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Effect of the Complexation with Cyclodextrins on the In Vitro Antiviral Activity of Ganciclovir Against Human Cytomegalovirus

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Abstract—The toxicity of the molecules currently used in the treatment of human cytomegalovirus (HCMV) in immunocompromised hosts often causes interruption of the therapy. Cyclodextrins (Cds), oligosaccharides possessing a hydrophobic cavity, have the property of forming inclusion complexes with a great number of molecules, improving their bioavailability and their biological properties. In this study, we have tested the ability of three native Cds to improve the antiviral effect of ganciclovir (GCV) on two HCMV strains: AD169, a reference susceptible strain, and RCl1, a GCV resistant strain. The efficacy of the GCV, expressed in IC_{50} values, showed no improvement in the presence of α-Cd, while the use of β- and γ-Cd improved by 6- and 4-fold, respectively, its antiviral activity tested on AD169 strain. The influence of β- or γ-Cd on GCV efficiency evaluated on RCl1 strain showed a decrease of the IC_{50}. Parallel NMR studies were undertaken in order to characterize formation of [GCV:Cd] complexes. The results showed that complexation between α- or γ-Cd and GCV did not occur. In contrast, spectra proved that β-Cd formed an inclusion complex with GCV. This complex was characterized in UV–Visible spectrophotometry and the influence of the β-Cd on the GCV penetration in cells was measured. The use of Cds as carriers of antiviral drugs would be a good alternative to traditional treatment, because it may allow the administration of lower doses and so continuous treatment by reducing the toxic effects of drugs.

Introduction

Human cytomegalovirus (HCMV) infections cause serious and often life-threatening diseases in immunocompromised hosts, particularly in organ and bone marrow recipients and in AIDS patients.[1] At present, two drugs, ganciclovir (GCV) and foscarnet, are available for the common treatment of HCMV infections. More recently, cidofovir,[2] a nucleotide analogue, and formivirsen,[3] an antisense drug, have been introduced for HCMV retinitis diseases, which resist other therapies. Despite a high initial response, many patients must interrupt the treatment because of the severe toxicity of the drugs or a progression of the disease.[4] The limited efficacy of antiviral treatments is due to the poor cellular penetration of the drug and to the fact that viral replication is intimately associated with the host cell biosynthesis.[5] In addition to their toxicity, the prolonged and alternated maintenance therapy with these drugs, which is often required for immunocompromised patients, favours the emergence of resistant HCMV strains.[6,7]

The carrying of antiviral molecules by cyclodextrins can avoid these problems by improving their bioavailability and their activity. Cyclodextrins (Cds) are cyclic oligosaccharides with a hydrophobic central cavity and a hydrophilic surface. The most common native Cds are α-, β- or γ-Cd constituted by respectively 6, 7, or 8 α-1,4-linked glucopyranose units (Fig. 1). Cds are able to form inclusion complexes with a great variety of molecules.[8,9] By complexation, they can increase the solubility, the stability, the bioavailability[10] and the cell absorption of the guest molecule.[11] In order to improve their pharmacological and biological properties, their molecular structures can be modified,[12] thus hydroxypropyl or galactosyl derivatives have the advantage of enhancing solubility and reducing haemolytic activity and nephrotoxicity.[13,14] Cds and their derivatives are currently being tested to improve drug pharmacological properties.[15–19]

The aim of this study was to test the capacity of α-, β- or γ-Cd to improve the antiviral efficacy of GCV against a susceptible reference HCMV strain, AD169, and a resistant strain, RCl1. Physico-chemical studies were undertaken in NMR and in UV–Visible spectrophotometry to characterize the complex formation between GCV and native Cds. The in vitro activity of the GCV in the absence and presence of native Cds was evaluated in

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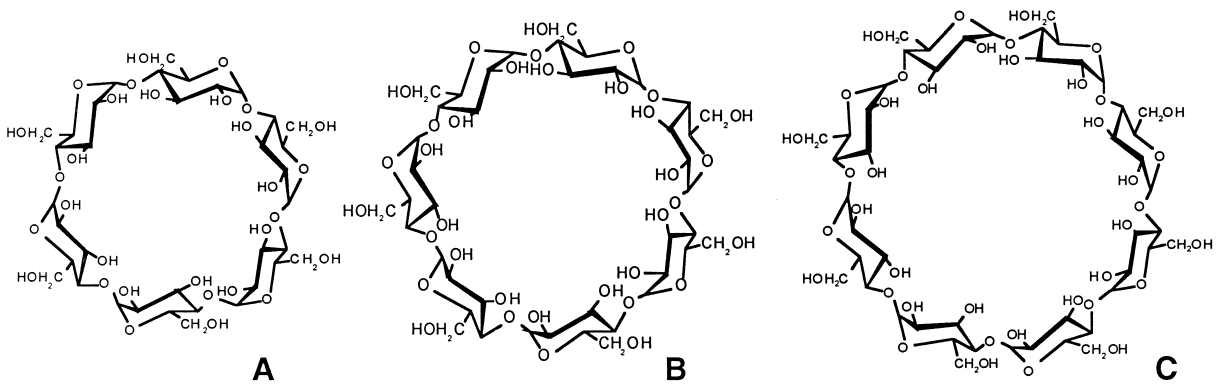

Figure 1. Chemical structure of cyclodextrins. In A α-Cd, in B β-Cd and in C γ-Cd.

terms of HCMV late antigen expression and HCMV replication. Modifications of GCV uptake in cells induced by the presence of Cds were measured.

Results

Characterization of the [GCV:Cds] complexes

Measurement of NMR spectra. In order to observe the formation of a complex between the GCV and the Cds, an NMR study was performed. The inclusion of a molecule in aqueous Cd solutions can be evidenced by modifications of the NMR spectra of guest and host molecules. The chemical structures of Cd and GCV molecules are presented in Figures 1 and 2. The ^{1}H NMR spectra of the three native Cds dissolved in D_2O in presence or absence of GCV are shown in Figure 3(A)–(C).

The α-Cd and γ-Cd spectra (Figs 3(A) and 3(C)) showed no spectral variation, indicating that α-Cd and γ-Cd did not form an inclusion complex with GCV.

The analysis of β-Cd spectrum (Fig. 3(B)) exhibited shifts especially for H3 and H5, which are protons located inside the cavity. These shifts resulted from interactions and inclusion of the GCV inside the cavity of the Cd.

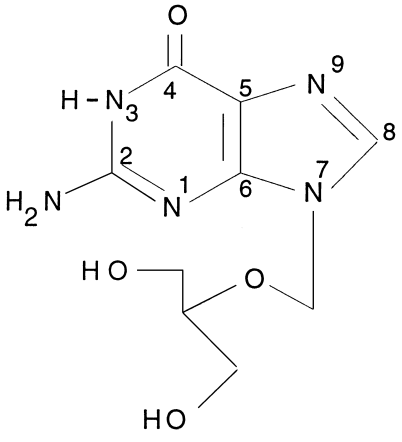

Figure 2. Chemical structure of ganciclovir.

The ^{1}H NMR study was inadequate to allow the determination of the structure of the inclusion complex and ^{13}C NMR study was then undertaken. This study (Table 1) showed a displacement of carbons of the GCV aromatic ring, in particular C-2, 4, 5, and 6 atoms. Shifts of other carbons exhibited no variation.

The results obtained with ^{1}H and ^{13}C NMR prove the existence of weak interactions between the GCV and the β-Cd and the formation of an inclusion complex.

Determination of stoichiometry of [GCV:β-Cd] complex. To determine the stoichiometry of the [GCV:β-Cd] complex, the continuous variation method was employed.[20] Figure 4 presents the Job plot of the Δabs × (GCV) versus the mole ratio of GCV obtained. The Job plot showed a maximum at r = 0.5, indicating 1:1 stoichiometry of complex.[20]

Stability constant. Absorption intensity at 253 nm of GCV at constant concentration was measured as a function of added β-Cd at room temperature. Absorption intensity of GCV increased with the increase of the β-Cd concentration before reaching a plateau. To estimate the simple (1:1) equilibrium constant, the y-reciprocal plot or Scott Plot is commonly used.[20–22] The plots of the Scott equation were linear (correlation coefficient (r) = 0.996) (Fig. 5), supporting the 1:1 complex formation. The stability constant is given by the Scott equation:[22]

$$b \times [\beta - Cd]/\Delta abs = [\beta - Cd]/[GCV] \times \Delta\varepsilon + 1/[GCV] \times K_{11} \times \Delta\varepsilon$$

where b is the path length, Δabs the absorbency change, [GCV] the total substrate concentration, K_{11} the stability constant, Δε the molar absorptivity change and [β-Cd] the ligand concentration.

The stability constant was given by K_{11} = (slope)/(y-intercept). The K_{11} value calculated was 4976 M^{-1}.

Cytotoxicity effects of GCV, Cds or [GCV:β-Cd] complex. The effect of GCV, Cds or complexes on the cellular viability was evaluated by measuring the enzymatic activity relative to unexposed control cells. Non-infected MRC5 cells were exposed to GCV, Cds, or complexes for 7 days in the same culture conditions as

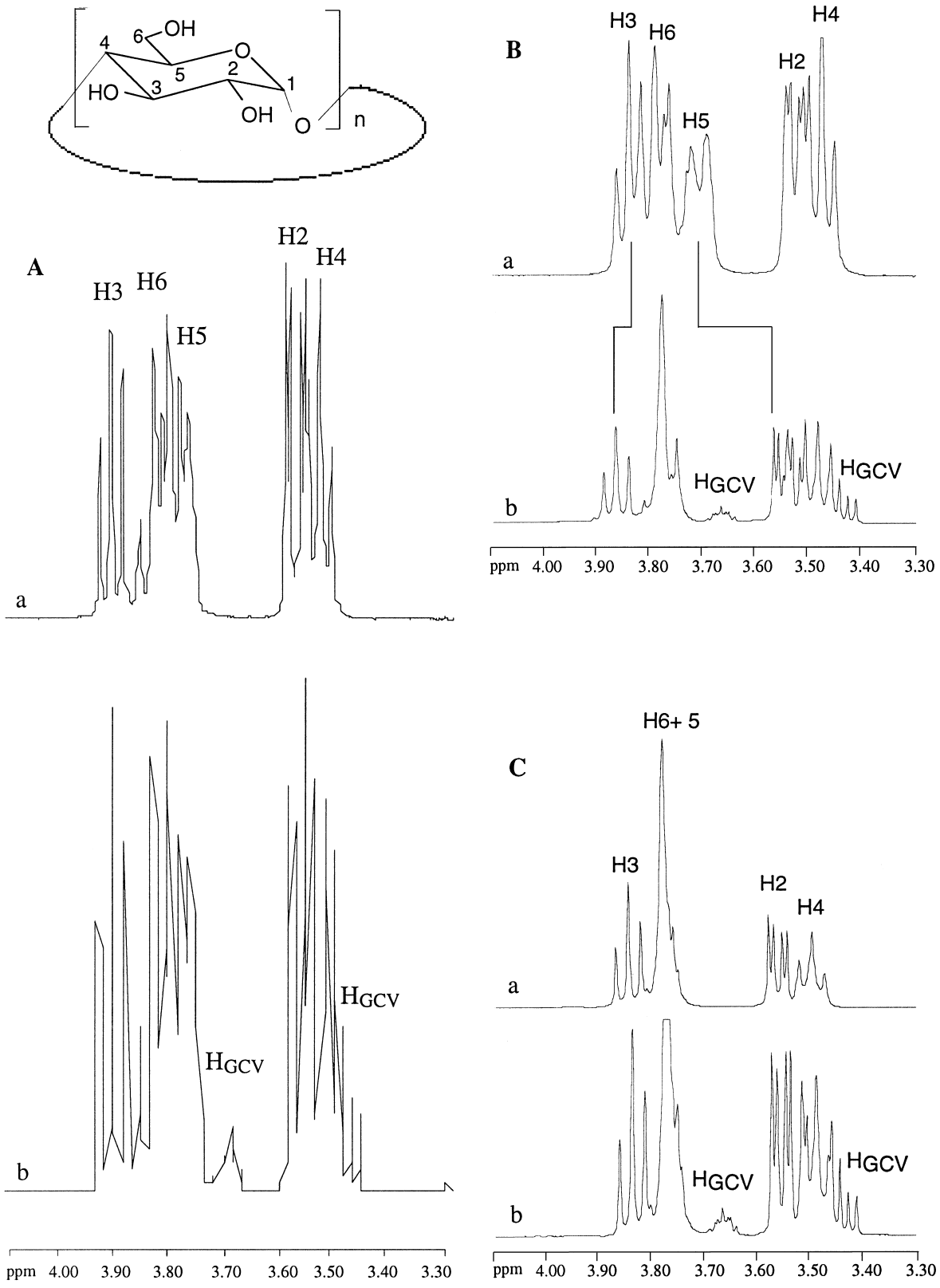

Figure 3. Partial ^{1}H NMR spectra of Cds and [GCV:Cd] complexes in D_2O obtained at 400 MHz and 25 °C. In A α-Cd, in absence (a) and in presence (b) of ganciclovir; in B β-Cd, in absence (a) and in presence (b) of ganciclovir and in C γ-Cd in absence (a) and in presence (b) of ganciclovir.

described in the Experimental. The results are presented in Table 2. Concentrations of GCV up to 0.14 mM showed no cytotoxic effect. The effects of α-, β- and γ-Cds were evaluated up to 2.56, 13.00, and 20.00 mM, respectively. The LC_{50} values indicated that native Cds had no inhibitory effect on the cell viability at the tested concentrations. [GCV:β-Cd] complex tested until 0.14 mM showed no cytotoxic effect.

Table 1. Change of ^{13}C NMR chemical shifts of ganciclovir in the presence of β-Cd in D_2O obtained at 400 MHz and 25 °C

Carbon atoms at GCV	δ (ppm)
C(2)	5
C(4)	17.9
C(5,6)	21.4
C(8)	−0.1

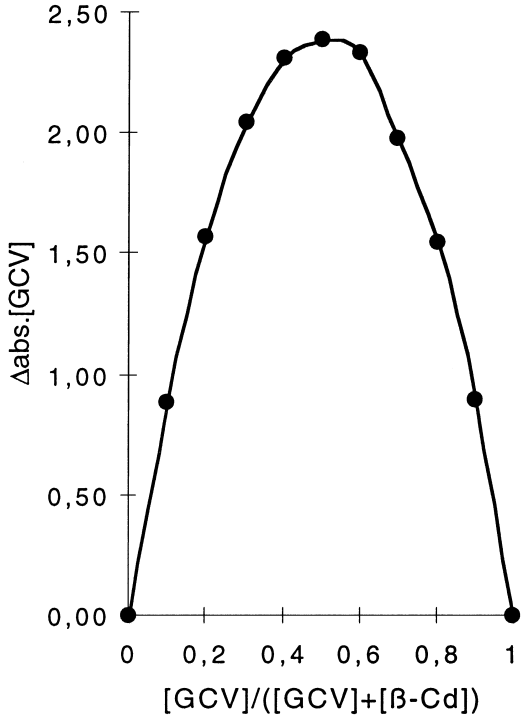

Figure 4. Continuous variation plot (Job's plot) for the intermolecular GCV/β-CD system.

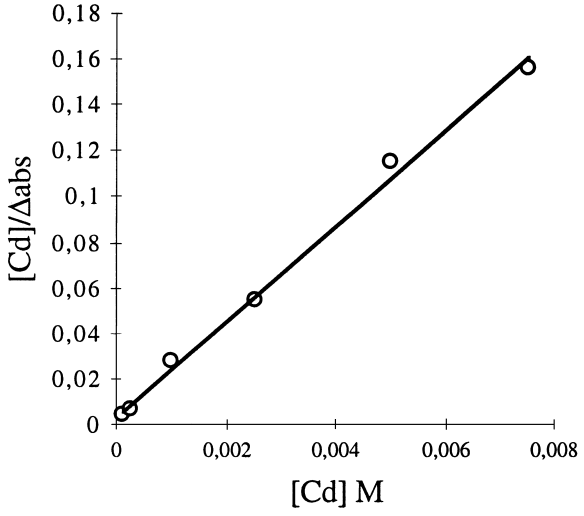

Figure 5. Representative y-reciprocal plot of the UV–visible absorption data for GCV. Absorption intentisity of GCV was measured at 253 nm at constant concentration (50 μM) in absence or in presence of increasing concentrations of β-Cd.

Table 2. Influence of GCV and Cds on the cell viability. Cells grown to confluence in 24-well plates (2.5×10^5 cells/well) were tested for 7 days with the GCV and different Cds at various concentrations. Cell viability was evaluated by their mitochondrial dehydrogenase activity (MTT assay)

Tested molecules	Lethal concentration 50 (LC_{50}) (mM)
GCV	> 0.140
α-CD	> 2.56
β-CD	> 13.00
γ-CD	> 20.00
[GCV:β-CD]	> 0.140

Antiviral activity of free or complexed GCV. The in vitro antiviral effect of free or complexed GCV was studied using two different methods of susceptibility evaluation on two HCMV strains. For both methods, CMV-infected MRC5 cells were exposed to different concentrations of free or complexed GCV for 6 days (ELISA) or 9 days (Plaque Reduction Assay).

The antiviral activity on AD169 and RCl1 strains of the GCV free and in presence of α-, β- and γ-Cds was evaluated by the plaque reduction assay (PRA) method (Table 3). The IC_{50} of the free GCV on the susceptible HCMV strain AD169 was evaluated as 1.20±0.10 μM. The antiviral effect of the GCV complexed with α-Cd (IC_{50} = 0.90±0.08 μM) was almost identical to the activity of the free GCV. On the contrary, its complexation with the β- or γ-Cds improved its efficacy. The IC_{50} decreased by 6- and 4-fold (IC_{50} = 0.20±0.03 μM and 0.30±0.03 μM), respectively.

The IC_{50} obtained by the PRA method with the free GCV on the RCl1 strain was 12.75 μM±2.07 (Table 3); it is thus a resistant strain. It is generally accepted that low level resistance to GCV is defined as 8 μM≤GCV IC_{50}≤ 30 μM and high level resistance as IC_{50} = 30 μM.[6] The efficiency of [α-Cd:GCV] was not tested on RCl1 strain, because of its inefficacy on AD169. The complexation of GCV by β-Cd produced an improvement of its efficacy (0.70±0.05 μM) against the complexation with γ-Cd

Table 3. Evaluation of the IC_{50} of the GCV in absence or in presence of Cds on HCMV strains by two methods. PRA method: MRC5 cells in 24-well plates (2.5×10^5 cells/well) were inoculated with viral suspension (moi = 100 PFU/well) and were treated by GCV and various complexes at different concentrations. After an incubation period of 7 days the antiviral activity of the drugs was evaluated. ELISA method: MRC5 cells in 96-well plates (4×10^4 cells/well) were inoculated with viral suspension (moi = 0.1 PFU/cell) and were treated by GCV or [GCV:β-Cd]. After an incubation period of 6 days, the antiviral activity was determined (see Experimental)

		IC_{50} (μM) PRA method	IC_{50} (μM) ELISA method
AD169	Free GCV	1.20±0.10	2.70±0.55
	[GCV:α-CD]	0.90±0.08	nd[a]
	[GCV:β-CD]	0.20±0.03	0.20±0.05
	[GCV:γ-CD]	0.30±0.03	0.30±0.01
RCl1	Free GCV	12.75±2.07	14.50±2.50
	[GCV:β-CD]	0.70±0.05	1.60±0.12
	[GCV:γ-CD]	4.12±0.48	nd

[a]nd: not done.

(4.12±0.48). IC_{50} of GCV obtained by the complexation with β- or γ-Cds was ≤8 μM.

In summary, the complexation of GCV by α-Cd does not cause improvement of its efficiency on AD169; on the other hand, the complexation by β- or γ-Cds increases the GCV antiviral activity on both HCMV strains; the use of β-Cd seems more effective than α-Cd.

The results obtained by both methods were approximately equivalent (Table 3), except for IC_{50} of free GCV on AD169: the one obtained in ELISA is 2-fold higher than the one in PRA. The IC_{50} obtained in ELISA was generally higher; this method seems more sensitive than the PRA method.

GCV uptake in MRC5 cells. Figure 6 shows the cellular uptake of GCV, either free or complexed with β-Cd. The complexation of the GCV with β-Cd increased its penetration into the MRC5 cells. This effect was observed from the first time points measured. Between 1 and 4 h, the complexed GCV penetrated the cells 3 times more efficiently than the free GCV and the maximum GCV levels persisted from 24 to 48 h. All the GCV entered the cells when complexed by the β-Cd, while the maximum uptake of free GCV is about 80% and the absorption level does not improve over the 96 h observation period (data not shown).

Discussion

In the present work, we characterized interactions between GCV and native Cds and we evaluated the in vitro antiviral activity of the GCV in the presence of Cds.

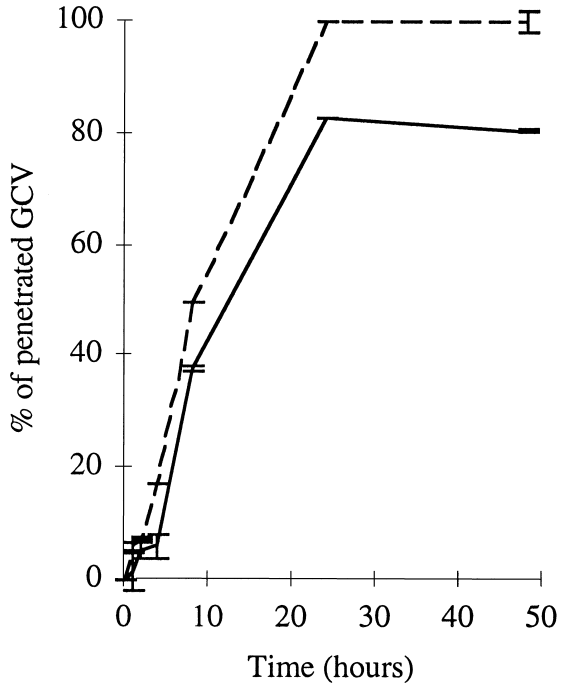

Figure 6. Measure of GCV uptake in MRC5 cells. —— penetration of free GCV; – – – – penetration of GCV complexed with β-Cd.

At first, we studied the influence of the three most often used Cds on the antiviral activity of GCV on the HCMV reference strain AD169 by two methods. The evaluation by the PRA method was done by counting of the infectious particles, whereas the ELISA method detected a late antigen production, which is dependent on DNA replication (inhibited by the GCV). The results obtained using the ELISA method were equivalent to those obtained by PRA. Consequently, both methods can be applicable to determine the susceptibility of HCMV strains to antiviral agents. The PRA was long considered as the reference method to determine the antiviral susceptibility of viral strains.[6] However, it is a time-consuming method and many other methods are currently being developed, such as DNA–DNA hybridization,[23] Fluorescent Immuno Assay, ELISA[24] or flow cytometry,[25] in order to decrease the time of assay and allow a standardization of the methods in laboratories. The ELISA method is faster, easier, with a more reliable spectrophotometric reading. Moreover, the PRA, with an evaluation by counting of PFU per well, did not allow working with high multiplicity of infection (moi). For these reasons, we have selected the ELISA method to determine antiviral susceptibility in further assays. This method could be used in clinical laboratories, for the routine evaluation of the HCMV isolates' susceptibility.

At first, we studied the influence of the three most used native Cds (α-, β- and γ-Cds) on the antiviral activity of GCV on the HCMV reference strain AD169. When γ-Cd was used as carrier, GCV activity was unaltered. Moreover NMR study showed that the GCV and the α-Cd did not form an inclusion complex. This Cd could not carry the GCV because its cavity is too small and it was not used for further experiments. On the contrary, the use of β- or γ-Cd as carrier improved the GCV efficacy. These biological effects could be due to the complexation of the GCV by the Cds. This hypothesis was confirmed by the NMR studies for the β-Cd, proving that the complexation with the GCV was possible and that the GCV was linked with the β-Cd by its aromatic group. However, the study revealed that γ-Cd and GCV did not form an inclusion complex. This Cd has a cavity too big to allow optimal binding with a molecule like GCV, which is a relatively small and hydrophilic molecule. The NMR results reveal the importance of the cavity size to get an adequate fit between the drug and the Cd. It is well known that β-Cd offers an optimum cavity environment for binding aromatic rings.[26] Most drug molecules tend to interact more favourably with β-Cd than α-Cd because the 6μ cavity diameter of the β-Cd accommodates aromatic groups found in many drug molecules. In contrast, the cavity diameter of α-Cd tends to be too small for a favourable fit. Interaction can also be seen between many drugs and γ-Cd; however, its cost has made its extensive use economically difficult[27] and its excessively broad cavity does not permit optimal binding with small molecules.

We characterized the complex formation between GCV and β-Cd. The ^1H NMR spectra exhibited spectral changes characteristic of the interaction between both molecules and the formation of an inclusion complex.

The ^{13}C NMR result allows us to hypothesize that the GCV is included in the Cd by its purine ring. The Job plot revealed that the stoichiometry of the [GCV:β-Cd] complex was 1:1, and the complex formation constant was determined to be 4976 M^{-1}. A schematic representation of the complex formed between GCV and β-Cd is proposed in Figure 7.

The effect of γ-Cd on the GCV activity is not due to the formation of an inclusion complex between GCV and γ-Cd. The effect of native Cds on the biological properties of drugs has already been tested in our laboratory. We have tested indeed the influence of α-, β- and γ-Cds on the anticellular activity of the antitumour drug, doxorubicin, and, similarly to the present case, α-Cd was unable to carry the doxorubicin and the β-Cd improved the doxorubicin activity without forming an inclusion complex with it.[28] This phenomenon has already been related by Nakanishi: enhanced sulphanilic acid absorption in presence of β-Cd was not due to formation of an inclusion complex between both molecules and that β-Cd may act directly on the mucus-free intestinal membrane.[29] The presence of β- and γ-Cds allowed a clear improvement of GCV efficacy on AD169 and RCl1 strains. These Cds had no effect on the lethality of MRC5 cells (Table 2), and they have no antiviral activity on the HCMV at the concentrations tested in the complexes (data not shown). We can conclude that the antiviral activity obtained with the complex [GCV:Cd] is not due to an increased toxic effect caused by the presence of the Cd or to an antiviral effect of the Cd itself.

The GCV is a nucleoside analogue, it requires an initial phosphorylation by a viral enzyme to be active and it inhibits the viral DNA polymerase (encoded by the gene sequence UL54 in HCMV). A resistance to this antiviral drug can result from alteration of the phosphotransferase (encoded by the gene sequence UL97), the viral DNA polymerase or both.[6] The resistant RCl1 strain exhibits low level of resistance to GCV ($IC_{50} = 14.50 \pm 2.50$) and its genetic profile displays a mutation of the UL97 gene.[30] This mutation, responsible for the GCV resistance, causes a decrease of the affinity of phosphotransferase for the GCV. An improvement of the bioavailability of this substrate can increase its phosphorylation in spite of alteration of the enzyme. The carrying of a molecule causes improvement of its bioavailability and cellular uptake and the decrease of the IC_{50} of the GCV could be explained by this phenomenon.

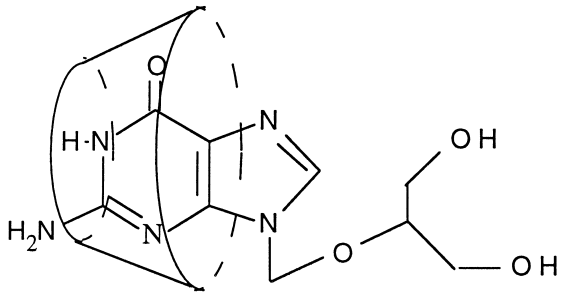

Figure 7. Proposed structure of the [GCV:β-Cd] complex.

The mechanism of action of this carrier is not well understood. The formation of an inclusion complex between the Cd and the guest molecule does not require covalent bonds, easily allowing the drug release. The Cds, which are relatively large with a hydrophilic surface, will only permeate biological membrane with some difficulty[12] and it is often accepted that Cds and complexes do not penetrate membranes and that Cds deliver the guest molecules to the surface of the biological membrane.[31] The Cds can act as simple carriers permitting an increase in the stability and the solubility and a slow release of the guest molecule, thus improving its bioavailability.[32] Thus, the carrier could increase the cellular uptake simply by modifying the distribution and concentration of the drug.[27] Alternatively, the carrying can directly increase the cellular uptake of the drug.[33,34] After dissociation of the complex, the free Cds may remove membrane lipid components, thereby modifying the transport properties of the membrane and facilitating absorption of the drug.[27,29,35] This effect would explain why the formation of an inclusion complex between the drug and the Cd is not necessary. The study of effect of the Cd on cell uptake showed that the β-Cd actually improved the GCV penetration in the MRC5 (Fig. 6). The β-Cd acted on the velocity of the penetration and on the amount of penetrated GCV. γ-Cd produces a similar effect (data not shown). The improvement of the bioavailability and cellular uptake of the drug by its complexation with Cds could explain the decrease in the IC_{50} of the GCV, particularly against the resistant strain. This phenomenon was already mentioned in the work of Bakker-Woudenberg et al. They proved that the encapsulation by liposomes of GCV and foscarnet improved their in vitro efficiency on the cytomegalovirus.[4,5]

Conclusions

Ganciclovir is the most commonly used drug for the treatment of HCMV infections and, often, its toxicity requires arrest of the treatment with the risk of inducing outbreak of this infection. Cd as drug carrier could allow a decrease in the dose of antiviral drugs and a more tolerable treatment. Furthermore, the carrier limits the toxicity of the drug, either by allowing the decrease of the dose or by reducing its toxicity itself as shown with piroxicam[36] and with pilocarpin.[37] In addition, in order to avoid the disadvantages of intravenous administration, GCV is currently proposed in tablet form as an alternative. However, its oral bioavailability is very poor (6%), and this is not a satisfactory solution. The complexation by a Cd could increase oral availability and permit a more effective use of the drug. Considering these properties, complexation with cyclodextrins could be a good alternative for the administration of these drugs, particularly in the case of immunocompromised patients infected with HCMV, who require lifelong treatments with toxic drugs.

The in vitro results are satisfying and we are currently developing a murine CMV model to confirm the in vivo efficacy of cyclodextrins as biovectors of antiviral agents (GCV, antisense oligonucleotides, etc).

Experimental

Chemicals

The native Cds were purchased from Wacker-Chemie GmbH (Lyon, France) for α- and γ-Cds, and from Roquettes Frères (Dijon, France) for β-Cd. 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).

Preparation of the complexes

The [GCV:Cd] complexes were prepared according to Higuchi and Connors.[38] GCV and Cd molecules were incubated in distilled water at a molar ratio of 1:10 for the biological assay and 1:1 for the NMR study, taking into account the Cd solubility in a final volume of 10 mL. The solution was equilibrated overnight at 20 °C by stirring at 200 rpm with a Rotatest 74401 apparatus (Bioblock-Scientific, Illkirch, France). The solution was then mixed v/v with Eagle's minimum essential medium (EMEM) (Polylabo, Strasbourg, France), 2-fold concentrated and immediately tested.

Complex characterization

NMR study. GCV and the different native Cds were incubated at a molar ratio 1:1 as previously described. The complexes were evaporated and lyophilized. The [GCV:Cd] complexes, the Cds and the GCV as powder were dissolved in deuterated water and the study was carried out in ^{1}H NMR for the complexes formed with α-, β- and γ-Cds and in ^{13}C NMR for [GCV:β-Cd] on a Brucker DRX-400 spectrometer.

Determination of stoichiometry of [GCV:β-Cd] complex. The continuous variation method was adopted to determine the stoichiometry of the complex.[20] The total concentration of the two species, ganciclovir and β-Cd, was kept constant, and the mole ratio was varied from 0 to 1. The differences of absorption intensity of ganciclovir in absence and in presence of β-Cd were measured for a given mole ratio by a UV–Visible spectrophotometer (UV mc^2, Safas, Monaco)

Absorption measurement in UV–Visible spectrophotometry. Absorption was measured at 253 nm of GCV at constant concentration (5×10^{-5} M) in absence and in presence of increasing concentrations of β-Cd (10^{-4}, 2.5×10^{-4}, 10^{-3}, 2.5×10^{-3}, 5×10^{-3} and 7.5×10^{-3} M) in water at room temperature.

Cells and virus

Human embryonic lung fibroblasts (MRC5) were grown in Eagle's minimum essential medium (EMEM) (Polylabo, Strasbourg, France) supplemented with 10% Foetal Calf Serum (FCS) (Dutscher, Brumath, France), 0.1% Amiklin (Bristol-Myers-Squibb) and 0.1% Clamoxyl (Smithkline Beecham Laboratories). Maintenance cell medium contained 2.5% FCS.

The HCMV strains studied were the strain AD169 (ATCC reference: VR-538), sensitive reference strain and a GCV resistant laboratory mutant RCl1, derived from AD169. It was obtained by exposure of AD169 to increasing concentrations of GCV.[30] Production and stocks of the HCMV strains were prepared from culture on MRC5 cells. The cells were infected with the virus and, after an adsorption period of 60 min, EMEM was added. They were incubated until a 90% cytopathic effect of cell layer occurred. At this time, the cells were frozen and defrozen, then the supernatant was collected, sonicated and centrifuged (10 min at 180 g). Supernatant was divided and stored at −180 °C. The HCMV titres were determined by Plaque Assay[39] on MRC5 cells in 24-well plates (Costar, Dutscher, Brumath, France) at confluence (2.5×10^5 cells/well). The cells were infected and, after an incubation period of 60 min, the inoculum was removed and replaced with 2 mL/well EMEM containing 2.5% FCS and 0.4% agarose (indubiose, Biosepra, Villeneuve-La-Garenne, France). Plaque Forming Units (PFU) were counted after 7 to 9 days by reverse optic microscopic observation.

Cytotoxicity assays

The toxicity of the molecules was evaluated by the modified MTT assay.[40] MRC5 cells were grown at confluence in 96-well plates (Costar, Dutscher, Brumath, France) at 4×10^4 cells per well and incubated with various concentrations of Cds or GCV for 7 days at 37 °C. After this incubation period, the cytotoxic effect of the molecules was evaluated by determination of the mitochondrial dehydrogenase activity using the MTT (1-[4,5-dimethylthiazol-2-yl]-3,5 diphenyl tetrazolium bromide) (Sigma Chemical Co., St Louis, MO, USA). The absorbance (540 nm) was measured by a titertek Multiskan MCC 340 MKII apparatus (Labsystems, Helsinki, Finland). A cytotoxic effect caused a decrease in purple formazan production compared to non-treated cells. The concentration of the molecule, which produced the death of 50% cells (LC_{50}), was then estimated. Results are shown as average values from three independent experiments performed in triplicate.

Susceptibility of the HCMV strains to antiviral agents

By plaque reduction assay (PRA). Susceptibility to antiviral drugs was tested using the PRA method described by Isenberg.[39] Monolayer MRC5 cells in 24-well plates (Costar, Dutscher, Brumath, France) at 2.5×10^5 cells/well were inoculated with HCMV suspension at an moi of 100 Plaque Forming Units (PFU)/well. After an incubation period of 60 min at 37 °C, the inoculum was removed and the cells were exposed to free or complexed GCV at various concentrations in medium with 0.4% agarose and incubated for 7 to 9 days at 37 °C. The virus titration was evaluated after a crystal violet staining, the number of PFU was determined using a reverse optic microscope and the concentration of the drug producing 50% of viral inhibition (IC_{50}) was estimated. Results are shown as average values from three independent experiments performed in triplicate.

By ELISA method. Monolayer MRC5 cells in 96-well plates (Costar, Dutscher, Brumath, France) at 4×10^4 cells/well were inoculated with an HCMV suspension at an moi of 0.1 PFU/cell. After an incubation period of 60 min at 37 °C, the inoculum was removed and the cells were incubated with free or complexed GCV at various concentrations for 6 days. After a fixation by an ethanol/acetone (95/5%) mixture during 30 min, cells were incubated for 30 min at 37 °C with Phosphate Buffered Saline (PBS) containing 0.5% Bovine Serum Albumin (BSA, Sigma Chemical Co., St Louis, MO, USA), followed by incubation for 60 min at 37 °C with a mouse monoclonal antibody directed against an HCMV late protein (Argène Biosoft, Varilhes, France). The cells were washed and incubated for 60 min at 37 °C with a peroxidase-conjugated polyclonal goat antibody (Diagnostic Pasteur, Marnes-La-Coquette, France). After washing, the cells were incubated for 15 min with MTB (methylthymol blue) (Sigma Chemical Co., St Louis, MO, USA). The reaction was stopped with 0.5 M H_2SO_4 and the absorbance was determined at 450 nm. The concentration of the drug producing a decrease of 50% in the viral production (IC_{50}) was estimated. Results are shown as average values from three independent experiments performed in triplicate.

Studies of GCV uptake in MRC5 cells. MRC5 cells at confluence were incubated with free GCV or GCV complexed by β-Cd in EMEM supplemented with 2.5% FCS and 0.1% of each antibiotic (Amiklin and Clamoxyl). The supernatant was removed several times (1, 2, 4, 8, 24, and 48 h) and the GCV concentration in the supernatant was assayed spectrophotometrically at 253 nm. The cells were washed and the GCV concentration was assayed in the supernatant. This procedure was repeated until the supernatant was free of GCV. From these data, the total amount of the absorbed GCV was calculated.

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References and Notes

1. Harada, K.; Eizuru, Y.; Isashiki, Y.; Ihara, S.; Minamishima, Y. *Arch. Virol.* **1997**, *142*, 215.
2. Lalezari, J. P.; Stagg, R. J.; Kuppermann, B. D.; Holland, G. N.; Kramer, F.; Ives, D. V.; Youle, M.; Robinson, M. R.; Drew, W. L.; Jaffe, H. S. *Ann. Intern. Med.* **1997**, *126*, 257.
3. Marwick, C. *JAMA* **1998**, *280*, 871.
4. Bergers, J. J.; Hengge, U. R.; Snijders, S. V.; Bakker-Woudenberg, I. A. J. M. *J. Controlled Release* **1997**, *47*, 163.
5. Bakker-Woudenberg, I. A. J. M.; Lokerse, A. F.; Ten Kate, M. *Scand. J. Infect. Dis.* **1991**, *S74*, 54.
6. Erice, A. *Clin. Microbiol. Rev.* **1999**, *12*, 286.
7. Stanat, S. C.; Reardon, J. E.; Erice, A.; Jordan, M. C.; Drew, W. L.; Biron, K. K. *Antimicrob. Agents Chemother.* **1991**, *35*, 2191.
8. Wenz, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 803.
9. Loftsson, T.; Brewster, M. E. *J. Pharm. Sci.* **1996**, *85*, 1017.
10. Duchêne, D.; Glomot, F.; Vaution, C. In *Cyclodextrins and Their Industrial Uses*; Duchêne, D., Ed.; Editions de Santé: Paris, 1987; Chapter 6, pp 215.
11. Kimura, E.; Bersani-Amado, C. A.; Sudo, L. S.; Santos, S. R. J.; Oga, S. *Gen. Pharmacol.* **1997**, *28*, 695.
12. Loftsson, T.; Stefansson, E. *Drug Dev. Ind. Pharm.* **1997**, *23*, 473.
13. Attioui, F.; Al-Omar, A.; Leray, E.; Parrot-Lopez, H.; Finance, C.; Bonaly, R. *Biol. Cell* **1994**, *82*, 161.
14. Leray, E.; Parrot-Lopez, H.; Auge, C.; Finance, C.; Bonaly, R.; Coleman, A. W. *J. Chem. Soc., Chem. Commun.* **1995**, *1019*.
15. Abdou, S.; Collomb, J.; Sallas, F.; Marsura, A.; Finance, C. *Arch. Virol.* **1997**, *142*, 1585.
16. Ahn, H. J.; Kim, K. M.; Choi, J. S.; Kim, C. K. *Drug Dev. Ind. Pharm.* **1997**, *23*, 397.
17. Montassier, P.; Duchêne, D.; Poelman, M. C. *Int. J. Pharm.* **1997**, *153*, 199.
18. Kaukonen, A. M.; Lennernas, H.; Mannerma, J. P. *J. Pharm. Pharmacol.* **1998**, *50*, 611.
19. Mura, P.; Bettinetti, G. P.; Manderioli, A.; Fauci, M. T.; Bramanti, G.; Sorrenti, M. *Int. J. Pharm.* **1998**, *166*, 189.
20. Connors, K. A. In *The Measurement of Complex Stability*; Wiley, J., Ed.; Wiley-Interscience: New York, 1987; p 21.
21. Shiotani, K.; Uehata, K.; Irie, T.; Hirayama, F.; Uekama, K. *Chem. Pharm. Bull.* **1994**, *42*, 2332.
22. Scott, R. L. *Rec. Trav. Chim.* **1956**, *75*, 787.
23. Dankner, W. M.; Scholl, D.; Stanat, S. C.; Martin, M.; Sonke, R. L.; Spector, S. A. *J. Virol. Methods* **1990**, *28*, 293.
24. Tatarowicz, W. A.; Lurain, N. S.; Thompson, K. D. *J. Virol. Methods* **1991**, *35*, 207.
25. McSharry, J. M.; Lurain, N. S.; Drusano, G. L.; Landay, A.; Manischewitz, J.; Notka, M. O.; Gorman, M.; Shapiro, H. M.; Weinberg, A.; Reichelderfer, P.; Crumpacker, C. *J. Clin. Microbiol.* **1998**, *36*, 958.
26. Gadre, A.; Rüdiger, V.; Schneider, H. J.; Connors, K. A. *J. Pharm. Sci.* **1997**, *86*, 236.
27. Rajewski, R. A.; Stella, V. J. *J. Pharm. Sci.* **1996**, *85*, 1142.
28. Al-Omar, A.; Abdou, S.; De Robertis, L.; Driguez, H.; Marsura, A.; Finance, C. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1115.
29. Nakanishi, K.; Nadai, T.; Masada, M.; Miyhajima, K. *Chem. Pharm. Bull.* **1992**, *40*, 1252.
30. Alain, S.; Mazeron, M. C.; Pepin, J. M.; Morinet, F.; Raskine, L.; Sanson-Le Pors, M. J. *Mol. Cell. Probes* **1993**, *7*, 487.
31. Uekama, K.; Hirayama, F.; Irie, T. *Drug Targeting Delivery* **1994**, *3*, 411.
32. Uekama, K.; Horikawa, T.; Yamanaka, M.; Hirayama, F. *J. Pharm. Sci.* **1994**, *46*, 714.
33. Bersani Amado, C. A.; Taniguchi, S. F.; Sudo, L. S.; Kimura, E.; Oga, S. *Gen. Pharmacol.* **1995**, *26*, 809.
34. Jarho, P.; Jarvinen, K.; Urtti, A.; Stella, V. J.; Jarvinen, T. *Int. J. Pharm.* **1997**, *153*, 225.
35. Irie, T.; Tsunenari, Y.; Uekama, K.; Pitha, J. *Int. J. Pharm.* **1988**, *43*, 41.
36. Cadel, S.; Bongrani, S. *Acta Physiol. Hung. Suppl.* **1990**, *75*, 45.
37. Järvinen, T.; Järvinen, K.; Urtti, A.; Thompson, D.; Stella, V. J. *J. Ocul. Pharmacol. Ther.* **1995**, *11*, 95.
38. Higuchi, T.; Connors, K. A. In *Advances in Analytical Chemistry and Instrumentation*; Reilley, C. N., Ed.; New York, 1965; Vol. 4; p 117.
39. Isenberg, H. D. In *Clinical Microbiology Procedures Handbook*; Isenberg, H. D., Ed.; American Society for Microbiology: Washington, 1992; Vol. 2, p 8.26.1.
40. Carmichael, J.; Degraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 936.