdV serotypes because clinical response of HAdV infections towards ribavirin treatment varied considerably.

Several HAdV prototypes (1, 2, 5, 11, 31, 34, 48) associated with disseminated infections and clinical isolates were tested. Predominating HAdV of species C were more sensitive to ribavirin (HAdV-2 and -5: $EC_{50} < 10\ \mu M$, EC_{99} 111 and 104 μM, respectively) than HAdV of other species, for example HAdV-31 (EC_{50} 56 μM, $EC_{99} > 500\ \mu M$). Differential ribavirin sensitivity of HAdV types may contribute to the variable outcome of ribavirin therapy. Rapid screening of antiviral agents with the rapid qPCR-based assay against a multitude of HAdV serotypes may also facilitate development of future antiviral agents.

Keywords: Adenovirus; Ribavirin; Phenotypic assay; Quantitative PCR; Immunosuppression

1. Introduction

The six species (A–F) of human adenoviruses (HAdV, genus *Mastadenovirus*, family Adenoviridae) with their 51 types are associated with a variety of diseases affecting all organ systems (Swenson et al., 2003). HAdV are an important cause of morbidity and mortality in the immunocompromised host (Bordigoni et al., 2001; Carrigan, 1997; Chakrabarti et al., 2002). In these patients HAdV can lead to severe organ infections such as, for example, nephritis or to life threatening disseminated disease with symptoms of septic shock and involvement of multiple organs (pneumonia, gastroenteritis, hemorrhagic cystitis, hepatitis, encephalitis) (Baldwin et al., 2000; Chakrabarti et al., 2002; La Rosa et al., 2001; Lion et al., 2003). The incidence of severe HAdV diseases is increasing with the growing number of immunocompromised patients. Children after allogeneic bone marrow transplantation are at high risk for disseminated disease with fatality rates as high as 50–80% (Bordigoni et al., 2001; Gavin and Katz, 2002; Seidemann et al., 2004; Venard et al., 2000). At present, there is no effective antiviral therapy available which could be considered as the gold standard to treat HAdV diseases. Several antiviral drugs have been used clinically with varying clinical outcome (Bordigoni et al., 2001; Gavin and Katz, 2002; Kojaoghlanian et al., 2003; La Rosa et al., 2001; Seidemann et al., 2004), but controlled clinical studies have not yet been performed due to multiple organizational problems.

Even determining the in vitro sensitivity of HAdV to antiviral agents is not an easy task because HAdV CPE reduction assays are difficult to interpret and may take more than 1 week with some slowly replicating HAdV serotypes (Mentel et al., 1997; Naesens et al., 2005). Therefore, we developed a phenotypic assay for testing the antiviral activity during the first round of replication using HAdV DNA content as an objective readout. As a prerequisite for this assay, the HAdV DNA replication kinetics of multiple HAdV serotypes was determined by

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doi:10.1016/j.antiviral.2006.03.009

a highly specific, quantitative PCR protocol (qPCR) with a TaqMan probe capable to detect all 51 HAdV types (Heim et al., 2003). From this data, an assay was established which holds promise to determine the antiviral activity of antiviral agents, which interact with the early steps of the replication cycle, against any HAdV serotype or clinical isolate including these which do not exhibit a clear CPE. This assay was evaluated with cidofovir and HAdV-C1, and more extensively with ribavirin and various HAdV serotypes.

In contrast to other nucleoside analogue antiviral drugs, ribavirin has a wide-spectrum activity against DNA and RNA viruses (Sidwell et al., 1972). Due to its chemical structure without any modification of the ribose moiety, it is frequently called an "atypical" nucleoside analogue. It holds an "orphan drug status" for the treatment of HAdV infection (Blasi, 2003). Several case reports, and small, non-controlled studies in immunosuppressed patients with HAdV disease indicated beneficial effects of ribavirin (Arav-Boger et al., 2000; Chakrabarti et al., 2002; Emovon et al., 2003; Gavin and Katz, 2002; Miyamura et al., 2000), but treatment failures were also reported occasionally (Bordigoni et al., 2001; La Rosa et al., 2001). Different HAdV serotypes may vary widely in their sensitivity to ribavirin as the 51 HAdV serotypes are genetically highly diverse (Swenson et al., 2003) and CPE-based assays indicated different susceptibilities of HAdV reference strains to ribavirin (Morfin et al., 2005). Fortunately, only a limited number of HAdV serotypes are strongly associated with disease in the immunocompromised host: HAdV-1, -2, and -5 of species C (HAdV-C1, -C2, -C5), HAdV-B11, and the closely related HAdV-B34 and -B35 (Seidemann et al., 2004; Swenson et al., 2003). Recently, HAdV-A31 was also described as a significant pathogen in immunocompromised patients (Bordigoni et al., 2001; Seidemann et al., 2004; Venard et al., 2000). Previously, human adenoviruses of species D (e.g. HAdV-D48) were found in highly immunodeficient, terminally ill AIDS patients (Schnurr and Dondero, 1993). Therefore, we focused on evaluating our assay with these clinically significant HAdV serotypes.

Significant differences in ribavirin sensitivity were observed. Drug sensitivity of clinical HAdV isolates from immunodeficient patients were found to be similar to HAdV prototype strains of the same serotype.

2. Materials and methods

2.1. Cell culture

Cell culture experiments were performed with A549 and HeLa cells, which were propagated under standardised conditions (37 °C, 5% carbondioxide). The cells were used between the 20th and 60th passage and harvested by trypsin/EDTA. Dulbecco's modification of minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 10 U penicillin G/ml, and 10 μg streptomycin/ml was used. For antiviral assays and HAdV DNA replication kinetics, cells were seeded on six-well plates at 10^6 cells/well in quadruplicates one day before each experiment (infection with HAdV and ribavirin application).

2.2. Antiviral agents

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was provided by ICN Pharmaceutics (Frankfurt, Germany). The dry substance was dissolved as 10 mM aqueous stock solution and stored at −20 °C for further use. Cidofovir was provided by Pharmacia & Upjohn (Kalamazoo, MI, USA). It was dissolved as 10 mM aequous stock solution and stored at −20 °C for further use.

2.3. Virus stock solutions

Human adenovirus (HAdV) prototype viruses of types C1, C2, C5, B11, A31, B34, and D48 (American Type Culture Collection, Manassas, VA) and adenoviruses isolated from immunosuppressed patients suffering from disseminated diseases (HAdV types C1 K, C2 J, and A31 W; German Adenovirus Reference Centre) were propagated on A549 cells (as well in HeLa cells in case of HAdV-A31) in 75 cm^2 cell culture flasks. Virus was harvested at >95% cytopathic effect (CPE) by freezing and thawing the cell culture flasks tree times. Supernatant was cleared by centrifugation (3000 × *g* for 5 min) and stored at −20 °C for further use. The concentration of HAdV stocks was determined both by $CCID_{50}$ method as proposed by Reed and Muench (Hierholzer and Killington, 1996) using A549 cells as well as by quantitative PCR (Heim et al., 2003).

2.4. Quantitative HAdV-PCR

The total intracellular HAdV DNA concentration was measured by a quantitative real-time PCR (qPCR) with a Taqman probe detecting a conserved region at the 5′ end of the hexon gene. This qPCR is generic for all HAdV types and was extensively validated for virus load diagnostics (interassay S.D. for the concentration in the range of 12–16%) (Heim et al., 2003).

2.5. Virus DNA-replication kinetics

Confluent monolayers of A549 cells in 16 wells (HeLa and A549 in case of HAdV-A31) were infected with HAdV at a multiplicity of infection (moi) of 10 $CCID_{50}$/cell for 1 h. Subsequently, cells were washed with PBS and incubated in fresh medium. After 3, 12, 24, and 48 h post infection (p.i.), four wells were harvested with trypsin/EDTA and total cellular DNA was extracted using the QiaAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Adenovirus DNA load was measured using qPCR (Heim et al., 2003).

2.6. Rapid qPCR-based antiviral activity assay

In order to test activity of antiviral agents, 24 wells with A549 cells (HeLa and A549 in case of HAdV-A31) were infected with HAdV as described above. After 1 h of incubation the virus inoculum was removed and the cell layers were washed with PBS. Fresh medium containing antiviral agents (0, 10, 50, 100, 200, and 500 μM ribavirin or 0, 1, 5, 25, 100, and 500 μM cidofovir, respectively) was added to each of four wells. The medium

was removed 24 h p.i. (24 and 48 h in case of HAdV-A31) and the cell layer was harvested by trypsin/EDTA. Total cellular DNA was extracted using the QiaAmp DNA Blood Mini Kit (Qiagen). The concentration of adenoviral DNA load was determined by qPCR (Heim et al., 2003).

2.7. CPE-based antiviral activity assay

A549 cells were seeded in 96-well plates at 1×10^4 cells/well and incubated for 24 h. Subsequently medium was aspirated and 100 μl fresh medium containing antiviral agents and 10^4 $TCID_{50}$ HAdV-C1 was added. Different concentrations of antiviral agents (cidofovir 0, 0.5, 1, 2, 4, 8, 16, and 32 μM; ribavirin 0, 5, 10, 20, 40, 80, 160, and 320 μM, each in 12 wells) were used. Cell culture media were changed at day 2 p.i. with fresh antiviral agents added to the medium. On day 5 p.i. cultures were inspected visually for CPE in a blinded fashion.

2.8. Cell-proliferation and viability assays

Reduction of cell proliferation by ribavirin was quantitatively evaluated by a XTT reduction assay (EZ4U assay, Biomedica, Vienna, Austria) (Wutzler et al., 2002). Briefly, 1×10^4 A549 cells/well were seeded in 96-well cell culture trays and incubated for a period of 4 days with various concentrations of ribavirin or cidofovir (0, 15, 31, 62, 125, 250, 500, and 1000 μM, each in 8 wells) in DMEM with 10% FBS. EZ4U was added to cell culture media and cells were incubated for 2 h at 37 °C. Absorbance was read at a wavelength of 450 nm in a standard photometer (reference wavelength 620 nm). A serial two-fold serial dilution of 1×10^5 A549 cells was used as a calibrator.

2.9. Statistical analyses

For each series of HAdV DNA replication kinetics, variability of total intracellular HAdV DNA was estimated by determination of the relative standard deviation. Statistical significance of the HAdV DNA concentration increase was determined by the Student's *t*-test. Inhibitory concentration 50%, 90%, and 99% (EC_{50}, EC_{90}, and EC_{99}) of ribavirin and cidofovir were calculated using the median effect plot (Chou and Talalay, 1984). Fifty percent of cytotoxic concentrations (CC_{50}) of ribavirin and cidofovir were calculated by non-linear regression. Confidence intervals of EC and CC values were calculated by the Monte Carlo method.

3. Results

3.1. Virus DNA-replication kinetics

In order to determine the interval required for HAdV DNA replication after infection of cells, viral DNA replication kinetics for each serotype were evaluated after infecting A549 cells at a high moi (10 $CCID_{50}$/cell). This approach was chosen to achieve an almost synchronous infection of the cells. The intracellular HAdV DNA concentration was determined after virus adsorption and internalization at 3 h p.i. as a basic value. Viral replication kinetics of all tested HAdV prototypes and clinical isolates are presented in Table 1. With exception of HAdV-C1 and HAdV-D48, increase of HAdV DNA was already significant at 12 h p.i. but increase of HAdV DNA was still <1 log. Most of the HAdV DNA replicated between 12 h and 24 h p.i.; only in case of HAdV-A31 DNA replication was considerably slower (Table 1). Because an attenuation phenotype may be suspected in case of the laboratory strain of HAdV-A31, a clinical isolate from an immunosuppressed patient suffering from a severe disseminated infection (HAdV-A31 W) was also tested. This clinical isolate also exhibited a slow DNA replication pattern similar to the results obtained with the laboratory strain of HAdV-A31 (Table 1). As slow DNA replication of HAdV-A31 may be cell type-dependent, DNA replication kinetics of the HAdV-A31 prototype and HAdV-A31 W were also tested in HeLa cells. Replication kinetics of both viruses were almost

Table 1
HAdV DNA replication kinetics displayed an increase of intracellular HAdV DNA concentration in A549 cells (except where indicated) at 12, 24, and 48 h p.i. compared to intracellular HAdV DNA concentration 3 h p.i.

	log increase (HADV DNA) 12 h p.i.	log increase (HADV DNA) 24 h p.i.	log increase (HADV DNA) 48 h p.i.
HAdV-C1	0.13 (S.D. 0.13)	3.38 (S.D. 0.07)***	4.26 (S.D. 0.04)***
HAdV-C1 K	1.57 (S.D. 0.16)***	4.21 (S.D. 0.70)***	5.43 (S.D. 0.06)***
HAdV-C2	0.28 (S.D. 0.11)**	2.93 (S.D. 0.12)***	4.66 (S.D. 0.07)***
HAdV-C2 J	0.65 (S.D. 0.11)***	2.99 (S.D. 0.11)***	4.40 (S.D. 0.04)***
HAdV-C5	0.17 (S.D. 0.06)**	2.89 (S.D. 0.06)***	4.37 (S.D. 0.02)***
HAdV-B11	0.68 (S.D. 0.07)***	3.03 (S.D. 0.07)***	4.08 (S.D. 0.11)***
HAdV-A31	0.54 (S.D. 0.11)***	0.66 (S.D. 0.11)***	2.23 (S.D. 0.07)***
.HAdV-A31W	0.58 (S.D. 0.13)**	1.37 (S.D. 0.1)***	2.32 (S.D. 0.33)***
HAdV-A31 (HeLa cells)	0.58 (S.D. 0.02)*	0.78 (S.D. 0.55)	3.33 (S.D. 0.07)***
HAdV-A31W (HeLa cells)	0.38 (S.D. 0.10)***	0.64 (S.D. 0.14)***	3.33 (S.D. 0.76)***
HAdV-B34	1.09 (S.D. 0.07)***	2.94 (S.D. 0.08)***	2.90 (S.D. 0.09)***
HAdV-D48	0.23 (S.D. 0.29)	3.29 (S.D. 0.27)***	3.43 (S.D. 0.06)***

Statistical significance of the HAdV DNA concentration increase compared to 3 h p.i. was determined by the Student's *t*-test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

identical in HeLa cells compared to A549 cells (Table 1). These results indicate that slow DNA replication seems to be a characteristic of HAdV-A31.

Clinical isolates HAdV-C1 K and HAdV-C2 J were also evaluated in order to determine whether there is a difference in capability of DNA replication between clinical isolates and laboratory strains of species HAdV-C. Similar to HAdV-A31, laboratory strains and clinical isolates of species HAdV-C had comparable DNA replication kinetics (Table 1).

3.2. Evaluation of qPCR-based antiviral activity testing

As many antiviral agents are nucleotide analogue or nucleoside analogue drugs which interfere with viral genome replication, we decided to determine their effects on HAdV DNA replication over a 24 h period post infection when most of the HAdV DNA replication takes place (Table 1). For all HAdV types tested (with exception of HAdV-A31) a HAdV DNA increase of more than 2 log was observed at 24 h p.i. (Table 1). Therefore, calculation of EC_{50}, EC_{90}, and EC_{99} values (50%, 90%, and 99% reduction of HAdV DNA replication, respectively) was feasible. This may be an advantageous feature of the assay because HAdV titers in disseminated disease are extremely high (e.g. 10^{10} copies/ml) (Claas et al., 2005; Heim et al., 2003; Seidemann et al., 2004) and reduction of virus loads by several magnitudes may be required for clinical effects. Therefore, EC_{99} values may be more helpful for predicting efficacy in vivo. As a first step of validation of our qPCR-based protocol, the well-established antiviral activity of the nucleotide analogue drug cidofovir against HAdV-C1 was tested. Reduction of HAdV DNA concentration was clearly concentration-dependent. Thus, the EC_{50} for cidofovir was determined to be <1 μM, the EC_{90} and EC_{99} were 3.1 μM (95% confidence interval (CI) 2–6 μM) and 18.8 μM (CI 95% 10–44 μM), respectively. As a second step antiviral activity of ribavirin against HAdV-C1 was tested in our qPCR-based protocol. For example, HAdV-C1 DNA replication was inhibited by 100 μM ribavirin at about 99.3% (Fig. 1A). Ribavirin also reduced increase of HAdV-C1 DNA in a concentration-dependent manner (Fig. 1A), and EC_{50}, EC_{90}, and EC_{99} values of <10 μM, 27.3 μM (CI 95% 15–54 μM) and 103 μM (CI 95% 52–230 μM) were calculated from this data (Table 2).

For comparison, a CPE-based antiviral assay also demonstrated activity of cidofovir and ribavirin against HAdV-1 but EC_{50} values were about 10-fold higher (11 and 64 μM, respectively), as compared to values determined by HAdV DNA quantification.

3.3. Antiviral activity of ribavirin against various serotypes of HAdV

Table 2 summarizes the in vitro inhibitory effect of ribavirin on DNA replication of HAdV prototypes and clinical isolates. Ribavirin had significant activity against all tested HAdV of species C (HAdV-C1, -C1 K, -C2, -C2 J, -C5) as determined by the qPCR-based assay (Table 2). Mean EC_{50}, EC_{90}, and EC_{99} of species HAdV-C were 9.3 μM (S.D. 1.8), 33.7 μM (S.D. 7) and 140.4 μM (S.D. 53.7). Comparison with cytotoxicity testing of ribavirin on A549 cells (CC_{50} value of 802 μM (CI 95% 662–977 μM)) indicated a specific inhibition of HAdV DNA replication not related to general cytotoxicity (SI > 80). For comparison, the CC_{50} values of cidofovir were even >1000 μM indicating a highly selective antiviral action (SI > 1336 for HAdV-C1). Antiviral activity of ribavirin against HAdV-B11 was slightly lower compared to HAdV-C species, but SI was still >80 (Table 2). For HAdV-A31, it was not feasible to determine

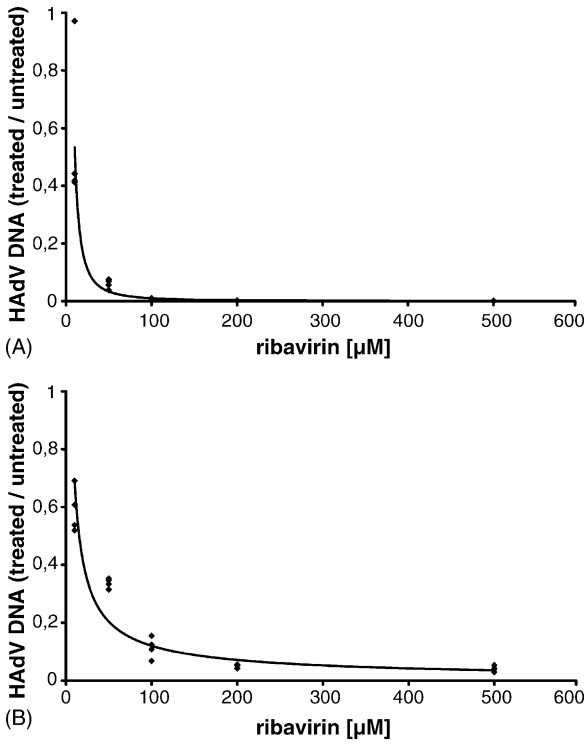

Fig. 1. Inhibition of HAdV DNA replication at 24 h p.i. by 0, 10, 50, 100, 200, and 500 μM ribavirin. (A) HAdV-C1 and (B) HAdV-B34.

Table 2
Antiviral activity of ribavirin against HAdV prototypes and clinical isolates in A549 cells (except where indicated)

	EC_{50} (μM)	EC_{90} (μM)	EC_{99} (μM)
HAdV prototype			
HAdV-C1	<10	27 (15–54)	104 (52–231)
HAdV-C2	<10	30 (23–42)	111 (79–159)
HAdV-C5	<10	28 (11–86)	104 (35–392)
HAdV-B11	<10	38 (14–131)	200 (63–883)
HAdV-A31	56 (19–277)	192 (55–1146)	nd[a]
HAdV-A31 (HeLa)	32 (12–127)	359 (98–2987)	nd[a]
HAdV-B34	16 (7–40)	143 (54–475)	>500
HAdV-D48	24 (9–84)	102 (33–448)	495 (133–2777)
Clinical isolates			
HAdV-C1 K	<10	41 (24–75)	229 (121–474)
HAdV-C2 J	13 (8–20)	42 (26–72)	155 (88–293)
HAdV-A31 W	17 (5–85)	196 (41–1718)	nd[a]
HAdV-A31 W (HeLa)	34 (15–90)	446 (157–1596)	nd[a]

Ribavirin concentrations for 50%, 90%, and 99% inhibition of viral DNA replication (EC_{50}, EC_{90}, and EC_{99}). Ninety-five percent confidence intervals indicated in brackets.

[a] Not determined due to insufficient increase of HAdV in untreated cultures.

an EC_{99} value at 24 h p.i., because the increase of HAdV-A31 DNA during the first 24 h p.i. was less than 2 log (Table 1). EC_{50} and EC_{90} values of the prototype HAdV-31 and a clinical isolate (HAdV-A31W) indicated that ribavirin had only low activity against HAdV-A31 resulting in SI values of 14 and 47, respectively. In order to calculate an EC_{99} value for HAdV-A31, the standard protocol was modified, and samples were taken at 48 h p.i. These results confirmed that the antiviral activity of ribavirin against HAdV-A31 (EC_{50} 20.8 μM (CI 95% 10–48 μM), EC_{90} 202.5 μM (CI 95% 84–596 μM), $EC_{99} > 500$ μM) and the clinical isolate HAdV-A31 W (EC_{50} 8.1 μM (CI 95% 2–90 μM), EC_{90} 104 μM (CI 95% 16–2718 μM), $EC_{99} > 500$ μM) was low compared to HAdV-C species (Table 2). As it was suspected that the low antiviral activity of ribavirin against HAdV-A31 was cell type-related because of its slow DNA replication in A549 cells, experiments were repeated with HeLa cells instead of A549 cells. However, almost identical results were obtained for the HAdV-A31 prototype and the clinical isolate HAdV-31 W (Table 2). Furthermore, antiviral activity of ribavirin against HAdV-B34 and -D48 was in the same range as activity against HAdV-A31 (Table 2), resulting in SI values of 50 and 33, respectively.

We did not test the activity of cidofovir against HAdV types other than HAdV-C1 because previous studies had demonstrated that cidofovir had almost identical activity against all HAdV serotypes tested (HAdV-C1, -C2, -E4, -C5, -B7, -D8, -D9, -B11, -B14, -D19, and -F41) (Gordon et al., 1991; Morfin et al., 2005).

4. Discussion

Development of a rapid and objective in vitro assay for testing antiviral agents against the 51 types of HAdV, which may exhibit different sensitivity to an antiviral agent was the first step of our study. CPE with rounding and grapelike clustering of swollen infected cells need to develop at least 48 h but may require up to 3 weeks, depending on the amount of virus (moi), HAdV serotype and cell line used (Kojaoghlanian et al., 2003; Morfin et al., 2005; Naesens et al., 2005; Swenson et al., 2003). This slows down testing of antiviral agents and almost precludes testing of clinical isolates for therapy decision making. Moreover, adenovirus CPE is sometimes hard to identify and hard to distinguish visually from non-specific cell alterations even by experienced laboratory personal (Mentel et al., 1996). Therefore, a fluorescent focus reduction assay has already been developed to test the antiviral activity of several drugs against HAdV (Mentel et al., 1996). This protocol made it much easier to identify the number of HAdV positive cells culture. However, this assay still required several days of time and specially trained lab personal. We developed a protocol, which uses the inhibition of HAdV DNA replication as an objective readout. This protocol takes advantage of a generic qPCR system which permits quantification of all 51 HAdV serotypes (Heim et al., 2003). Moreover, screening of new antiviral drugs that interact with the early steps of HAdV replication up to DNA replication by a qPCR-based protocol may be much faster and more objective compared to conventional CPE-based assays.

As a first step of validation of our qPCR-based protocol, the antiviral activity of the nucleotide analogue drug cidofovir against HAdV-C1 was tested. Thus, the EC_{50} for cidofovir was determined to be 0.6 μM (CI 95% 0.4–0.9). Although this value indicated a higher activity than our results with a classical CPE reduction assay (EC_{50} 11 μM), both results compared favorably to previously published data that varied also considerably. The activity of cidofovir against HAdV-C1 in A549 cells as determined by plaque reduction assay (EC_{50} 0.6 μM) (Gordon et al., 1991) was more than 10-fold higher as determined by antigen detection (EC_{50} 31 μM) (Morfin et al., 2005). The latter study stated also some variability depending on the cell line used (HEp-2 cells, EC_{50} 67 μM; PLC cells, EC_{50} 10 μM). Moreover, it was reported that other factors such, as for example, multiplicity of infection (moi) and time between inoculation and analysis of antiviral effects may influence EC_{50} results (Naesens et al., 2005). A high multiplicity of infection (10 $CCID_{50}$/cell) was used in our qPCR-based protocol to achieve an almost synchronous infection of cells. Therefore, antiviral activity can be quantified during the first round of HAdV replication. However, infection with a high moi may lead to decreased antiviral effects (Naesens et al., 2005) and consequently to a virtually lower activity of an antiviral agent compared to an assay using a low moi. Nevertheless, antiviral activity of cidofovir was demonstrated in our qPCR-based protocol with comparatively low EC_{50} values. This indicates that direct measurement of HAdV DNA replication, the putative target of cidofovir, is more sensitive for detecting the antiviral activity of cidofovir (and probably other nucleotide/nucleoside analogue antiviral agents) than measuring indirect parameters such as CPE or antigen concentration.

Similarly to cidofovir, antiviral activity of ribavirin against HAdV varied considerably, probably also depending on cell line, moi, and incubation time (Morfin et al., 2005; Naesens et al., 2005; Sidwell et al., 1972). In the first paper, antiviral activity of ribavirin against HAdV was reported but no detailed data on different HAdV types were presented (Sidwell et al., 1972). A more recent study showed activity of ribavirin against HAdV C species but not other species of HAdV (Morfin et al., 2005), whereas another recent study did not detect antiviral activity even against HAdV-C2 (Naesens et al., 2005). Regarding the more than 1 log differences between EC_{50} values of cidofovir determined by slightly different protocols (Morfin et al., 2005; Naesens et al., 2005), it is plausible that with a probably less active substance such as ribavirin contradictory results on antiviral activity may be generated. However, it is remarkable that a species-specific activity of ribavirin against HAdV-C was observed both by Morfin et al. (2005) and in this study (Table 2). As for cidofovir, antiviral activity of ribavirin against HAdV-C1 DNA replication ($EC_{50} < 10$ μM) was higher than determined by a CPE-based assays (EC_{50} 62 μM) although a lower moi was used in the latter assay. This result may suggest that ribavirin inhibits directly or indirectly HAdV DNA replication. Unfortunately, the mode of antiviral action of ribavirin against HAdV is unknown. A direct inhibition of HAdV DNA polymerase by ribavirin seems to be quite unlikely because ribavirin is nucleoside analogue and not a deoxynucleoside analogue. However,

this mode of action is not impossible because precise substrate specificity of HAdV DNA polymerase is unknown.

Recently, it was demonstrated that ribavirin directly interacts the RNA polymerase of poliovirus as a false substrate (Crotty et al., 2000). In case of poliovirus, an increased mutation rate by ribavirin leading to error catastrophe seems to be more important for antiviral effect than direct inhibition of the polymerase (Crotty et al., 2000). This may also explain previous results showing that ribavirin is more active against persistent Coxsackievirus infections than acute infections (Heim et al., 1997). Moreover, ribavirin resistant mutants emerge indicating the specific interaction of ribavirin with poliovirus polymerase (Heim et al., 1997; Vignuzzi et al., 2005). Ribavirin may also interfere with priming of HAdV DNA replication by terminal protein (Temperley and Hay, 1992) or inhibit capping of early transcripts of HAdV as described for other viruses (Goswami et al., 1979; Scheidel and Stollar, 1991) and thus interfere with expression of HAdV early proteins necessary for efficient HAdV DNA replication. HAdV type specific expression levels of early proteins or type-related susceptibility of HAdV DNA polymerase to ribavirin could explain the variable sensitivity of HAdV types to ribavirin. By contrast, inhibition of IMP dehydrogenase by ribavirin resulting in decreased cellular dGTP pools (Streeter et al., 1973) can probably not explain type-related sensitivity of different HAdV types. However, this does not preclude that IMP dehydrogenase inhibition may contribute to the antiviral activity of ribavirin against HAdV. Recently, Zhang et al. (2003) suggested, that ribavirin interferes with the virus-induced interferon signaling pathway and intenses the interferon activity.

Another topic of this study was to establish the in vitro activity of ribavirin against several clinically important HAdV serotypes strains and clinical isolates. Different activity of ribavirin against HAdV types (Table 2) (Morfin et al., 2005) may explain the different outcomes of HAdV infected patients treated with ribavirin. So far, controlled and randomized clinical studies on antiviral treatment of HAdV disease with ribavirin or cidofovir are not available and treatment options for the life-threatening disseminated HAdV disease are very limited (Baldwin et al., 2000; Bordigoni et al., 2001; Carrigan, 1997; Chakrabarti et al., 2002; Kojaoghlanian et al., 2003; La Rosa et al., 2001; Ljungman, 2004; Seidemann et al., 2004). Nevertheless, smaller studies and case reports were suggestive of potentially beneficial effects of ribavirin in immunocompromised patients (Arav-Boger et al., 2000; Gavin and Katz, 2002; Kojaoghlanian et al., 2003; Ljungman, 2004; Schleuning et al., 2004) but ribavirin therapy was not always successful. (Bordigoni et al., 2001; Lankester et al., 2004). It was suspected that treatment failures may be related to massively compromised immune status, and the importance of host factors in antiviral treatment of disseminated adenovirus infections can be surmised. HAdV DNA loads in clinical blood samples of massively immunocompromised bone marrow recipients suffering from life-threatening disseminated HAdV disease may reach concentrations of $>1 \times 10^{10}$ HAdV DNA copies/ml blood (Heim et al., 2003; Lankester et al., 2004; Lion et al., 2003; Seidemann et al., 2004).

Therefore, a prerequisite for a successful treatment seems to be a significant inhibition of HAdV replication by at least 1 log (90%) or several logs (for example 99% or 99.9%). Antiviral activity is usually indicated by calculating EC_{50} values. However, our qPCR experiments were also designed to evaluate the capacity of ribavirin to inhibit HAdV replication almost completely (for example by 99%) by determining EC_{99} values. This parameter seems to be a better predictor for antiviral efficacy in disseminated HAdV infections with high virus loads, since EC_{99} values of different HAdV types varied widely (Table 2). These results suggest that clinical failure of ribavirin therapy may not only depend on host factors but also on the sensitivity of the individual HAdV type causing the infection (Venard et al., 2000).

Besides prototype stains of HAdV types, clinical HAdV isolates from patients with disseminated HAdV disease were tested in this study. EC_{50}, EC_{90}, and EC_{99} values were in the same range as HAdV prototypes (Table 2). These results suggest that typing of clinical HAdV isolates may be sufficient to predict ribavirin sensitivity. Nevertheless, ribavirin sensitivity testing of clinical isolates seems to be a sensible strategy because typing of clinical isolates usually requires more than 1 week. By contrast, results on ribavirin sensitivity of clinical isolates can be obtained by the qPCR-based protocol as early as 30 h after isolating the virus. For providing sensitivity testing results more rapidly, the clinical isolate inoculum should be quantified by qPCR instead of the $CCID_{50}$ method. Comparison of qPCR with $CCID_{50}$ results of clinical isolates revealed a mean difference of HAdV DNA (copies/ml) to $CCID_{50}$/ml of 3 log (S.D. 1.7). Therefore, we suggest an inoculum with a concentration of 10^4 HAdV DNA copies/cell, approximately equal to a moi of 10 $CCID_{50}$/cell. However, threshold EC_{50} values describing sensitivity or resistance of clinical HAdV isolates have to be determined in clinical treatment studies.

Although controlled clinical studies for ribavirin treatment of HAdV disease are not available, there is ample clinical data on pharmacokinetics, tolerated doses and side effects. Ribavirin peak plasma concentrations (160.8 μM) were even above EC_{99} values of highly sensitive HAdV-C viruses (Table 2) and can be reached after a single dose of 2400 mg intravenous ribavirin (Laskin et al., 1987). Trough levels 8 h after infusion were 10.2 μM, still above EC_{50} values of HAdV-C (Table 2) (Laskin et al., 1987). These pharmacokinetic results clearly support clinical studies with i.v. ribavirin for disseminated HAdV-C infections. With long term oral application of 400 mg ribavirin twice daily, mean peak concentration of 6.61 μM (S.D. 0.5) to 11.07 μM (S.D. 2.3) were reached, approximately EC_{50} concentrations of species C HADV (Table 2) (Lertora et al., 1991). No significant toxicities related to ribavirin were apparent, except hemolytic anemia which did not require discontinuation of ribavirin (Lertora et al., 1991). Hemolytic anemia is caused by accumulation of ribavirin in red blood cells because red blood cells lack 5′ nucleotidase and alkaline phosphatase (Page and Connor, 1990). In vitro, ribavirin had no or only minor cytotoxic effects on A549 cells (CC_{50} 802 μM, 95% confidence interval 662–977 μM). This result is in accordance with the low cytotoxicity of ribavirin in HeLa and Vero cells, as determined by 50%

inhibition of plating efficiency (4000 μM) (Kirsi et al., 1984) and CC_{50} values for HEp-2 cells (400 μM) and PLC cells (2200 μM) (Morfin et al., 2005). However, a higher cytotoxic activity was observed in human embryonic lung fibroblasts (HEL) (CC_{50} 30 μM) (Naesens et al., 2005). The latter CC_{50} value indicated a significant cytotoxicity of ribavirin, which seems to be considerably higher than in all other cell lines and also much higher than anticipated by side effects of clinical studies.

In conclusion, rapid and objective results generated by a qPCR-based antiviral activity assay confirmed the high activity of cidofovir and indicated the serotype-dependent activity of ribavirin against the most predominant HAdV of species C. The established qPCR-based protocol may also speed up the screening of new antiviral agents against HAdV. Virus load testing in diagnostic blood samples, typing of clinical isolates, and in future sensitivity testing of clinical HAdV isolates may also help to design clinical studies for evaluating antiviral agents against HAdV.

Acknowledgment

We thank Jens Hainmueller, MPA (Harvard University, John F. Kennedy School, Department of Government, Boston, MA) for help with biostatistics.

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