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In vitro antiviral efficacy of the ganciclovir complexed with β-cyclodextrin on human cytomegalovirus clinical strains

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Abstract

The toxicity of the compounds currently used in the treatment of human cytomegalovirus (HCMV) infections in immunocompromised hosts may force the treatment to be discontinued. The aim of this study was to improve the antiviral activity of ganciclovir (GCV), one the most widely used drug, by complexing it with β-cyclodextrin. Cyclodextrins (cds) have the property to form inclusion complexes with a great number of molecules and to enhance bioavailability and biological properties of these molecules. In this study, we investigated the in vitro antiviral activity of complexed GCV against several strains of HCMV: AD169, a reference strain, RCL-1, a laboratory mutant resistant to GCV, and four clinical isolates. The complexed GCV was more effective than free GCV against all HCMV strains tested. Cds as carriers for antiviral drugs would represent a useful adjunct to classical treatment procedures. They may make it possible to administer lower doses, thus reducing the toxic side effects of the drugs.

Keywords: Human cytomegalovirus; Drug carrier; Cyclodextrins; Ganciclovir-resistance

1. Introduction

Human cytomegalovirus (HCMV) infections cause serious and often life-threatening diseases in immunocompromised hosts, particularly in organ (i.e. bone marrow) recipients and AIDS patients (Erice, 1999). Despite a reduction in the incidence of AIDS-related opportunistic infections in patients under highly active anti-retroviral treatment, HCMV retinitis development or progression remains a threat (MacDonald et al., 2000). At present, few drugs are available for the treatment of HCMV infections, namely, ganciclovir (GCV), foscarnet and cidofovir. More recently, an antisense oligonucleotide, fomivirsen has been introduced for the treatment of HCMV retinitis (Perry and Balfour, 1999). The currently used drugs suffer from poor oral bioavailability (De Jong et al., 1998; Jacobson et al., 1999), the emergence of drug-resistant HCMV strains (Erice,

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1999) and dose-limiting toxicity (Perry and Davis, 1997; Lalezari et al., 1997). GCV is most widely used in clinical practice, both for induction and maintenance therapy and for prophylaxis (Kariya et al., 2000). As a nucleoside analogue, its ultimate target is the DNA polymerase. However, GCV requires intracellular phosphorylation for activity. The first phosphorylation is achieved by a virus-encoded phosphotransferase. This means that GCV resistance can be generated by mutations in two viral genes: the DNA polymerase gene (UL54) and the phosphotransferase gene (UL97). Mutations related to GCV resistance have been well established (Harada et al., 1997; Baldanti et al., 1998; Erice, 1999).

To enhance drug bioavailability, several strategies may be followed, such as the use of prodrugs or carriers. GCV derivatives (i.e. valganciclovir or an elaidic acid ester of the GCV) have proven more efficacious than GCV in vitro and in vivo (De Jong et al., 1998; Andrei et al., 2000). The use of carriers of antiviral molecules represent another alternative to improve the bioavailability and the activity of the drugs concerned. Liposomes, nanoparticles or polymer microspheres have already been studied as anti-CMV agent carriers (Merodio et al., 2000), heightening the in vitro or in vivo efficiency of GCV or foscarnet (Bakker-Woudenberg et al., 1990; Bergers et al., 1997; Veloso et al., 1997; Cheng et al., 2000). However, these carriers have proven difficult to use (Barrat, 2000); thus we selected cyclodextrins (cds) in order to carry GCV. They are cyclic oligosaccharides with a hydrophobic central cavity and a hydrophilic surface. The most common native cd is the β-cd composed of 7 α-1,4-linked glucopyranose units. Cds are capable of forming inclusion complexes with a great variety of molecules (Loftsson and Brewster, 1996). Complexation induces greater solubility, stability and bioavailability (Duchêne et al., 1987) together with better cellular absorption of the target molecule (Kimura et al., 1997). Cds and their derivatives are currently tested to improve the pharmacological properties of some drugs (Abdou et al., 1997; Montassier et al., 1997; Kaukonen et al., 1998; Mura et al., 1998; Al-Omar et al., 1999; Loftsson and Jarvinen, 1999; Matsuda and Arima, 1999). We have used cds as biovectors for GCV in previous studies, and we have shown that the complexation of GCV with β-cd improves its in vitro activity against HCMV laboratory strains AD169 and the RCL-1 (Nicolazzi et al., 2001).

The aim of this study was to evaluate the in vitro antiviral efficacy of GCV carried by the β-cd against clinical HCMV isolates. Clinical HCMV isolates were phenotypically and genotypically characterized for their susceptibility towards GCV. The GCV:β-cd complex was evaluated in terms of inhibition of CMV late antigen expression.

2. Materials and methods

2.1. Chemicals

The native β-cd was purchased from Roquettes Frères (Dijon, France). The GCV (Cymevan®) was purchased from Roche (Neuilly-sur-Seine, France).

The reagents were dissolved in distilled water and sterilized by filtration through a 0.22 μM membrane (Millipore, Molsheim, France).

2.2. Preparation of the complexes

The [GCV:β-cd] complexes were obtained in the conditions described by Higuchi and Connors (1965). GCV and β-cd molecules were incubated in distilled water at a molar ratio of 1:10, taking into account the β-cd solubility in a final volume of 10 ml. These conditions were chosen in a previous study (Nicolazzi et al., 2001). The solution was equilibrated overnight at 20 °C by stirring at 200 rpm with a Rotatest 74401 apparatus (Bioblock-Scientific, Illkirch, France). The formation of an inclusion complex was confirmed by Nuclear Magnetic Resonance measurements (Nicolazzi et al., 2001). The solution was then mixed v/v with Eagle's minimum essential medium (EMEM) (Polylabo, Strasbourg, France).

2.3. Cells and virus

Human embryonic lung fibroblasts MRC-5 (Mérieux) were used for virus isolation. Cells were grown in Eagle's minimum essential medium (EMEM) (Polylabo) supplemented with 10% fetal calf serum (FCS) (Dutscher, Brumath, France), 0.1% Amiklin (Bristol-Myers Squibb) and 0.1% Clamoxyl (Smith-Kline Beecham Laboratories). The maintenance medium was the same, but contained 2.5% FCS.

The laboratory strains were AD169 (ATCC reference: VR-538) and a GCV-resistant laboratory mutant RCL-1 derived from AD169 (Alain et al., 1993). Four clinical isolates of HCMV from four unrelated patients were obtained from the Virology Laboratory of CHU of Nancy (Table 1).

Stocks of the HCMV strains were prepared on cultured MRC-5 cells, as previously described (Nicolazzi et al., 2001).

2.4. Susceptibility of the HCMV strains to antiviral agents

Susceptibility to antiviral drugs was determined using ELISA. Monolayer MRC-5 cells in 96-well plates (Costar, Dutscher, Brumath, France) at 4×10^4 cells/well were inoculated with a HCMV suspension at a m.o.i. of 0.1 PFU/cell. After incubation (60 min, 37 °C), the inoculum was removed and the cells were incubated at 37 °C with free or complexed GCV at various concentrations. After 6 days, late HCMV proteins were detected by an ELISA, using McAb SL-20 (Argene Biosoft), as previously described (Nicolazzi et al., 2001). The drug concentration leading to a 50% decrease in viral production (IC_{50}) was estimated. Results are expressed as mean of three independent experiments performed in triplicates.

Table 1
HCMV isolates

Sample	Specimens	Context
539	Urine	Renal transplantation
731	Urine	Renal transplantation
1558	Urine	Congenital
2288	Tracheal sample	Bone marrow transplantation

2.5. Preparation of DNA samples

The infected cell layer from a 25 cm^2 flask was disrupted by freezing followed by thawing. Pelleted cells and virions were resuspended and incubated for 1 h at 50 °C in TES buffer with 200 μg/ml proteinase K (Sigma, St. Louis) and 1% SDS (Sigma). DNA was extracted using a standard phenol/chloroform procedure (Perbal, 1991).

2.6. DNA amplification

Amplification of CMV genome segments by polymerase chain reaction (PCR) were performed according to Erice et al. (1997) for polymerase UL54 gene and according to Chou et al. (1995) for phosphotransferase UL97 gene. The mixture contained 2 mM MgCl, 50 mM KCl, 0.2 mM dNTP, 0.2 μM of each primer and 1 unit of *Thermus aquaticus* (Taq) DNA polymerase (Life Technology, Cergy Pontoise, France) in Tris buffer (pH 8). The amplification reaction consisted of 35 cycles: denaturation for 1 min at 95 °C, annealing for 2 min at 55 °C and extension for 1 min at 72 °C.

2.7. Sequencing of PCR products

The UL97 gene of HCMV strains was amplified using oligonucleotide sets CPT1088, CPT1713, CPT1285 and CPT1878 (Chou et al., 1995), which permit the sequencing from codon 450 to 623. The HCMV DNA polymerase gene was amplified by PCR using oligonucleotide sets Z 4957, Z 4958, Pol 2401 and 3727 (Erice et al., 1997; Harada et al., 1997) which permit the sequencing from codon 370 to 1000. The amplified fragments were purified using chelex minicolumns (Wizard PCR purification system, Promega, France). After elution with 50 μl of H_2O, the DNA yield was determined by spectrophotometry. Ninety nanograms of purified DNA were used in the sequencing reaction (Prism 401388, Applied Biosystems, Foster City, CA), which was performed according to the Manufacturer's recommendations. The products were analyzed with a DNA sequencing system (373A model, Applied

Table 2
Genotypic characterization of human cytomegalovirus isolates

Sample	Ganciclovir IC_{50} (μM)	UL97 sequence analysis	UL54 sequence analysis
Laboratory strains			
AD 169	2.70 ± 0.55	Wild-type	Wild-type
RCL-1	14.50 ± 2.50	G594A	S545L
Clinical isolates			
1558	3.25 ± 0.62	Wild-type	Wild-type
539	6.45 ± 0.82	Wild-type	Wild-type
731	6.70 ± 0.55	Wild-type	S655L, N685S
2288	18.25 ± 2.25	Wild-type	S655L, N685S

Wild-type: AD169 sequence.

Biosystems). The sequences were determined twice for each sample, using sense or antisense primers. They were then aligned and compared with the AD169 reference sequence using the Navigator Program (Applied Biosystems).

3. Results

3.1. Susceptibility of clinical isolates to ganciclovir

The IC_{50} determined by an ELISA detecting the production of a late antigen for the reference strain AD169 was 2.70 μM. The laboratory mutant RCL-1 exhibited an IC_{50} of 14.50 μM. As shown in Table 2, three clinical isolates were GCV-susceptible (isolates 1558, 539, 731), with IC_{50} values ranging from 3.25 to 6.70 μM, one isolate (2288) was resistant with an $IC_{50} = 18.25$ μM.

3.2. Sequencing results

3.2.1. Analysis of CMV UL97 region

Analysis of CMV UL97 by sequencing revealed the presence of UL97 gene mutation in only one strain (Table 2), the laboratory mutant. Strain RCL-1 contained a single mutation, an Ala–Gly mutation at codon 594. The UL97 sequences of the four clinical isolates were identical and contained the same sequence as compared to the reference strain AD169.

3.2.2. Analysis of CMV UL54 region

Analysis of CMV UL54 by sequencing revealed polymerase gene mutations in 2 clinical isolates and in the laboratory mutant (Table 2). A Ser–Leu mutation at codon 655 and an Asp–Ser mutation at codon 685 were detected in two clinical isolates (731, 2288) and a Leu–Ser mutation at codon 545 was detected in strain RCL-1.

3.3. Antiviral activity of the GCV complexed with β-cyclodextrin

The antiviral activity of GCV complexed with β-cd was compared with that of GCV (Table 3). Complexation of GCV with the β-cd increased the antiviral efficiency of GCV. GCV complex formation with β-cd reduced the IC_{50} value by a factor of 10 for strains AD169, RCL-1 and 1558, but only 2.6-fold for strain 539 and no change at all for strain 731.

4. Discussion

In the present study, we evaluated the in vitro antiviral efficacy of GCV complexed with the β-cd against clinical HCMV isolates. First, we isolated HCMV strains from patients and we determined their in vitro susceptibilities to GCV. It is generally accepted that a HCMV strain is resistant to GCV if the IC_{50} is greater than 8 μM and highly resistant if greater than 30 μM (Smith et al., 1997; Erice, 1999).

We characterized, in parallel, these isolates genotypically by sequencing the UL97 and UL54 genes, of which certain mutations are associated by the GCV resistance. The mutations (N685L and S655L) found in the genome of the two clinical isolates 731 and 2288 were only intrinsic variations unrelated to GCV resistance. They were found in both GCV-susceptible and GCV-resistant strains (Smith et al., 1997; Chou et al., 1999). We observed a discrepancy between susceptibility and sequencing results: for one HCMV isolate (strain 2288), the high value of IC_{50} obtained was not associated with GCV resistance-associated mutations in the UL97 and UL54 genes. This isolate could be made up of a predominant population of GCV-susceptible virus associated with a minor population of resistant virus. This minor resistant virus subpopulation may have remained undetectable by sequencing.

However, the laboratory mutant RCL-1 presented mutations in the UL97 and UL54 genes, that are known to be associated with GCV resistance (Mazeron, 1997; Cihlar et al., 1998; Erice, 1999).

The results of the antiviral tests demonstrated that, whatever the status of the HCMV isolate's susceptibility to GCV, complexation of GCV with β-cd yielded greater efficiency. IC_{50} values obtained for the GCV β-cd complexes were, indeed, lower than the IC_{50} values for free GCV for all HCMV strains except for one isolate (strain 731), for which the IC_{50} of free GCV and complexed GCV were equivalent.

In our previous studies, we have studied the influence of the cd complexation of GCV on its biological properties. The increased antiviral activity seemed to be due to an improved GCV bioavailability thanks to enhanced cellular uptake of GCV when complexed with β-cd (Nicolazzi et al., 2001). The cellular uptake of GCV could be ascribed to increased stability and solubility, and/or slower release from the cells (Uekama et al., 1994), as well as enhanced transport through the MRC-5 system (Irie and Uekama, 1999).

The increase of the cellular uptake of the drug by its complexation with cds could explain the improvement of the antiviral efficacy of GCV, particularly against those strains against which it showed high IC_{50} values (strains RCL-1 and 2288). Through improving the cellular uptake of GCV a lower IC_{50} value against the drug-resistant strain RCL-1 could be achieved. In contrast, complexation of GCV with β-cd did not increase the efficacy of GCV against isolate 731, which already had marked susceptibility to GCV.

The development of cds as biovectors to ameliorate the bioavailability of GCV should make it possible to lower the dosage of GCV and thus reduce the drug's toxicity. This avoids discontinuing treatment, which could otherwise favor development of drug resistance.

The physicochemical characteristics of the [GCV:β-cd] complex were determined in a previous study (Nicolazzi et al., 2001). We are currently studying the efficacy of cds as biovectors of antiviral agents (GCV, antisense oligonucleotides…) via the oral route on a murine CMV model.

Table 3
Susceptibility testing results of HCMV isolates

Sample	IC_{50} of free GCV (μM)	IC_{50} of [GCV: β-cd] (μM)	Improvement in IC_{50}
AD169	2.70 ± 0.55	0.20 ± 0.05	13.5
RCL-1	14.50 ± 2.50	1.60 ± 0.12	9.1
1558	3.25 ± 0.62	0.20 ± 0.06	16.2
539	6.45 ± 0.82	2.50 ± 0.51	2.6
731	6.70 ± 0.55	5.80 ± 0.51	1.1
2288	18.25 ± 2.25	0.75 ± 0.80	24.3

Elisa method: MRC5 cells in 96-well plates (4×10^4 cells/well) were inoculated with HCMV suspension (m.o.i. = 0.1 PFU/cell) and treated by GCV or [GCV:β-cd]. The antiviral activity was determined, after an incubation period of 6 days.

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