ELSEVIER

Antiviral Research 56 (2002) 73–84

Antiviral Research

www.elsevier.com/locate/antiviral

Sequence changes in the human adenovirus type 5 DNA polymerase associated with resistance to the broad spectrum antiviral cidofovir

Paul R. Kinchington [a,b,*], Trinita Araullo-Cruz [a], Jean-Paul Vergnes [a], Kathleen Yates [a], Y. Jerold Gordon [a]

[a] *Department of Ophthalmology, 1020 Eye and Ear Institute, University of Pittsburgh, 203 Lothrop Street, Pittsburgh, PA 15213, USA*
[b] *Department of Molecular Genetics and Biochemistry, University of Pittsburgh, 203 Lothrop Street, Pittsburgh, PA 15213, USA*

Received 20 June 2001; accepted 11 June 2002

Abstract

Although there is currently no FDA approved antiviral treatment for adenovirus (Ad) infections, the broad spectrum antiviral cidofovir (CDV) has demonstrated potent inhibitory activity against many Ad serotypes in vitro and in an in vivo ocular replication model. The clinical potential of CDV prompted the assessment for the emergence of CDV resistance in Ad5. Serial passage of Ad5 in increasing concentrations of CDV resulted in derivation of four different Ad5 variants with increased resistance to CDV. CDV resistance was demonstrated by ability to replicate viral DNA in infected cells at CDV concentrations that inhibit the parental virus, by ability to form plaques in CDV concentrations of >20 μg/ml and by increased progeny release following infection and growth in media containing CDV. Using marker rescue, the loci for CDV resistance in variant R1 was shown to be mediated by one residue change L741S, one of two mutations within the R1 encoded DNA polymerase. The CDV-resistant variants R4, R5 and R6 also contained mutations in their respective DNA polymerase sequences, but these were different from R1; variant R4 contained two changes (F740I and V180I), whereas both R5 and R6 variants contained the non-conserved mutation A359E. R6 contained additional alterations L554F and V817L. The location of the R1 change is close to a region of the DNA polymerase which is conserved with other polymerases that is predicted to involve nucleotide binding.

Keywords: Antiviral drugs; Adenovirus; Cidofovir; Drug resistance

1. Introduction

The 49 known serotypes of adenovirus cause several common human diseases which involve the respiratory tract, conjunctiva, gastrointestinal tract, urinary bladder and liver. While the majority of these diseases are self-limiting and have few

* Corresponding author. Tel.: +1-412-647-6319; fax: +1-412-647-5880
E-mail address: kinchingtonp@msx.upmc.edu (P.R. Kinchington).

0166-3542/02/$ - see front matter
PII: S0166-3542(02)00098-0

long term consequences, there is considerable morbidity and up to 28% mortality associated with adenoviral infections in patients with underlying immune compromise, such as those with an underlying HIV infection or those undergoing haematopoietic stem-cell grafts (Howard et al., 1999). The morbidity and mortality associated with immune compromised infections would clearly be limited by effective antiviral intervention against adenoviruses (Baldwin et al., 2000; Hoffman et al., 2000). Adenoviruses are also the most common infectious cause of ocular disease worldwide (Gordon et al., 1996a). The most serious manifestation, epidemic keratoconjunctivitis (EKC), is highly infectious, often involves both eyes, and may cause significant patient morbidity and some vision loss as a result of the formation of sub-epithelial immune infiltrates (SEI) (Gordon et al., 1996a).

The high morbidity and potential sight-threatening consequences have prompted the exploration of effective antivirals against adenovirus infections. To date, there is no FDA approved antiviral for treatment of adenovirus disease. Antivirals such acyclovir (ACV) or ganciclovir (GCV), which are activated through viral nucleotide/protein kinase activities in herpesvirus-infected cells that mediate an initial phosphorylation step, are not effective against adenoviruses because adenoviruses do not encode virus specific kinase activities. However, several antivirals based on acyclic phosphonyl nucleotide analogs have activity against adenoviruses (de Clercq, 1997), and the antiviral cidofovir (CDV) has been the most developed. As an analog of dCMP, CDV does not require the initial viral mediated phosphorylation, and inhibits replication of many DNA viruses including adenoviruses, herpesviruses, papillomaviruses and poxviruses (de Clercq, 1997; Lea and Bryson, 1996; Safrin et al., 1999; Jabs, 1997; Chatterjee et al., 1992; Bronson et al., 1990). CDV also has activity against certain tumors (Andrei et al., 1998; de Clercq et al., 1999). CDV is FDA-approved for systemic treatment of human cytomegalovirus (hCMV) retinitis in AIDS patients, and is a first line alternative in cases where resistance to GCV develops (Kendle and Fan-Havard, 1998; Emery, 1998; Plosker and Noble, 1999; Kiehl and Basara, 2001). CDV has also shown potential application for treatment of AIDS related progressive multifocal leukoencephalopathy (De Luca et al., 2001) and has been evaluated in topical use for treatment of papilloma virus infections (Snoeck et al., 2001), herpesvirus skin infections (Afouna et al., 1999) as well as for herpes simplex virus (HSV) and hCMV infections resistant to other antivirals (Martinez and Luks-Golger, 1997; Snoeck et al., 1996; Platzbecker et al., 2001). Regarding adenoviruses, CDV inhibits all serotypes tested (Gordon et al., 1991a,b, 1992; Romanowski and Gordon, 2000). Low and infrequent dosing with CDV inhibits adenoviral replication in an in vivo ocular replication model in the NZ white rabbit, reducing the duration of virus shedding and the peak titers obtained on the ocular surface (Gordon et al., 1992, 1991a, 1994a; Romanowski et al., 1997, 1999; Romanowski and Gordon, 2000). The broad spectrum nature of CDV indicates possible use for both adenoviral and herpesvirus ocular infections (Romanowski et al., 1999; Gordon et al., 1994b). While recent evidence has suggested that off-label clinical use of topical CDV for ocular adenovirus and herpesvirus infections is not recommended, as CDV may lead to lacrimal canalicular blockage (Romanowski et al., 2001b), there remains considerable potential for the use of CDV to treat life-threatening systemic adenovirus infections in immune compromised patients (Bordigoni et al., 2001; Hoffman et al., 2000; Legrand et al., 2001).

One of the most important issues to be addressed in the development and assessment of any antiviral is the emergence of antiviral resistance. Drug resistance poses a formidable challenge in the clinical setting, and resistance to CDV has proven to be a complicating problem in the treatment of hCMV retinitis in AIDS patients that have co-developed CDV resistance following emergence of GCV resistance (Erice, 1999; Emery, 1998, 2001). CDV-resistant hCMV and HSV have been isolated in vitro following serial passage in CDV or GCV (Cihlar et al., 1998; Andrei et al., 2000; Lurain et al., 1992). An initial report from us detailed development of CDV antiviral resistance in vitro in adenovirus serotype 5 following passage in CDV (Gordon et al., 1996b). In the Ad5-rabbit

model of ocular disease, the Ad5 CDV-resistant variants showed no significant drop in pathogenicity, and demonstrated preferential replication in CDV treated infected eyes (Romanowski et al., 2001a). In this work, we report the characterization of the DNA sequence changes which are associated with CDV resistance in Ad5.

2. Materials and methods

2.1. Cells, viruses and experimental drugs

Three CDV-resistant variants (designated R1, R2 and R3) that were derived from stocks of the ATCC reference strain of Ad5 (designated Ad-P) have been described previously (Gordon et al., 1996b). All three variants were subsequently found to be genotypically identical to R1 in respect to the DNA polymerase genes and thus only R1 virus was characterized in these studies. The variants R4, R5 and R6 were independently developed from three plaque purified isolates of the ATCC strain (American Type Culture Collection, Manassas, VA) designated Ad-P4, Ad-P5 and Ad-P6, respectively, and were derived in a similar fashion. Briefly, parental stocks were infected at low multiplicity (0.01–0.1 plaque forming units (pfu)/cell) onto A549 monolayers and grown ion media with 7 μg/ml CDV for 10 days. Progeny virus was obtained by multiple freeze-thaw and serially passed at low multiplicities in media containing CDV (Table 1). Serial passage at higher CDV concentrations was initiated following efficient growth at the lower CDV concentration. The final stocks of the CDV-resistant variants were prepared from isolated plaques growing in CDV, and these were used to determine IC_{50} values shown in Table 1. The Ad5 temperature sensitive (ts) mutant H5ts149, which contains a lesion in the DNA polymerase coding sequence, resulting in substitution of $leucine_{411}$ to phenylalanine (Roovers et al., 1990), was kindly supplied by Dr C.S. Young (Department of Microbiology, Columbia University, New York, NY). All virus stocks were grown in A549 cells prepared in Minimal Essential Media containing 6% fetal bovine serum and antibiotics as detailed previously (Gordon et al., 1991a), except that stocks of H5ts149 were prepared at 32 °C. CDV for molecular studies was provided by Gilead Sciences (Foster City, CA).

Table 1
IC_{50} of parental and CDV-resistant variants

	[CDV]Passage	CDV-IC_{50} (μg/ml)
Ad-P	–	5.1±1.4
R1	15_6, 20_{10}, 30_4, pp	36.5±5.8
Ad-P4	pp, 0_2	3.8±1.8
R4	7_1, 10_1, 14_1, 20_{10}, 30_9	25.0±4.24
Ad-P5	pp, 0_2	3.6±1.58
R5	7_1, 10_1, 14_5, 20_9, pp	23.7±2.08
Ad-P6	pp, 0_2	3.95±1.81
R6	7_1, 10_1, 14_1, 20_{10},30_9, pp	28.9±1.89

Passage history is given as the concentration of CDV used and the number of passages at that concentration in subscript. For example, R1 was passed six times at 15 μg/ml, followed by 10 passages at 20 μg/ml, and four passages at 30 μg/ml; pp, plaque picked. IC_{50} was determined by the concentration required to reduce the number of plaques at 10 days postinfection by 50%. [CDV] is given in μg/ml.

2.2. DNA preparation and analysis

DNA for cloning and the DNA replication assay was prepared from A549 cells infected with 0.1 pfu/cell and grown in the absence or presence of 20 μg/ml of CDV for 96 h. Whole infected cell DNA was extracted following cell lysis and digestion in 10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% SDS and 200 μg/ml proteinase K at 55 °C for 3 h, followed by phenol/chloroform extraction and ethanol precipitation. For characterization of the R1 and Ad-P DNA polymerase coding sequences, DNA-plasmid clones were prepared using a 3.7 kb BglII DNA fragment, representing sequences between 3328 and 8914 (with respect to the adenovirus type 5 genomic sequence in the NCBI database, accession number NC 001406), cloned into the BamHI site of pGem3z (Promega Corp, Madison, WI). DNA clones of R4, R5, R6 and the respective parental strains were derived using a DNA fragment generated by SphI and KpnI digestion, representing sequences from 5137 to 8416. The complete DNA polymerase coding region is transcribed from right to left in the adenovirus genome and is encoded by sequence

from 8370 (ATG start codon) to 5191 (stop codon). DNA sequencing of both strands of the cloned fragments was determined using the T7, SP6 primers and 10 synthetic primers derived from the Ad5 DNA sequence that enabled redundant sequencing of both strands of the entire DNA polymerase from each virus, using the University of Pittsburgh core sequencing facility. DNA sequencing of the DNA polymerase of viruses isolated from the marker rescue experiments were obtained from four PCR-amplified overlapping regions representing the complete DNA polymerase sequences. For DNA replication analysis, A549 cells infected at 0.1 pfu/cell with R1 and parental virus were grown for 18 h in the presence or absence of 20 μg/ml CDV, and DNA was prepared as just outlined. Equivalent levels of each infected cell DNA were digested with PstI, subjected to agarose gel electrophoresis and blotted to membranes using standard protocols (Sambrook et al., 1989). Adenovirus DNA was visualized following probing with α-^{32}P-dCTP radiolabeled purified serotype 2 DNA (Gibco BRL Laboratories Inc., Gaithersburg, MD) labeled using a random primer extension kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

2.3. Marker rescue

Marker transfer was carried out using procedures detailed previously (Volkert et al., 1989). Briefly, infectious Ad5 H5ts149 DNA–terminal protein (DNA–TP) complexes were prepared from purified adenovirus H5ts149 virions grown at 32 °C in A549 cells that were harvested at 80% visible cytopathic effect. Virus was released from infected cells by multiple freeze-thaw cycles, followed by membrane extraction using 1,1,2-trichlorofluoroethane. Virus in the aqueous phase was fractionated on discontinuous CsCl gradients for 26 h at 60,000 × g, and virus present at the interface between densities of 1.3 and 1.5 μg/ml was collected, dialyzed and then disrupted in DNA release buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 4 M guanidinium HCl, 2 mM 2-mercaptoethanol). DNA–TP complexes were fractionated on continuous 10–40% sucrose gradients made in the same buffer and centrifuged to equilibrium for 60,000 × g for 18 h. Fractions containing DNA were identified by spectrophotometry at 260 nm, dialyzed against 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and concentrated by centrifugal filtration. Transfection of the DNA into A549 cells was carried out using a modified calcium phosphate precipitation method (Graham et al., 1977), with 1–3 μg of DNA–TP complex and 1 μg of linearized plasmid containing the cloned BglII fragment from Ad5 variant R1 and carrier A549 DNA. A549 cells were exposed to the precipitated DNA for 4–6 h at 37 °C, when media was replaced and co-transfected cells were then incubated at 32 °C until 20% cytopathic effect was observed. Virus progeny from the co-transfection was titrated at 32, 37 and 39 °C, either in the absence or presence of 20 μg/ml of CDV. For genetic and growth analysis, well-isolated plaques obtained at the 39 °C titration in the presence of CDV were grown and DNA prepared as just detailed prior to PCR amplification for DNA sequence analysis.

2.4. Progeny yield analysis

The progeny yield of viruses grown in several CDV concentrations was determined by establishing six identical sets of confluent A549 cells for each virus, which were infected in parallel at an approximate multiplicity of 1/cell. Following 2 h absorption at 37 °C, cells were washed twice in warm media to remove residual unbound virus, and subsequently incubated for 48 h in media containing 0, 10, 20, 30, 40 and 50 μg/ml CDV. Subsequently, infected cells were scraped from the tissue culture dish into their own media, frozen/thawed × 4 and subsequently titrated on fresh monolayers of A549 cells in duplicate to obtain progeny titers.

3. Results

3.1. Isolation and characterization of Ad5 CDV-resistant variants

Six CDV-resistant viruses were isolated in total. Variants R1, R2 and R3, whose isolation was

detailed previously (Gordon et al., 1996b) were subsequently found to contain the same mutations in the DNA polymerase genes (see below), and only the CDV-resistant variant R1 was further studied here. The parallel isolation of three genotypically identical viruses suggested a common origin (such as their presence in the parental stocks) and, therefore, the subsequent isolation of CDV-resistant variants designated R4, R5, and R6 were derived from plaque-purified parental stocks of the adenovirus ATCC strain (designated Ad-P4, Ad-P5 and Ad-P6, respectively). This approach forced the de novo development of CDV resistance over the possible selection for preexisting CDV-resistant variants. Table 1 shows the IC_{50} (μg/ml) for each virus and the respective parent, based upon the concentration of CDV required for quantitative reduction of plaque numbers to 50% of that in the absence of CDV. In general, the CDV-resistant variants demonstrated an approximately sixfold increase in IC_{50} level over that of the parental Ad5 viruses. At CDV concentrations of 20 μg/ml, while plaque numbers were not affected for resistant viruses, plaque size was smaller in media containing CDV than without. To demonstrate CDV resistance through other methods, we assayed the accumulation of viral DNA in Ad5-P and R1-infected cells in the presence or absence of 20 μg/ml CDV at 18 h postinfection. At 2 h postinfection (reflecting input virus), DNA was not detected in any samples by Southern blot (data not shown). However, at 18 h postinfection in the presence of CDV, R1-infected cells accumulated 26% of the viral DNA that accumulated in the absence of CDV (Fig. 1). In contrast, the same concentration of CDV resulted in accumulation of less than 1% of the Ad-P DNA accumulating in untreated controls. The DNA showed no alterations in the restriction profile, and CDV resistance was not accompanied with alterations in plaque morphology other than size. Similar results were obtained for all other CDV-resistant variants (not shown).

The effect of CDV on virus growth of parental and CDV-resistant viruses was further demonstrated by determining progeny virus yield titers obtained from infected cells at 48 h postinfection and incubated in various concentrations of CDV

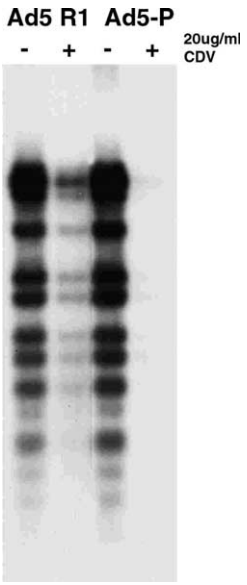

Fig. 1. Southern blot of adenoviral DNA accumulating in infected cells at 18 h postinfection, in the presence (+) and absence (−) of 20 μg/ml of CDV in the media. Cells were infected at 0.1 pfu/cell. One microgram of total infected cell DNA was digested with PstI and electrophoresed in each lane. Adenoviral DNA was identified by using a ^{32}P-dCTP-radiolabeled adenovirus type 2 DNA probe, and bands were quantified by phosphorimager. The DNA fragments shown range in size from 3.6 kbp to 430 bp.

(Fig. 2). Compared to growth in the absence of CDV, the adenovirus parental strain Ad-P demonstrated a 1 log reduction in virus growth at 20 μg/ml CDV and a greater than 3 log loss of virus production titers at CDV concentrations of 40 μg/ml or greater. The plaque-purified parental isolates (Ad-P4, 5, 6) demonstrated almost identical reductions at each CDV concentration (not shown). In contrast, all CDV-resistant viruses demonstrated a lower rate of decline in progeny titers with increasing CDV concentrations, and demonstrated a 1.6–2.2 log difference in progeny titers from those obtained from the parental strain at 50 μg/ml CDV. These data further demonstrate the CDV resistance of the R1, R4, R5 and R6 viruses.

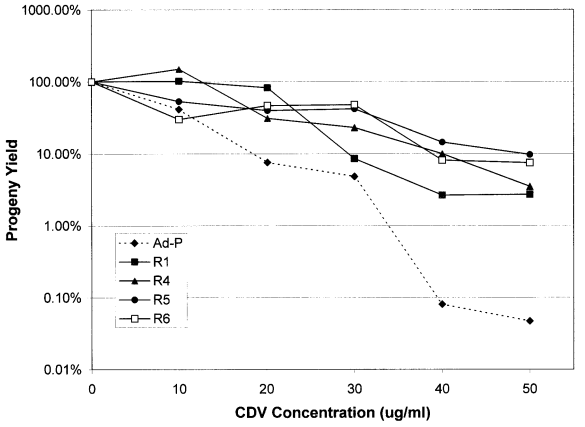

Fig. 2. A progeny virus assay for the adenovirus parents Ad-P and the four CDV-resistant variants. Cell monlayers were infected with adenovirus and incubated in the absence or in the presence of media containing 10–50 μg/ml CDV for 48 h. The titers of the virus produced at each concentration were the mean of duplicate titrations. The graph shows the log reduction in virus progeny over CDV concentrations from 10 to 50 μg/ml, as a percentage of the progeny virus titer determined following growth in the absence of CDV (set as 100%). All titrations were performed in the absence of CDV.

3.2. CDV resistance is directed by the Ad5 DNA polymerase

Resistance to CDV was strongly suspected to be directed by amino acid changes within the Ad5 encoded DNA polymerase. CDV is a monophosphate nucleotide analog that, upon diphosphorylation by cellular enzymes, competes for dCTP incorporation into DNA by the viral encoded DNA polymerase. Resistance to CDV in HSV-1 (Andrei et al., 2000) and hCMV (Cihlar et al., 1998) correlates with mutations in the respective viral DNA polymerases. However, to establish 'proof of principle' that CDV resistance is directed by Ad5 DNA polymerase, marker transfer studies were carried out with the DNA polymerase from CDV-resistant virus R1. A cloned DNA fragment containing the complete R1 DNA polymerase gene was co-transfected with infectious DNA–TP complexes derived from the ts Ad5 mutant H5ts149, which contains a single point mutation at residue 411 of the DNA polymerase gene as the basis of its temperature sensitivity (Roovers et al., 1990). DNA recombination events resulting in replacement of the H5ts149 DNA polymerase with the R1 polymerase were expected to rescue the ts phenotype and simultaneously transfer resistance to the H5ts149 genome (except where DNA recombination events occurred between and separated the ts locus and the CDV resistance locus). Following co-transfection and growth at 32 °C, progeny virus was harvested and titrated at 32, 37 and 39 °C in the absence or presence of CDV (Fig. 3). H5ts149 formed plaques efficiently at 32 °C, and formed almost no plaques at 37 and 39 °C, and at all temperatures formed no plaques in the presence of CDV. On the other hand, R1 plaque efficiency and size increased with increasing temperature, and plaques formed both in the absence and presence of CDV (plaque size was smaller on CDV treated cultures but were of similar number). Progeny virus resulting from the co-transfection of the R1 DNA polymerase with H5ts149 DNA contained non-ts virus, as indicated by plaque formation at 39 °C. Of these, 32% of the co-transfection plaque titer determined at 39 °C in the absence of CDV was detected following incubation at 39 °C in the presence of CDV. Marker rescue events with DNA fragments of similar size derived from other regions of the R1 genome did not result in any CDV-resistant viruses or rescue of the ts phenotype (not shown). Those viruses from the co-transfection showing a CDV sensitive, non-ts phenotype likely resulted from crossover events during recombination that separated the ts mutation and the locus encoding resistance. We conclude that a portion of marker-rescued virus contained the mutations driving CDV resistance, establishing that the DNA polymerase from R1 encodes CDV resistance. This was confirmed by sequence analysis (see below). This strongly implicates the DNA polymerase genes in mutants R4, R5 and R6 is also the locus for CDV resistance in those viruses.

3.3. Identity of changes in the DNA polymerase associated with CDV resistance

The complete DNA sequences of the DNA polymerases from all CDV-resistant and parental viruses was determined. The sequence of the reference ATCC strain (Ad-P) and the plaque

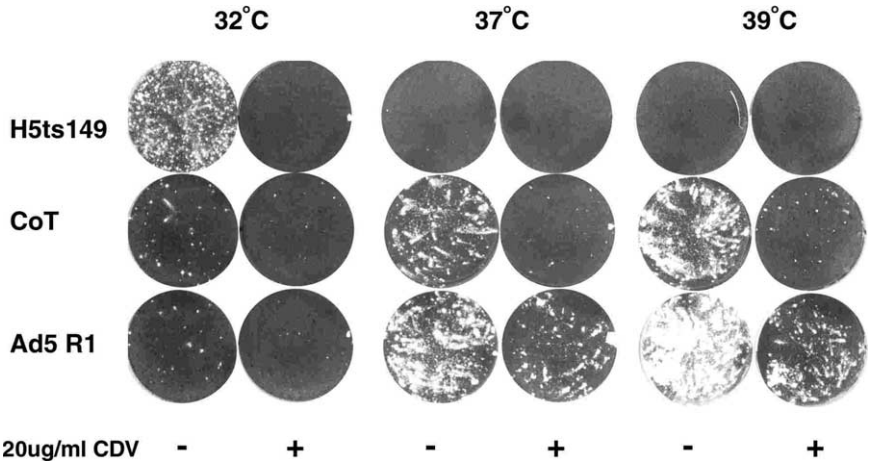

Fig. 3. Plaques of A549 infected cells grown at 32, 37 and 39 °C in the presence (+) or absence (−) of 20 μg/ml CDV, with either the ts virus H5ts149, the Ad5 R1 CDV-resistant mutant (Ad5-R1), or were progeny virus resulting from co-transfection of Ad5 H5ts149 with a DNA fragment from the R1 containing the DNA polymerase (CoT). Cells were inoculated with approximately 100–150 pfu/cell (determined for CoT and R1 at 37 °C and for H5ts149 at 32 °C). Plaques were visualized by crystal violet staining at 6 days postinfection.

purified parental strains was identical to the published Ad5 genomic sequence found in the NCBI database (NC 001406). CDV-resistant variant R1 (and previously detailed variants R2 and R3) contained two base pair changes at positions 1041 and 741 (Table 2). Variants R4, R5 and R6 contained different sequence changes in their DNA polymerases. R4 contained two amino acid mutations, with one notably at position 740, immediately adjacent to the CDV resistance-defining mutation of the R1 virus (see below). An additional silent base pair change was found at position 5992 of R4. Variants R5 and R6 both contained a relatively non-conserved mutation at residue 359, and variant R6 containing two additional mutations (Table 2).

Table 2
Amino acid changes in the adenovirus DNA polymerase gene of CDV-resistant mutants

Variants R1, R2, R3	L741S[a], S1041R
Variant R4	F740I, V180I
Variant R5	A359E
Variant R6	A359E, L554F, V817L

Amino acids are given with respect to the standard one letter code. The first is that in the parent, followed by the residue position within the adenovirus type 5 DNA polymerase, followed by the resulting amino acid in the mutant.

[a] This altered residue is sufficient for CDV resistance.

Characterization of the complete DNA polymerase sequence of six well-isolated marker rescued viruses isolated from non-ts and CDV-resistant plaques revealed that five of the marker-rescued viruses contained both mutations found in the R1 polymerase and contained a wild type leucine at residue 411, the ts locus of H5ts149. However, one of the viral isolates (RP3) contained the mutation at residue 741 and a wild-type serine at residue 1041. This result implied that the change at residue 741 is sufficient for CDV resistance. To determine if the isolate with only one change resulted in different CDV sensitivity as compared to the viruses containing two mutations, five of the plaque-isolated viruses from the co-transfection were directly compared in a progeny virus yield assay to parental virus (Fig. 4; carried out at 37 °C). Parental virus (Ad-P) showed CDV sensitivity with an approximate 1 log drop in progeny at 20 μg/ml CDV, and greater than 3 log drop at 50 μg/ml CDV. Of the five marker rescued isolates, including the single mutation variant RP3, all demonstrated resistance to CDV and a reduced rate of loss of progeny virus with increasing CDV concentrations. Their behavior in

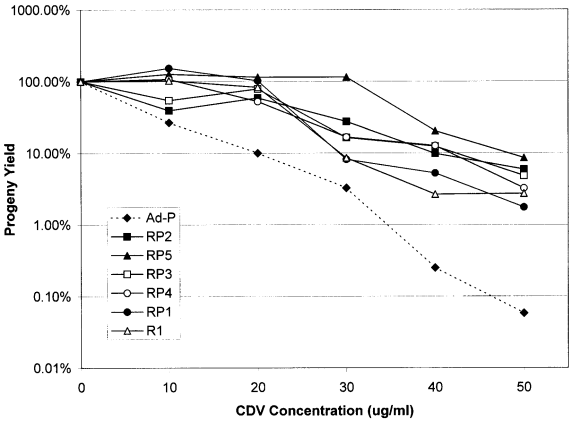

Fig. 4. A progeny virus assay for the adenovirus parent (Ad-P) the R1 CDV-resistant variant and for five plaque purified CDV-resistant variants isolated at 39 °C from the marker rescue studies. The assay was carried out as detailed in Section 2 and in the legend to Fig. 2. Isolates R1, RP1, RP2, RP4 and RP5 all contain two amino acid changes at residues 1041 and 741 of the DNA polymerase, whereas isolate RP3 contains only the mutation at residue 741.

this assay closely matched that of the R1 virus with both mutations. This data strongly implies that the changes at position 741 are sufficient for CDV resistance, and that the changes at position 1041 have no discernable additional effects.

Fig. 5 shows the nucleotide changes in the four CDV-resistant DNA polymerases in relation to domains of the adenovirus DNA polymerase gene which are conserved with other DNA polymerases (Earl et al., 1986; Wang et al., 1989). The mutations at residues 741 (found in R1) and at 740 (found in R4) are located immediately adjacent to a region designated region III, which has been implicated in nucleotide binding (Earl et al., 1986; Wang et al., 1989). A comparison of the known DNA sequence of adenovirus serotypes reveals that the sequence surrounding the 741 mutation (SSFLET) is completely conserved at this location in adenovirus types 2, 5, 4, 7, and 17, and is only slightly altered in adenovirus types 40 and 12 (TSFVET in type 40 and SSFIET in type 12). The positions of most other mutations identified lie outside the conserved regions.

4. Discussion

The study of the mechanisms of antiviral resistance offers important insights into the nature of drug–virus interactions and viral pathogenesis. Practically, the development of resistance remains an important consideration in the preclinical evaluation of any antiviral. As CDV has potent activity against multiple adenovirus serotypes in vitro and in vivo (de Oliveira et al., 1996; Gordon et al., 1991a,b; Romanowski and Gordon, 2000), it was proposed as a candidate for the treatment of adenoviral disease. Recent formulations for its use include a topical form for adenovirus ocular infections (Romanowski and Gordon, 2000), or as a systemic treatment for life-threatening adenovirus infections in stem cell transplant recipients (Bordigoni et al., 2001; Hoffman et al., 2000; Legrand et al., 2001). Here, we demonstrate that adenovirus type 5 can develop CDV resistance following growth of virus in sub-inhibitory concentrations of CDV. As far as we are aware, the CDV-resistant Ad5 variants reported here represent the first drug resistant adenoviruses described.

The determinants of CDV resistance were unequivocally established in one variant (R1) to be sufficiently directed by a single base change at residue 741 of the DNA polymerase. This change was located immediately adjacent to a domain conserved in other DNA polymerases that is suspected to be involved in nucleotide binding. The proof that the adenovirus DNA polymerase encoded CDV resistance was not surprising, as CDV resistance in HSV-1 and CMV is directed by changes in the viral DNA polymerases (Andrei et al., 2000; Snoeck et al., 1996; Emery, 1998; Cihlar et al., 1998; Chou et al., 2000). In addition, the adenovirus genome does not contain candidate genes that could conceivably affect nucleotide metabolism of CDV, such as are found in herpesvirus genomes. The suspected mechanism of action of CDV is that it is diphosphorylated by cellular enzymes and is then incorporated into growing DNA strands following competition with dCTP on the viral DNA polymerase (Cihlar and Chen, 1996; Xiong et al., 1997). We speculate that CDV resistance most likely develops by altering the binding/nucleotide processing abilities of CDV-

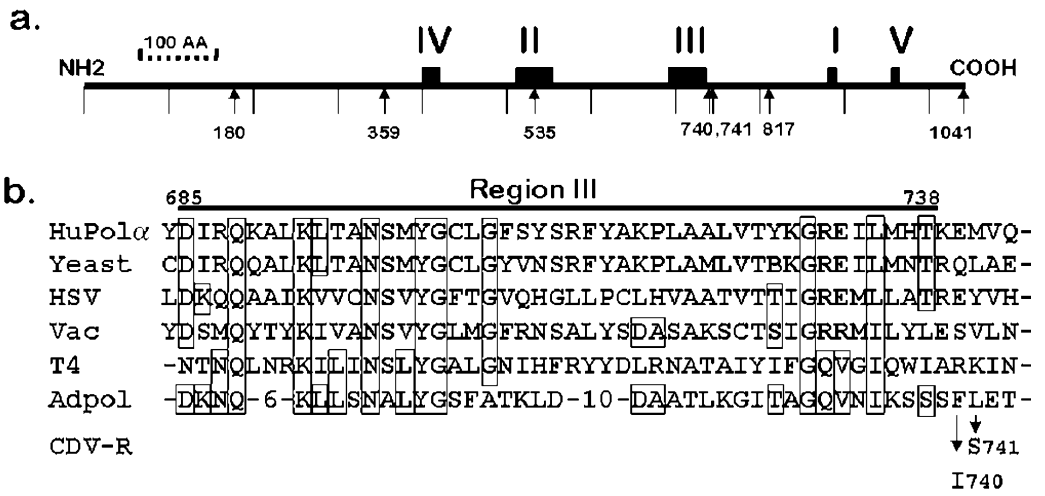

Fig. 5. (a) A diagrammatic representation of the adenovirus DNA polymerase, showing the relative positions of the five conserved elements within the DNA polymerase gene that contain amino acid sequences in most other DNA polymerases. The dashed line represents 100 amino acids, and lines below the polymerase divide the coding sequence into 100 residue sections. NH2 and COOH represent the amino and carboxyl ends of the protein, respectively. The relative positions of the mutations seen in this work are also indicated below the linear representation of the DNA polymerase. (b) An expansion of conserved region III, showing the amino acids of other polymerases aligned as detailed in Gordon et al. (1992). Amino acids in other polymerases which are identical to the adenovirus DNA polymerase at the same position are boxed. Amino acids are shown in standard single letter format. Numbers in the domains indicate the number of residues missing in gaps introduced into the amino acid sequence for alignment. The position of the changes identified in this work are shown beneath each of the conserved regions. The source of the DNA polymerases is shown to the left; vac, vaccinia; T4, bacteriophage T4; HSV, herpes simplex virus type 1; Hupolα, human DNA polymerase alpha; Adpol, adenovirus type 5 DNA polymerase; CDV-R, changes in CDV-resistant viruses.

PP on the DNA polymerase so that CDV-PP is less efficiently recognized over dCTP, but the resolution of this possibility will require biochemical purification and characterization of the parental and altered DNA polymerases. Therefore, while the direct association of the changes found in the DNA polymerases of R4, R5 and R6 with CDV resistance was not proven in these studies, we consider it highly unlikely that changes elsewhere in the adenovirus genome could be responsible. With this assumption, our data indicates that CDV resistance can arise by changes in at least two regions of the DNA polymerase, as characterized by the residue 741 mutation in R1 and the residue 359 change in R5. The contribution of the other changes in the DNA polymerases to CDV resistance will require their individual introduction into the DNA polymerase and the assessment of CDV sensitivity, which was considered beyond the scope of the current study. Clearly, changes not related to CDV resistance can arise during the extensive passage of the viruses to derive CDV resistance, as shown by the non-coding base change identified at base pair 5992 in CDV-resistant mutant R4.

The question remains as to the possible development of CDV resistance in vivo following future clinical use of CDV to treat adenovirus disease. Unfortunately, the development of CDV for topical treatment of adenovirus and HSV-1 ocular infections has been halted, due to toxicity and market considerations. Persistent epiphora associated with lacrimal canalicular blockade, a complication first noted in the rabbit model with adenovirus type 5 (Gordon et al., 1994a), was observed in a limited number of patients following off label usage of CDV (Romanowski et al., 2001b). However, the expected need for long term usage of systemic CDV to treat disseminated adenovirus infections in pediatric transplant and HIV infected patients (Legrand et al., 2001; Bordigoni et al., 2001; Hoffman et al., 2000) favors the emergence of clinical resistance. In herpesviruses, de novo CDV-resistant HSV and CMV have been derived in vitro, and clinically important

HSV and hCMV viruses that have developed resistance to ACV and GCV also show cross resistance to CDV, particularly when ACV/GCV resistance is a result of changes within the DNA polymerase (Andrei et al., 2000; Emery, 1998; Erice, 1999; Chou et al., 2000; Cihlar et al., 1998; Harada et al., 1997; Lurain et al., 1992). However, not all DNA polymerases defining GCV and ACV resistance are CDV-resistant, and CDV remains one option following development of GCV resistance (Jabs, 1997; Jabs et al., 1998a,b; Smith et al., 1998; Chou et al., 1997; Bowen et al., 1999).

While we only detailed the development of resistance for adenovirus type 5, serotypes 1 and 6, which also replicate in the rabbit ocular model, are also effectively limited by ocular treatment with CDV. Types 3, 7, 8, 19 and 37 are also effectively inhibited by CDV in vitro. It may be pertinent for future studies to focus on the development of resistance in additional adenovirus serotypes, particularly those most commonly associated with long term systemic disease.

Acknowledgements

We thank Eric Romanowski for critical reading of the manuscript. This work was supported by NIH Grants EY08227, NIH Core Grant for Vision Research EY08098, The Eye and Ear Foundation of Pittsburgh, and a Lou Wassermann Award to PRK from Research to Prevent Blindness Inc.

References

Afouna, M.I., Mehta, S.C., Ghanem, A.H., Higuchi, W.I., Kern, E.R., DeClercq, E., El-Shattawy, H.H., 1999. Influence of the treatment protocol upon the in vivo efficacy of cidofovir (HPMPC) and of acyclovir (ACV) formulations in topical treatment of cutaneous HSV-1 infection in hairless mice. J. Pharm. Sci. 88, 530–534.

Andrei, G., Snoeck, R., de Clercq, E., Esnouf, R., Fiten, P., Opdenakker, G., 2000. Resistance of herpes simplex virus type 1 against different phosphonylmethoxyalkyl derivatives of purines and pyrimidines due to specific mutations in the viral DNA polymerase gene. J. Gen. Virol. 81, 639–648.

Andrei, G., Snoeck, R., Piette, J., Delvenne, P., de Clercq, E., 1998. Antiproliferative effects of acyclic nucleoside phosphonates on human papillomavirus (HPV)-harboring cell lines compared with HPV-negative cell lines. Oncol. Res. 10, 523–531.

Baldwin, A., Kingman, H., Darville, M., Foot, A.B., Grier, D., Cornish, J.M., Goulden, N., Oakhill, A., Pamphilon, D.H., Steward, C.G., Marks, D.I., 2000. Outcome and clinical course of 100 patients with adenovirus infection following bone marrow transplantation. Bone Marrow Transplant. 26, 1333–1338.

Bordigoni, P., Carret, A.S., Venard, V., Witz, F., Le Faou, A., 2001. Treatment of adenovirus infections in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin. Infect. Dis. 32, 1290–1297.

Bowen, E.F., Cherrington, J.M., Lamy, P.D., Griffiths, P.D., Johnson, M.A., Emery, V.C., 1999. Quantitative changes in cytomegalovirus DNAemia and genetic analysis of the UL97 and UL54 genes in AIDS patients receiving cidofovir following ganciclovir therapy. J. Med. Virol. 58, 402–407.

Bronson, J.J., Ferrara, L.M., Hitchcock, M.J., Ho, H.T., Woods, K.L., Ghazzouli, I., Kern, E.R., Soike, K.F., Martin, J.C., 1990. (*S*)-1-(3-Hydroxy-2-(phosphonylmethoxy)propyl)cytosine (HPMPC): a potent antiherpesvirus agent. Adv. Exp. Med. Biol. 278, 277–283.

Chatterjee, S., Burns, P., Whitley, R.J., Kern, E.R., 1992. Effect of (*S*)-1-[(3-hydroxy-2-phosphonyl methoxy)propyl] cytosine on the replication and morphogenesis of herpes simplex virus type 1. Antiviral Res. 19, 181–192.

Chou, S., Marousek, G., Guentzel, S., Follansbee, S.E., Poscher, M.E., Lalezari, J.P., Miner, R.C., Drew, W.L., 1997. Evolution of mutations conferring multidrug resistance during prophylaxis and therapy for cytomegalovirus disease. J. Infect. Dis. 176, 786–789.

Chou, S., Miner, R.C., Drew, W.L., 2000. A deletion mutation in region V of the cytomegalovirus DNA polymerase sequence confers multidrug resistance. J. Infect. Dis. 182, 1765–1768.

Cihlar, T., Chen, M.S., 1996. Identification of enzymes catalyzing two-step phosphorylation of cidofovir and the effect of cytomegalovirus infection on their activities in host cells. Mol. Pharmacol. 50, 1502–1510.

Cihlar, T., Fuller, M.D., Mulato, A.S., Cherrington, J.M., 1998. A point mutation in the human cytomegalovirus DNA polymerase gene selected in vitro by cidofovir confers a slow replication phenotype in cell culture. Virology 248, 382–393.

de Clercq, E., 1997. Acyclic nucleoside phosphonates in the chemotherapy of DNA virus and retrovirus infections. Intervirology 40, 295–303.

de Clercq, E., Andrei, G., Balzarini, J., Hatse, S., Liekens, S., Naesens, L., Neyts, J., Snoeck, R., 1999. Antitumor potential of acyclic nucleoside phosphonates. Nucleosides Nucleotides 18, 759–771.

De Luca, A., Giancola, M.L., Ammassari, A., Grisetti, S., Cingolani, A., Larussa, D., Alba, L., Murri, R., Ippolito, G., Cauda, R., Monforte, A., Antinori, A., 2001. Potent anti-retroviral therapy with or without cidofovir for AIDS-associated progressive multifocal leukoencephalopathy: ex-

tended follow-up of an observational study. J. Neurovirol. 7, 364–368.
de Oliveira, C.B., Stevenson, D., LaBree, L., McDonnell, P.J., Trousdale, M.D., 1996. Evaluation of Cidofovir (HPMPC, GS-504) against adenovirus type 5 infection in vitro and in a New Zealand rabbit ocular model. Antiviral Res. 31, 165–172.
Earl, P.L., Jones, E.V., Moss, B., 1986. Homology between DNA polymerases of poxviruses, herpesviruses, and adenoviruses: nucleotide sequence of the vaccinia virus DNA polymerase gene. Proc. Natl. Acad. Sci. USA 83, 3659–3663.
Emery, V.C., 1998. Cytomegalovirus drug resistance. Antivir. Ther. 3, 239–242.
Emery, V.C., 2001. Progress in understanding cytomegalovirus drug resistance. J. Clin. Virol. 21, 223–228.
Erice, A., 1999. Resistance of human cytomegalovirus to antiviral drugs. Clin. Microbiol. Rev. 12, 286–297.
Gordon, Y.J., Aoki, K., Kinchington, P.R., 1996. In: Pepose, J.S., Holland, G.N., Wilhelmus, K.R. (Eds.), Ocular Infections and Immunity, 1st ed.. Mosby, St. Louis, pp. 877–894.
Gordon, Y.J., Araullo-Cruz, T.P., Johnson, Y.F., Romanowski, E.G., Kinchington, P.R., 1996. Isolation of human adenovirus type 5 variants resistant to the antiviral cidofovir. Invest. Ophthalmol. Vis. Sci. 37, 2774–2778.
Gordon, Y.J., Araullo-Cruz, T., Romanowski, E., Myers, B., Santora, D., Lin, M., Kowalski, R., 1991. Replication of ocular isolates of human adenovirus is serotype-dependent in rabbit corneal organ culture. Curr. Eye Res. 10, 267–271.
Gordon, Y.J., Romanowski, E.G., Araullo-Cruz, T., 1994. Topical HPMPC inhibits adenovirus type 5 in the New Zealand rabbit ocular replication model. Invest. Ophthalmol. Vis. Sci. 35, 4135–4143.
Gordon, Y.J., Romanowski, E.G., Araullo-Cruz, T., 1994. HPMPC, a broad-spectrum topical antiviral agent, inhibits herpes simplex virus type 1 replication and promotes healing of dendritic keratitis in the New Zealand rabbit ocular model. Cornea 13, 516–520.
Gordon, Y.J., Romanowski, E., Araullo-Cruz, T., de Clercq, E., 1992. Pretreatment with topical 0.1% (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine inhibits adenovirus type 5 replication in the New Zealand rabbit ocular model. Cornea 11, 529–533.
Gordon, Y.J., Romanowski, E., Araullo-Cruz, T., Seaberg, L., Erzurum, S., Tolman, R., de Clercq, E., 1991. Inhibitory effect of (*S*)-HPMPC, (*S*)-HPMPA, and 2′-nor-cyclic GMP on clinical ocular adenoviral isolates is serotype-dependent in vitro. Antiviral Res. 16, 11–16.
Graham, F.L., Smiley, J.R., Russell, W.C., Naish, R., 1977. Characteristics of a human cell line transformed by DNa from human adenovirus type 5. J. Gen. Virol. 36, 59–72.
Harada, K., Eizuru, Y., Isashiki, Y., Ihara, S., Minamishima, Y., 1997. Genetic analysis of a clinical isolate of human cytomegalovirus exhibiting resistance against both ganciclovir and cidofovir. Arch. Virol. 142, 215–225.
Hoffman, J.A., Shah, A.J., Ross, L.A., Kapoor, N., 2000. Adenoviral infections and a prospective trial of cidofovir in pediatric hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant. 7, 388–394.
Howard, D.S., Phillips, I.I.G., Reece, D.E., Munn, R.K., Henslee-Downey, J., Pittard, M., Barker, M., Pomeroy, C., 1999. Adenovirus infections in hematopoietic stem cell transplant recipients. Clin. Infect. Dis. 29, 1494–1501.
Jabs, D.A., 1997. Cidofovir. Arch. Ophthalmol. 115, 785–786.
CMV Retinitis and Viral Resistance Study Group, Jabs, D.A., Enger, C., Dunn, J.P., Forman, M., 1998. Cytomegalovirus retinitis and viral resistance: ganciclovir resistance. J. Infect. Dis. 177, 770–773.
The Cytomegalovirus Retinitis and Viral Resistance Study Group, Jabs, D.A., Enger, C., Forman, M., Dunn, J.P., 1998. Incidence of foscarnet resistance and cidofovir resistance in patients treated for cytomegalovirus retinitis. Antimicrob. Agents Chemother. 42, 2240–2244.
Kendle, J.B., Fan-Havard, P., 1998. Cidofovir in the treatment of cytomegaloviral disease. Ann. Pharmacother. 32, 1181–1192.
Kiehl, M.G., Basara, N., 2001. Cidofovir for cytomegalovirus-preemptive therapy in stem cell transplant recipients. Blood 98, 1626.
Lea, A.P., Bryson, H.M., 1996. Cidofovir. Drugs 52, 225–230.
Legrand, F., Berrebi, D., Houhou, N., Freymuth, F., Faye, A., Duval, M., Mougenot, J.F., Peuchmaur, M., Vilmer, E., 2001. Early diagnosis of adenovirus infection and treatment with cidofovir after bone marrow transplantation in children. Bone Marrow Transplant. 27, 621–626.
Lurain, N.S., Thompson, K.D., Holmes, E.W., Read, G.S., 1992. Point mutations in the DNA polymerase gene of human cytomegalovirus that result in resistance to antiviral agents. J. Virol. 66, 7146–7152.
Martinez, C.M., Luks-Golger, D.B., 1997. Cidofovir use in acyclovir-resistant herpes infection. Ann. Pharmacother. 31, 1519–1521.
Platzbecker, U., Bandt, D., Thiede, C., Helwig, A., Freiberg-Richter, J., Schuler, U., Plettig, R., Geissler, G., Rethwilm, A., Ehninger, G., Bornhauser, M., 2001. Successful preemptive cidofovir treatment for CMV antigenemia after dose-reduced conditioning and allogeneic blood stem cell transplantation. Transplantation 71, 880–885.
Plosker, G.L., Noble, S., 1999. Cidofovir: a review of its use in cytomegalovirus retinitis in patients with AIDS. Drugs 58, 325–345.
Romanowski, E.G., Araullo-Cruz, T., Gordon, Y.J., 1997. Topical corticosteroids reverse the antiviral effect of topical cidofovir in the Ad5-inoculated New Zealand rabbit ocular model. Invest. Ophthalmol. Vis. Sci. 38, 253–257.
Romanowski, E.G., Bartels, S.P., Gordon, Y.J., 1999. Comparative antiviral efficacies of cidofovir, trifluridine, and acyclovir in the HSV-1 rabbit keratitis model. Invest. Ophthalmol. Vis. Sci. 40, 378–384.
Romanowski, E.G., Gordon, Y.J., 2000. Efficacy of topical cidofovir on multiple adenoviral serotypes in the New Zealand rabbit ocular model. Invest. Ophthalmol. Vis. Sci. 41, 460–463.

Romanowski, E.G., Gordon, Y.J., Araullo-Cruz, T., Yates, K., Kinchington, P.R., 2001. The antiviral resistance and pathogenicity of cidofovir-resistant adenovirus variants in the new Zealand hite rabbit ocular model. Invest. Ophthalmol. Vis. Sci. 42, 1812–1815.

Romanowski, E.G., Yates, K.A., Gordon, Y.J., 2001. Antiviral Prophylaxis with twice daily topical cidofovir protects against challenge in the adenovirus type 5/new zealand rabbit ocular model. Antiviral Res. 52, 275–280.

Roovers, D.J., Young, C.S., Vos, H.L., Sussenbach, J.S., 1990. Physical mapping of two temperature-sensitive adenovirus mutants affected in the DNA polymerase and DNA binding protein. Virus Genes 4, 53–61.

Safrin, S., Cherrington, J., Jaffe, H.S., 1999. Cidofovir. Review of current and potential clinical uses. Adv. Exp. Med. Biol. 458, 111–120.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning, 2 ed.. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 9.31–9.57.

Smith, I.L., Taskintuna, I., Rahhal, F.M., Powell, H.C., Ai, E., Mueller, A.J., Spector, S.A., Freeman, W.R., 1998. Clinical failure of CMV retinitis with intravitreal cidofovir is associated with antiviral resistance. Arch. Ophthalmol. 116, 178–185.

Snoeck, R., Andrei, G., de Clercq, E., 1996. Patterns of resistance and sensitivity to antiviral compounds of drug-resistant strains of human cytomegalovirus selected in vitro. Eur. J. Clin. Microb. Infect. Dis. 15, 574–579.

Snoeck, R., Bossens, M., Parent, D., Delaere, B., Degreef, H., Van Ranst, M., Noel, J.C., Wulfsohn, M.S., Rooney, J.F., Jaffe, H.S., de Clercq, E., 2001. Phase II double-blind, placebo-controlled study of the safety and efficacy of cidofovir topical gel for the treatment of patients with human papillomavirus infection. Clin. Infect. Dis. 33, 597–602.

Volkert, F.C., Munz, P.L., Young, C.S., 1989. A genetic investigation of the mechanism of adenovirus marker rescue. Virology 173, 77–88.

Wang, T.S., Wong, S.W., Korn, D., 1989. Human DNA polymerase alpha: predicted functional domains and relationships with viral DNA polymerases. FASEB J. 3, 14–21.

Xiong, X., Smith, J.L., Chen, M.S., 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob. Agents Chemother. 41, 594–599.