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In vitro inhibition of human cytomegalovirus replication by calcium trinatrium diethylenetriaminepentaacetic acid

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Abstract

Desferrioxamine (DFO) has been shown to inhibit human cytomegalovirus (CMV) replication in vitro. In the present study, we compared antiviral effects of DFO in human foreskin fibroblast (HFF) cells against several CMV strains with those of other chelators that interact with iron and other ions from different pools. DFO, a hydrophilic chelator, that may chelate both intracellular and extracellular ions inhibited production of CMV late antigen at 50% effective concentrations (EC_{50}s) ranging from 6.2 to 8.9 μM. EC_{50}s for calcium trinatrium diethylenetriaminepentaacetic acid (CaDTPA) ranged from 6.1 to 9.9 μM. EC_{50}s for 2,2′-bipyridine (BPD), a hydrophobic chelator, which diffuses into cell membranes ranged from 65 to 72 μM. Concentrations which inhibited BrdU incorporation into cellular DNA by 50% (IC_{50}s) ranged from 8.2 to 12.0 μM (DFO), from 65 to 89 μM (BPD), and from 139 to 249 μM (CaDTPA). CaDTPA was the only chelator which completely inhibited production of infectious virus in HFF and vascular endothelial cells at concentrations which had no significant effects on cellular DNA synthesis and growth. Addition of stoichiometric amounts of Fe^{3+} in the culture medium of HFF cells completely eliminated antiviral effects of DFO while antiviral effects of CaDTPA and BPD were only moderately affected. Fe^{2+} and Cu^{2+} were stronger inhibitors of CaDTPA than Fe^{3+}; however, Mn^{2+} and Zn^{2+} completely suppressed antiviral effects of CaDTPA. The results show that CaDTPA is a novel nontoxic inhibitor of CMV replication. The antiviral activity of CaDTPA is suppressed by metal ions with a decreasing potency order of $Mn^{2+}/Zn^{2+} > Fe^{2+} > Cu^{2+} > Fe^{3+}$.

Keywords: Human cytomegalovirus; Desferrioxamine; Diethylenetriaminepentaacetic acid; 2,2′-Bipyridine; Chelators

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1. Introduction

Two anti-CMV compounds have recently been approved for treatment of patients with CMV disease, ganciclovir (GCV) (Collaborative DHPG Treatment Study Group, 1986; Laskin et al., 1987) and phosphonophormic acid (PFA; foscarnet) (Singer et al., 1985; Walmsley et al., 1988). However, prolonged use of ganciclovir is often associated with serious side effects such as anemia, neutropenia and irreversible testicular damage (Felsenstein et al., 1985; Erice et al., 1987; Collaborative DHPG Treatment Study Group, 1986; Laskin et al., 1987; Hecht et al., 1988), while treatment with PFA may result in nephrotoxicity and hypocalcemia (Walmsley et al., 1988; Youle et al., 1988). Another complication resulting from increased use of both GCV and PFA is an increased emergence of drug-resistant CMV strains (Stanat et al., 1991; Knox et al., 1991). Therefore, additional efficacious, nontoxic drugs with a different mechanism of action for use against CMV infections are needed.

2-acetylpyridine 5-[(dimethylamino)thiocarbonyl]thiocarbonohydrazone (1110U81), originally developed as a specific inhibitor of herpes simplex virus-encoded ribonucleotide reductase (Spector et al., 1989; Porter et al., 1990), was shown to inhibit CMV replication in vitro (Hamzeh et al., 1993). Although both sequence homology studies and enzyme purification studies failed to show that CMV encodes an active ribonucleotide reductase enzyme, antiviral effects of 1110U81 could stem from its ability to bind iron. We have found that desferrioxamine (DFO), a trihydroxamic acid commonly used in therapy as a chelator of ferric ion in disorders of iron overload (Pippard and Callender, 1983), is a nontoxic inhibitor of CMV replication in vitro (Cinatl et al., 1994). Since anti-CMV activity of DFO was completely prevented by co-incubation with $FeCl_3$, it is probable that DFO exerts its primary effects by chelating ferric ion and subsequently inhibiting CMV replication. Furthermore, DFO at a pharmacologically attainable concentration of 10 μM decreases the expression of a member of the superimmunoglobulin family ICAM-1 (intercellular adhesion molecule 1) and E-selectin ELAM-1 (endothelial leucocyte adhesion molecule-1) in human umbilical vein endothelial cells (Cinatl et al., 1995). These findings suggest a novel mechanism which could account for immunomodulatory activity of the drug observed in vivo and encourage general interest in iron chelators as anti-CMV agents and immunomodulators.

In the present study, we compared antiviral activity of DFO with other iron chelators including diethylenetriaminepentaacetic acid (DTPA) and 2,2′-bipyridine (BPD) that interact with different iron pools. DTPA, a hydrophilic extracellular chelator, was the most potent antiviral substance among the agents used. Since DTPA also binds ions other than iron, we investigated effects of different ions on its antiviral activity.

2. Materials and methods

2.1. Cells and viruses

Human foreskin fibroblasts (HFF) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) were established as described previously (Jaffe et al., 1973) and grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 20 ng/ml basic fibroblast growth factor. Cultures of human peripheral blood lymphocytes (PBL) were obtained from buffy coats by separation on Percoll (Biochrom, Berlin, Germany) and phytohemagglutinin stimulation of separated mononuclear cells at a cell concentration of 10^6/ml in RPMI 1640 medium containing 10% FBS.

CMV laboratory strains including AD169, Towne and Davis were purchased from American Type Culture Collection (Rockville, MD, USA). The viruses were propagated in EMEM supplemented with 4% FBS (maintenance medium). Virus titre was determined by examination of immediate early antigen-forming units (I.E.F.U.) produced in maintenance medium as described previously (Braun and Schacherer, 1988). In our hands, this method has a sensitivity comparable to that of plaque-forming unit assay. Briefly,

medium of infected cultures at 5-fold dilutions was incubated with confluent HFF monolayers in 96-well plates (Nunc, Wiesbaden, Germany). At 24 h after infection, immunoperoxidase staining of cells using monoclonal antibody directed against 72-kDa immediate early (IE) antigen of CMV (DuPont, Bad Homburg, Germany) was performed. Stained nuclei were counted microscopically and virus titre expressed as numbers of I.E.F.U. per ml.

2.2. Compounds

DFO (Desferal) was obtained from Ciba-Geigy (Basel, Switzerland) and prepared fresh (on the day of each experiment) in dimethylsulfoxide. Complexes of trinatrium DTPA with different ions including calcium trinatrium DTPA (CaDTPA), magnesium trinatrium DTPA (MgDTPA) and zinc trinatrium DTPA (ZnDTPA) (each dissolved in distilled water at a concentration of 100 mg/ml) were kindly provided by Astrapin (Pfaffen-Schwabenheim, Germany). DTPA complexed to different ions was used since uncomplexed chelator showed significant toxicity for cultured cells. CaDTPA (Antiron) is currently used in some countries in clinical trials for the treatment of patients with iron overload. 2,2′-bipyridine (BPD) obtained from Sigma (Deisenhofen, Germany) was prepared fresh in dimethylsulfoxide. $FeCl_3$, $FeCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $MgCl_2$ and $CaCl_2$ and other chemicals were obtained from Sigma.

2.3. Antiviral assays

The effects of compounds on the replication of CMV were measured by enzyme-linked immunosorbent assay (ELISA) and viral yield reduction assay.

(i) CMV ELISA was based on a detection of CMV late antigen protein in infected HFF cells and performed as described previously (Tatarowicz et al., 1992) with minor modifications. Briefly, selected drug concentrations in quadruplicate and CMV strains at an MOI of 0.01 were added to each well of 96-well plates containing confluent HFF. Following a 6–7-day incubation, the cells were fixed with methanol–acetone (6:4) and incubated with mouse monoclonal antibody directed against 67-kDa CMV late nuclear protein (DuPont). Horseradish–peroxidase-conjugated goat anti-mouse IgG was used as a secondary antibody. The substrate used was POD blue (Boehringer, Mannheim, Germany). The absorbance was measured at 450 nm (against reference wavelength 690 nm). Drug effects were calculated as a percentage of the reduction in absorbance in the presence of each drug concentration compared with absorbance obtained with virus in the absence of the drug.

(ii) CMV virus yield assay was performed using confluent HFF and HUVEC layers grown in polystyrene culture flasks. HFF were infected with CMV at MOI ranging from 0.01 to 1 I.E.F.U. per cell and HUVEC at MOI 10 I.E.F.U. per cell. After a 90 min incubation period, the cells were washed 3 times with phosphate-buffered saline and a medium containing 4% FBS without or with different concentrations of drugs was added. In order to determine the inhibition of CMV replication in HFF, virus yield was measured in a single-cycle assay format (i.e. 3 days after infection). In some experiments, CaDTPA was added at different times after infection of HFF. In HUVEC cultures, virus yield was determined 14 days after infection. To liberate cell associated virus, cultures were frozen and thawed twice and the medium was clarified by centrifugation ($600 \times g$ for 10 min.). Virus yield was determined as I.E.F.U./ml after titration in HFF monolayers (see above).

(iii) In order to investigate antiviral mechanisms of CaDTPA other than inhibition of virus replication, effects of cell pretreatment before virus infection, effects on virus adsorption and direct virucidal effects were measured. To assess effects of cell pretreatment, HFF were incubated with CaDTPA at concentrations ranging from 10 to 200 μM for 24 h, 48 h and 72 h before virus infection. After pretreatment with CaDTPA, cells were washed with PBS and incubated with AD 169 virus strain (MOI 0.1) for 90 min in order to allow virus adsorption. After adsorption, the cells were washed with PBS and maintenance medium without the drug was added. Virus titre was deter-

Table 1
Effects of different chelators on the production of CMV late antigen in HFF and on DNA synthesis in HFF, HUVEC and PBL

Compound	EC_{50} (μM)			IC_{50} (μM)		
	AD169	Towne	Davis	HFF	HUVEC	PBL
DFO	6.2 ± 0.71	6.8 ± 0.95	8.9 ± 0.68	9.1 ± 1.3	8.2 ± 0.72	12 ± 2.5
BPD	65 ± 8.1	72 ± 9.4	68 ± 6.9	65 ± 8.3	73 ± 6.9	89 ± 11
CaDTPA	8.2 ± 0.96	6.1 ± 0.71	9.9 ± 1.2	152 ± 21	139 ± 19	249 ± 25
MgDTPA	9.3 ± 0.76	8.1 ± 1.1	8.7 ± 0.68	140 ± 18	145 ± 14	232 ± 24
ZnDTPA	>200	>200	>200	>400	>400	>400

Values are mean ± S.D. from two independent experiments.

mined 3 days after infection. To assess effects on virus adsorption, HFF were incubated with different concentrations of CaDTPA during the 90 min adsorption period with AD 169 strain. After adsorption, cells were washed with PBS, incubated in maintenance medium without the drug and virus titre was determined 3 days after infection. To measure virucidal effects of CaDTPA, AD169 strain (10^6 I.E.F.U./ml) were incubated with different concentrations of the drug at 37°C for 1 h, 2 h or 4 h. After incubation, virus was titrated on HFF monolayers.

2.4. Measurement of cellular DNA synthesis

Effects of the drugs on DNA synthesis were measured in HFF, HUVEC and PBL cultures. HFF were seeded at a density 4×10^4 cells per cm^2 in 96-well plates in EMEM containing 10% FBS. PBL were seeded at a density 5×10^5 per 1 ml RPMI supplemented with 10% FBS and 50 U/ml of recombinant interleukin 2. The drugs were added at different concentrations 2 days after seeding. DNA synthesis was measured 24 h after drug addition by quantitative determination of 5-bromo-2-deoxyuridine (BrdU) incorporated into cellular DNA using the ELISA method. A peroxidase labelled monoclonal antibody to BrdU and other chemicals were obtained as assay kit from Boehringer-Mannheim. The procedure was performed according to the manufacturer's instructions. The results were expressed as percentages of absorbance of untreated control cultures.

2.5. Measurement of cell proliferation

To measure effects on cell growth HFF were seeded at a density 2×10^4 per cm^2 in culture flasks in EMEM containing 10% FBS without or with different drug concentrations. Viable cells were counted using a hemocytometer 2, 4 and 6 days after drug addition. Cell viability was determined by trypan blue exclusion method.

2.6. Electron microscopy

HFF were infected with AD169 strain at MOI 1 and incubated with 200 μM CaDTPA or without the drug. Three days after infection, cells were processed for electron microscopy as described previously (Cinatl et al., 1994). Briefly, cells were pelleted and fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Durupan-Epon. Thin sections were contrasted with uranyl acetate and lead citrate and viewed with a Joel, JEM, 2000 CX microscope.

3. Results

3.1. Effects of chelators on CMV L-antigen production

Table 1 shows antiviral activities of DFO, BPD, CaDTPA, MgDTPA and ZnDTPA against different CMV strains in HFF cells by using the measurement of CMV L-antigen production by ELISA. DFO inhibited L-antigen production at

50% effective concentrations (EC_{50}s) which ranged from 6.2 μM to 8.9 μM. EC_{50}s for BPD were 10 times higher than those for DFO and ranged from 65 μM to 72 μM. CaDTPA and MgDTPA had EC_{50}s at similar molar concentrations as DFO, which ranged from 6.1 μM to 9.9 μM for CaDTPA and from 8.1 μM to 9.3 μM for MgDTPA. ZnDTPA had no antiviral effects at concentrations up to 200 μM (maximum concentration tested).

3.2. Effects of chelators on cellular DNA synthesis and growth

Effects of chelators on DNA synthesis in different cell types are shown in Table 1. DFO was the most potent inhibitor in all cell types. Concentrations which inhibited BrdU incorporation into cellular DNA by 50% (IC_{50}s) were 9.1 μM for HFF, 8.2 μM for HUVEC and 12 μM for PBL. IC_{50}s of BPD were at least 5 times higher than those of DFO, i.e. 65 μM for HFF, 73 μM for HUVEC and 89 μM for PBL. IC_{50}s of both CaDTPA and MgDTPA were at least 15 times higher than those of DFO, i.e. IC_{50}s of CaDTPA were 152 μM for HFF, 139 μM for HUVEC and 249 μM for PBL; IC_{50}s of MgDTPA were 140 μM for HFF, 145 μM for HUVEC and 232 μM for PBL cells. CaDTPA and MgDTPA were more potent inhibitors of DNA synthesis in HFF and HUVEC than in PBL. ZnDTPA had no effect on DNA synthesis in all cell types at a concentration as high as 400 μM (maximum concentration tested). The results in Table 1 show that CaDTPA and MgDTPA are the only chelators which have a significant selectivity index (ratio IC_{50}/EC_{50}) of about 15, when inhibition of DNA synthesis in replicating cells was used to assess IC_{50}. Since both CaDTPA and MgDTPA show similar antiviral and cytostatic activities, CaDTPA was used in most further investigations.

For further evaluation of the selectivity index of CaDTPA, we investigated effects of the drug on HFF growth in a time period of 6 days. CaDTPA at concentrations of 50 μM and 100 μM had no significant effect on the growth of HFF. CaDTPA at concentrations of 150 μM and 200 μM inhibited cell growth by 45% and 75%, respectively. Concentrations up to 200 μM had no effect on cell viability (data not shown).

3.3. Effects of CaDTPA on virus yields

In HFF cultures infected with AD169 strain at MOI 0.01, CaDTPA completely inhibited virus replication at a concentration of 50 μM as shown by a measurement of virus yields in a single-cycle assay. CaDTPA at a concentration of 6.25 μM inhibited of virus yield by 90% (Table 2).

Increasing MOI of AD 169 strain up to 0.1 I.E.F.U. per cell had no significant effect on antiviral activity of the drug (Table 2). In contrast, the antiviral activity of CaDTPA was decreased in cultures infected at MOI 1; a CaDTPA concentration as high as 200 μM was required to completely inhibit production of infectious virus (Table 2).

To evaluate whether antiviral activity of CaDTPA is common to different cell types, we tested its effects on virus yields in HUVEC, since

Table 2
Effects of CaDTPA on CMV (AD169 strain) replication in HFF cells infected at different MOIs

CaDTPA (μM)	Virus titre (log I.E.F.U./ml)		
	MOI	MOI	MOI
	0.01	0.1	1
0	3.21 ± 0.041	4.99 ± 0.056	6.08 ± 0.048
6.25	2.32 ± 0.029	3.95 ± 0.022	6.15 ± 0.045
12.5	1.98 ± 0.048	3.23 ± 0.042	5.32 ± 0.025
25	1.11 ± 0.033	2.36 ± 0.039	3.71 ± 0.039
50	0	0	2.51 ± 0.042
100	0	0	1.08 ± 0.044
200	0	0	0

HFF cells were infected with AD169 strain at different MOIs. Different concentrations of CaDTPA were added to a maintenance medium immediately after virus adsorption. Virus yields were determined 72 h after infection. Values are mean ± SD from three independent experiments.

Table 3
Effects of time of drug addition on the inhibition profile of CaDTPA

CaDTPA (μM)	Virus titre (log I.E.F.U./ml)			
	0 h	16 h	32 h	48 h
0	5.08 ± 0.039	–	–	–
6.25	4.32 ± 0.028	4.55 ± 0.051	4.81 ± 0.046	4.98 ± 0.028
25	2.45 ± 0.041	2.71 ± 0.042	3.86 ± 0.037	3.96 ± 0.031
100	0	0	2.32 ± 0.042	4.08 ± 0.026

HFF cells were infected with AD169 strain at MOI 0.1. CaDTPA was added at different concentrations to a maintenance medium immediately after virus adsorption (0 h) or 16 h, 32 h and 48 h after virus infection. Virus yields were determined 72 h after infection. Values are mean ± S.D. from three independent experiments.

endothelial cells represent an important target for CMV in vivo (Myerson et al., 1984; Toorkey and Carrigan, 1989; Grefte et al., 1993). In the cells infected at MOI 10, CaDTPA at a concentration of 50 μM completely inhibited virus replication (data not shown).

CaDTPA pretreatment had no effect on viral titre, did not influence virus adsorption and had no direct virucidal activity (data not shown). On the other hand, treatment of infected HFF throughout a period preceding replication of viral DNA was not required for the antiviral activity; CaDTPA retained its full antiviral activity when added 16 h after infection and maintained significant antiviral activity even when added 48 h post-infection (Table 3).

3.4. Virus morphogenesis

Electron microscopy investigations suggest that CaDTPA inhibit CMV morphogenesis probably in the assembly and/or budding of nucleocapsid from the nuclear membrane. Numerous viral nucleocapsids were present both in CaDTPA treated and untreated HFF (Figs. 1 and 2). However, while in untreated cells numerous cytoplasmic enveloped viral particles, dense bodies and extracellular mature virus particles were observed (Fig. 1), the cytoplasmic phase of CMV morphogenesis and virus production was completely suppressed in treated cells (Fig. 2).

3.5. Effects of different ions on antiviral activity of different chelators

In a previous study, we demonstrated that stoichiometric amounts of ferric ion completely inhibited antiviral effects of DFO as measured in plaque reduction assay (Cinatl et al., 1994). Therefore, we tested whether Fe^{3+} may also prevent antiviral effects of chelators other than DFO. Fe^{3+}, at stoichiometric amounts, completely inhibited antiviral effects of DFO as determined by measurement of virus titre, while antiviral effects of BPD were influenced to a significantly lower extent (data not shown). The addition of stoichiometric amount of Fe^{3+} to the culture medium had only little effect on antiviral activity of CaDTPA (Table 4).

Since other ions than Fe^{3+} also form complexes with CaDTPA (Sillén and Martell, 1971), we tested effects of Zn^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+} and Ca^{2+} on antiviral activity of the drug. The inhibitory effects of CaDTPA on virus production were completely prevented by coincubation with stoichiometric amounts of Zn^{2+} and Mn^{2+} (Table 4). Stoichiometric amounts of Fe^{2+} and Cu^{2+} did not completely prevent antiviral effects of CaDTPA; however, Fe^{2+} showed stronger antagonistic activity than Cu^{2+} (Table 4). Mg^{2+} and Ca^{2+} had no significant effects on antiviral activity of CaDTPA (data not shown). The ions added to a culture medium without CaDTPA at concentrations of up to 100 μM did not influence virus replication significantly (data not shown).

Fig. 1. Electron microscopic observations of CMV-infected, untreated (control) HFF cell. Overview of parts of nucleus (n) with numerous nucleocapsids (arrow) and the cytoplasma (p) with enveloped virions and dense bodies (A). Nucleocapsids in the nucleus have lucid as well as dense cores (arrow) (B). Enveloped particles in the cytoplasm have a characteristic appearance of CMV virions (arrowheads) and dense bodies are mostly enveloped (arrows) (C). Extracellular virus particles are present (D). Magnification, × 16,000 (A); × 37,000 (B and C); × 30,000 (D).

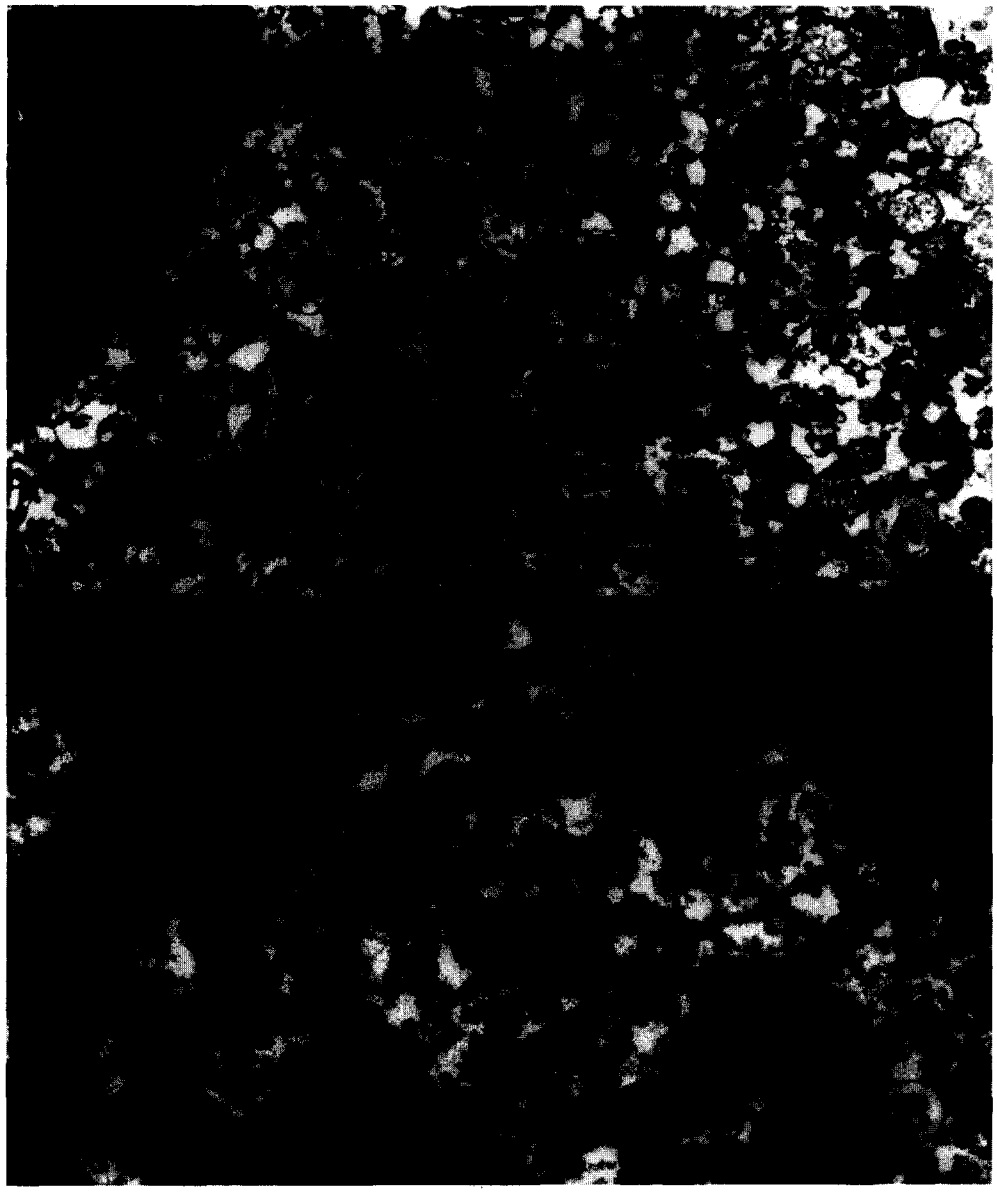

Fig. 2. Electron microscopic observations of CMV-infected cell in HFF culture treated with 200 μM CaDTPA. Overview of parts of nucleus (n) with numerous nucleocapsids and cytoplasm (p) with no evidence of viral particles or dense bodies (A). Nucleocapsids in the nucleus have mostly dense cores (B). Magnification, × 12,500 (A); × 60,000 (B).

4. Discussion

Previously, we demonstrated that DFO at non-toxic concentrations inhibits CMV replication in cultured fibroblast cells (Cinatl et al., 1994). The antiviral activity of DFO was prevented by coin-

cubation with stoichiometric amounts of Fe^{3+}, which suggests that DFO exerts its primary effect by chelating ferric ion and subsequently inhibiting CMV replication. DFO is a hydrophilic chelator which binds iron and other ions from both intracellular and extracellular pools (Peters et al., 1966; Bridges and Cudkowicz, 1984). To study the relationship between intracellular and extracellular chelatable iron pools and CMV replication, we tested antiviral effects of other iron chelators including BPD and DTPA which distribute to different cellular compartments. BPD, a hydrophobic chelator, partitions into cell membranes and binds iron as it passes through this lipid environment (Nunez et al., 1983; Bridges and Cudkowicz, 1984). DTPA, a hydrophilic chelator, has access only to the extracellular spaces and binds iron and other ions from the extracellular pool (Aisen and Listowsky, 1980). The results showed that: (i) DTPA (both CaDTPA and MgDTPA) is a nontoxic inhibitor of CMV replication; (ii) zinc and manganese suppress antiviral activity of DTPA more than other metal ions; (iii) complete inhibition of virus replication in HFF and HUVEC was observed at concentrations that can be achieved in vivo.

CaDTPA was a significantly more selective anti-CMV agent than DFO or BPD. CaDTPA showed a significant therapeutic index when IC_{50} was measured in dividing cells. BPD and DFO inhibited CMV replication at concentrations which were inhibitory for cellular DNA synthesis and cell growth. The ability of CaDTPA to chelate ions only from extracellular spaces, which may be less deleterious for cell metabolism than chelation from intracellular compartments (as observed for other chelators), may be one possible explanation for such differences between CaDTPA and other chelators. In fact, several studies have demonstrated that impaired DNA synthesis in cells treated with CaDTPA is a secondary reaction to toxic effects such as damage of the plasma membrane (Taylor and Jones, 1972; Lücke-Huhle, 1976).

Table 4
Effect of different metal ions on anti-CMV activity of CaDTPA

Metal ion	Virus titre (log I.E.F.U./ml)	Inhibition (%)
0	2.45 ± 0.036	>99.99
Fe^{3+}	2.79 ± 0.034	99.59
Zn^{2+}	4.98 ± 0.042	0
Mn^{2+}	5.04 ± 0.032	0
Fe^{2+}	3.89 ± 0.033	94.73
Cu^{2+}	3.21 ± 0.034	98.92

HFF cells were infected with AD169 strain at MOI 0.1. After virus adsorption, infected cultures were incubated in maintenance medium with 25 μM CaDTPA without or with different metal ions. Each ion and CaDTPA were used at a molar ratio of 1:1. Virus titres were determined 72 h after infection. Values are mean ± S.D. from three independent experiments. Virus titre of control (untreated) cultures ranged from 9.6×10^4 to 2.7×10^5 I.E.F.U./ml.

In addition to different distribution, the chelators show also differences in their affinity for different ions, which may be characterized by the so-called conditional stability constant (K_M). DFO and BPD have remarkably high affinity for iron and low affinity for calcium, copper, zinc and aluminium (Sillén and Martell, 1971; Polson et al., 1985; Nunez et al., 1983). However, DFO is selective for Fe^{3+} while BPD is selective for Fe^{2+} (Bridges and Cudkowicz, 1984; Nunez et al., 1983). Therefore, antiviral effects of BPD in HFF cells were influenced only moderately by Fe^{3+}. The finding that antiviral effects of CaDTPA are significantly more affected by Fe^{2+} than Fe^{3+} suggests that, in our culture system, CaDTPA forms more stable complexes with Fe^{2+} than with Fe^{3+}. DTPA forms stable complexes not only with iron but also with other metals including zinc, manganese, copper, yttrium and indium (Muller-Eberhard et al., 1963; Waxman and Brown, 1969; Sillén and Martell, 1971; Catsch and Hartmuth-Hoene, 1975). DTPA has a much lower K_M for calcium and magnesium than for most other metal ions (Sillén and Martell, 1971). Therefore, exchange of the central Ca^{2+} in CaDTPA against other metal ions may result in more stable complexes. Addition of zinc or manganese to a culture medium completely inhibited antiviral activity of CaDTPA against CMV. In this case, complex-exchange rate of zinc or manganese against other ions may be completely blocked with a lack of antiviral effects as a conse-

quence. The possibility that the binding of zinc blocks the activity of DTPA is also suggested by the fact that ZnDTPA did not inhibit virus production. On the other hand, effects of metal ions on antiviral activity of CaDTPA can not be simply explained by different K_Ms. DTPA has a significantly higher K_M for Fe^{3+} than for Zn^{2+} or Mn^{2+}; however, Fe^{3+} had only moderate effects on antiviral activity of CaDTPA.

Previously, we found that inhibition of CMV by DFO is selective for CMV while replication of other viruses including herpes simplex, adenovirus and poliovirus was not influenced (Cinatl et al., 1994). Similarly, CaDTPA at concentrations up to 200 μM had no activity against herpes simplex virus in HFF cells (data not shown). The mechanism by which chelators exert their antiviral activity against CMV is not clear. In general, chelators could inhibit virus replication through their effects on a variety of cellular and viral enzymes which require metal ions for their activity including those involved in a synthesis of DNA and RNA (Chesters, 1992; Back et al., 1993). However, it seems very improbable that the inhibition of metalloenzymes by CaDTPA accounts for CMV inhibition in HFF cells since the chelator does not influence directly intracellular ion pools and its antiviral concentrations would show stronger interference with cellular metabolism. Other mechanisms may stem from effects of chelators on the plasma membrane of treated cells. Different ions have been demonstrated to have important effects on cells by direct interaction with the membrane (Hefner and Storey, 1981; Kar et al., 1992). It is possible that chelation of ions associated with the external surface of the plasma membrane by CaDTPA may result in altered membrane properties. It should be also noted that different ions are involved in signal transduction (similar to that of growth factor and hormones) which is important for initiation of replication of viral DNA (Huang and Kowalik, 1993). On the other hand, it is also possible that chelators directly interact with membrane-associated proteins or lipids. In this case, antiviral activity of CaDTPA would not result from a depletion of ions required for CMV replication and suppression of antiviral activity by metal ions would stem simply from inactivation of the chelator. To test this hypothesis, uninfected and CMV-infected cells should be grown in medium and fetal bovine serum that are depleted of the tested metal ions, especially Zn^{2+} and Mn^{2+}.

Pharmacological studies showed that nontoxic doses of CaDTPA yield peak plasma levels of up to 200 μM (Cleton et al., 1963), i.e. at least 4 times higher than those which completely inhibited CMV replication in vitro. Subsequent disappearance from the plasma occurred in an exponential fashion with a half-time of about 50 min which may be the main limiting factor in clinical use of CaDTPA as an antiviral agent. In patients with iron overload, the subcutaneous infusion of 2–4 g of CaDTPA proved to be effective in urinary iron excretion without serious side effects (Pippard et al., 1986; Wonke et al., 1989). In conclusion, the results showed that CaDTPA is an effective anti-CMV agent in cultured cells at concentrations that can be achieved in vivo. Moreover, the findings suggest that CaDTPA and other chelators inhibit CMV replication by a novel mechanism.

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