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2-chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine-1-carboxamide (CMV423), a new lead compound for the treatment of human cytomegalovirus infections

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Abstract

Human cytomegalovirus (HCMV) remains one of the major pathogens in immunocompromised patients (AIDS and transplants) and the main cause for congenital infections leading from slight cognitive defects up to severe mental retardation. The drugs that are currently available for the treatment of HCMV infections, i.e. ganciclovir, foscarnet and cidofovir, are all acting at the level of the viral DNA polymerase. Here we describe an entirely new molecule, the 2-chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine-1-carboxamide (CMV423), that shows very potent in vitro activity against HCMV. CMV423 is highly active against HCMV reference strains and clinical isolates, but also against those strains, isolated from patients or emerging after in vitro selection, that are resistant to either ganciclovir, foscarnet or cidofovir. CMV423 also showed activity in two ex vivo models, that are both highly relevant for the pathophysiology of HCMV, the retinal pigment epithelial and the bone marrow stromal cell assays. Viral antigen expression analysis by flow cytometry, as well as time of addition experiments, confirmed that CMV423 acts on a step of the viral replicative cycle that precedes the DNA polymerase step and, most likely, coincides with the immediate early (IE) antigen synthesis. Finally, CMV423 combined with either ganciclovir, foscarnet or cidofovir in checkerboard experiments demonstrated a highly synergistic activity.

Keywords: Human cytomegalovirus; CMV423; Immediate early antigens

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1. Introduction

Human cytomegalovirus (HCMV) is a member of the herpesvirus family that is responsible for a broad range of infections in immunocompromised as well as non-immunocompromised persons (Eddleston et al., 1997; Hebart et al., 1998; Reddy et al., 1999). The infections can be due to both primary and secondary infections, the latter appearing after reactivation of the virus from a latent state.

The physiopathology of HCMV infection is complex, the viral replication as such, and also the immune reactions, being responsible for different disorders (Zaia, 1991; Lagneaux et al., 1996b; Sweet, 1999). The compounds used for the treatment of HCMV infections are ganciclovir (Noble and Faulds, 1998), foscarnet (Chrisp and Clissold, 1991) and cidofovir (Snoeck et al., 1988; Naesens et al., 1997), which are all known to function as DNA polymerase inhibitors, the viral DNA polymerase being an enzyme produced during the early (E) phase of the viral replicative cycle. During the immediate early (IE) phase of the viral replicative cycle, pathological events are induced by the virus that are not controlled by the classic antiviral therapies (Lagneaux et al., 1996b). The expression of IE proteins alone can quickly modify host cell behaviour (Grundy, 1998).

Furthermore, there is a need to develop new antivirals, based on new molecular targets, since the broad use of ganciclovir and foscarnet has led to the emergence of drug-resistant viruses with mutations at the level of the DNA polymerase (Sullivan et al., 1993) as well as UL97, a phosphotransferase responsible for the first step of ganciclovir phosphorylation (Lurain et al., 1994; Littler et al., 1992; Abraham et al., 1999).

Here we describe CMV423, a tetrahydroindolizine derivative as the lead compound of a series for the treatment of HCMV infections. This molecule showed a potent and selective activity against various laboratory and clinical HCMV strains, including HCMV strains known to be resistant to ganciclovir, foscarnet and the acyclic nucleoside phosphonate analogues cidofovir and adefovir. CMV423 has an original mechanism of action, targeted at the IE expression of HCMV replication.

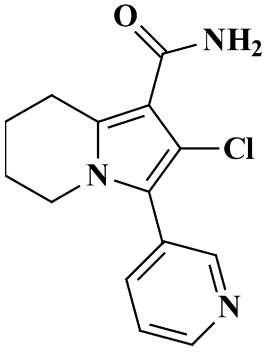
2. Materials and methods

2.1. Compounds

The origin of the compounds was as follows: ganciclovir [GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine, Cymevene®, Cytovene®], Roche; foscarnet (PFA, phosphonoformate sodium salt, Foscavir®) and dextran sulfate (DS), MW 5000, Sigma Chemicals, St. Louis, MO; cidofovir [CDV, HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl]cytosine, Vistide®] and adefovir [PMEA, 9-(2-phosphonylmethoxyethyl)adenine], Gilead Sciences, Foster City, CA; and CMV423 (2-chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine-1-carboxamide) (Fig. 1), Aventis Pharma, Vitry sur Seine, Paris, France. The structure and purity of CMV423 were confirmed by NMR and HPLC. The synthesis of this compound as well as other related compounds will be published elsewhere.

2.2. Viruses

HCMV reference strains Davis and AD-169 were obtained from the American Type Culture Collection (ATCC). The clinical isolates were obtained from various institutions. The different viruses were inoculated on HEL cells and passaged



CMV423

Fig. 1. Structure of CMV423.

a minimum of ten times before preparation of virus stocks, consisting of cell-free virus in the form of supernatant of infected cells. The different strains were isolated from the urine, throat swab, broncho-alveolar lavage or blood of either immunocompromised patients (AIDS or transplants) or patients with congenital infection. The different HCMV-resistant strains used in the present study were either clinical isolates or strains emerging in vitro after serial passages of the HCMV reference strain AD-169 in the presence of increasing concentrations of the test compounds.

For herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), reference strains KOS and Lyons were, respectively, used, while for varicella-zoster virus (VZV), two thymidine kinase-positive (TK^+) (OKA and YS) and two thymidine kinase-negative (TK^-) (07-1 and YS-R) were used. The human herpesvirus type 6 (HHV-6) variant A GS strain growing in T-lymphoblastoid cell line HSB-2 was used for the evaluation of the antiviral activity while the activity against human herpesvirus type 8 (HHV-8) was tested on exponentially growing BCBL-1 cells.

2.3. Plaque reduction assay and cytopathicity (CPE) reduction assay

The viral assays used have been extensively described elsewhere (Snoeck et al., 1988; Neyts et al., 2000). Briefly, confluent human embryonic lung (HEL) fibroblasts (ATCC CCL137), grown in 96-well microtiter plates, were infected with the different HCMV strains at 20 plaque forming units (PFU) (plaque reduction assay) or 100 or 200 PFU [CPE (cytopathic effect) reduction assay] per well. After a 2 h incubation period, the supernatant containing the remaining virus was removed and replaced by 10–12 concentrations of the test compounds in duplicate. After an incubation period of 7 days, the effect of the drugs on either plaque formation or cytopathicity was determined by microscopic examination after ethanol fixation and Giemsa staining. Antiviral activity was expressed as the IC_{50} or the concentration required to reduce plaque formation or virus-induced cytopathicity by 50%. IC_{50} were estimated from graphic plots of the percentage inhibition of virus growth as a function of the concentration of the test compounds. The antiviral activity of CMV423 against HSV-1, HSV-2, VZV, HHV-6 and HHV-8 has been determined as previously reported (Andrei et al., 1992, 1995; Neyts et al., 2001).

2.4. Cytotoxicity assay

Cytotoxicity (CC_{50}) measurements were based on the inhibition of HEL cell growth. HEL cells were seeded at a rate of 3×10^3 cells per well into 96-well microtiter plates and allowed to proliferate for 24 h in MEM containing 20% FCS, 1% L-glutamine and 0.3% sodium bicarbonate. Then, medium containing 2% FCS, 1% L-glutamine and 0.3% sodium bicarbonate, containing different concentrations of the test compounds was added. After 3 days of incubation at 37 °C in 5% CO_2, when the cell monolayer was about 70% confluent, the cell number was determined with a Coulter counter (Coulter Electronics, England). The minimum cytotoxic concentration was calculated as the CC_{50} or concentration required to reduce cell growth by 50% relative to the number of cells in the untreated controls (Snoeck et al., 1988).

2.5. Virus yield assay

After incubation of the virus-infected HEL cells with the different concentrations of the test compounds, under the appropriate conditions, supernatants containing free viruses were harvested and stored at −80 °C. Virus titers were determined by virus plaque assay, using 10-fold dilution steps. Antiviral activity was expressed as IC_{90} or IC_{99}, that are concentrations required to reduce viral yield by 90 or 99%, respectively.

2.6. HCMV antigen expression assay

At different times after infection, depending on the antigen that was analysed, the HCMV-infected and uninfected HEL cells were fixed in acetone at 4 °C. After resuspension in PBS containing 5% gelatin, the cells were incubated at 37 °C in the presence of the desired monoclonal antibody [mAb E13 (Biosoft), directed against IE proteins 1 and 2; and mAb CCH_2 (Dako), which reacts with the

delayed early DNA-binding protein p52]. After a washing step, the cells were incubated for 30 min with a fluorescein-labeled rabbit anti-mouse IgG and finally fixed in a solution of 0.5% formaldehyde in PBS. The cells were then analysed with a fluorescence-activated cell sorter, as described earlier (Andrei et al., 1991).

2.7. Time of addition assay

The test compounds at varying concentrations were added in duplicate at different time points after infection. After 7 days, the antiviral activity was determined. The last time point for which an activity comparable to that of the control (compounds present from time zero) was observed, was interpreted as the step of the virus cycle at which test compounds interacted.

2.8. Time of removal assays

Under the same conditions as described above for the antiviral activity assays, the different concentrations of the test compounds were added 2 h after infection and removed at different time points. The antiviral activity was recorded as described above. The last time point, for which an activity comparable to that of the control was registered, corresponds to the shortest time of incubation needed for the test compounds to reach intracellular concentration of their active metabolite(s) affording antiviral activity.

2.9. Checkerboard combinations

As previously described (Snoeck et al., 1992), the checkerboard combinations method was used to study the combined inhibitory effects of the different drugs on HCMV-induced cytopathicity. The combination effects were analysed by the isobologram method. In this analysis, the IC_{50} was used for the calculation of the fractional inhibitory concentration (FIC). While the FIC index, which corresponds to the FIC values of the compounds combined ($FIC_x + FIC_y$) is equal to 1, the combination is additive; when it is between 1.0 and 0.5, the combination is partially synergistic; when it is <0.5, the combination is synergistic; when it is between 1.0 and 2.0, the combination is partially antagonistic; and when it is >2.0, the combination is antagonistic.

2.10. Ex vivo HCMV models

2.10.1. Retinal pigment epithelial cell model

- Indirect immunofluorescence: to follow HCMV IE antigen expression in retinal pigment epithelial cells (RPE), indirect immunofluorescence was performed using either mAb E13 directed against exon 2 of IE proteins 1 and 2, or mAb F4a and 87-55/02/2 [(Behring, Germany), a generous gift from Dr Walter] which detect p149 and pp150 late proteins, respectively.
- Western blot analysis: RPE cells were infected with HCMV (strain AD-169) at a multiplicity of infection (MOI) of 0.1 or 0.01, in the presence of different concentrations of CMV423. Cells were then washed twice with PBS and refed with medium containing drugs, which were maintained throughout the experiment. At 8 days post infection, the cells were lysed by addition of Lamelli's electrophoresis buffer and the samples were submitted to Western blot analysis.

2.10.2. Bone marrow (BM) stromal cell assay

After informed consent, BM from normal volunteer donors was collected by sternal aspiration and BM-mononuclear cells (BM-MNC) were isolated and confluence of adherent cells was usually achieved after 4–6 weeks of culture. Stromal cells were infected with HCMV (AD-169 or clinical strains) by adding the virus at an MOI of 0.025 and 0.1 for 2 h at 37 °C in MEM medium. Stromal cells were washed twice to remove residual particles. Cells were then treated with different concentrations of the compound, and further incubated at 37 °C in 5% CO_2 atmosphere in MEM medium containing 15% FCS. Expression of different viral antigens was evaluated by flow cytometry, as described above.

3. Results

3.1. Antiviral activity spectrum

CMV423 was evaluated against a broad range of herpesviruses. CMV423 showed relatively low activity (IC_{50} ranging from 0.18 to 18.1 μM) against reference strains of HSV-1 and HSV-2 when tested in a CPE reduction assay. In a plaque assay performed on HEL fibroblasts CMV423 did not interfere with the replication of VZV. Activity against HHV-6 was demonstrated by different methods: CPE assay, detection of HHV-6 antigen p41 by Western blotting and HHV-6 DNA detection by slot blot analysis. In the different assays performed, the inhibition of HHV-6 replication was found to be concentration-dependent: CMV423 displayed an IC_{50} value of 0.1–0.3 μM, as compared with about 4 μM for both foscarnet (PFA) and cidofovir (CDV). When evaluated against HHV-8, CMV423 conferred 50% inhibition of viral DNA synthesis in BCBL-1 cells at a concentration of 13 μM.

3.2. Anti-HCMV activity

When evaluated against HCMV reference strains and HCMV clinical isolates in plaque reduction assays (viral inoculum 20 PFU) in HEL cells, CMV423 showed IC_{50} values ranging from 0.0040 to 0.0070 μM, as compared with 0.16–0.26 μM for CDV, 1.8–3.5 μM for GCV and 12–55 μM for PFA. About 40 different clinical strains isolated from different groups of patients (transplant recipients, AIDS and congenitally infected patients) were examined. The antiviral activity of CMV423 was markedly affected by the size of the viral inoculum, more so than the reference compounds: a significant decrease in antiviral activity was observed at the higher MOIs (Table 1).

Cytotoxicity (CC_{50}), as measured by inhibition of cell growth, showed that CMV423 was slightly more cytotoxic than the reference drugs GCV, PFA and CDV, in HEL cells. However, when the selectivity index (SI), or ratio CC_{50}/IC_{50} was determined, CMV423 proved to be the most potent and selective inhibitor of HCMV replication (Table 1).

When evaluated in the virus yield assay on HEL cells, CMV423 had an IC_{99} that appeared to be dependent on the viral inoculum titer for both reference strains and clinical isolates (Table 2). For the reference compounds tested in parallel, the dependence of the IC_{99} on the inoculum was not as marked as for CMV423.

CMV423 proved active against a panel of HCMV strains that were resistant to a broad range of antiviral drugs. HCMV strains selected in vitro or clinical isolates resistant to either GCV, CDV, PFA still retained their sensitivity to CMV423 (Table 3).

Starting from HCMV reference strains that were passaged in the presence of increasing concentrations of CMV423, no virus-drug resistance for CMV423 could be detected even after 24 months.

When CMV423 was tested in combination with either GCV, PFA or CDV, the three drugs licensed for the treatment of HCMV in the clinic, it displayed a highly synergistic activity, as demonstrated by the isobologram technique (Fig. 2). Whether Davis or AD-169 strains were used, the FIC values for the three combinations were located under the isoline of 0.5. The same three drugs, when combined with CMV423 and tested for their CC_{50} towards growing HEL cells, proved additive rather than synergistic, suggesting that the highly synergistic antiviral activity was not due to increased CC_{50} but, plausibly, the result of interference with two different steps of the viral replicative cycle (data not shown).

3.3. Mechanism of anti-HCMV action

3.3.1. Time of addition

The ability of CMV423 to inhibit the viral replication was lost when the compound was added between 24 and 48 h post infection (Fig. 3), while for GCV and CDV, two compounds that are known to interact with the viral DNA polymerase reaction, anti-HCMV activity was lost essentially when the compounds were added at 72 or 96 h post infection. Thus, GCV and CDV lost their anti-HCMV activity if added at later times than CMV423, suggesting that CMV423

Table 1
Anti-HCMV activity of CMV423

Compound	Viral inoculum[a] (PFU/well)	IC_{50} (μM)[b]			CC_{50} (μM)[c]	SI[d]		
		AD-169 strain	Davis strain	Clinical isolates		AD-169 strain	Davis strain	Clinical isolates
CMV423	20	0.007±0.004	0.004±0.007	0.005±0.004	101	14429	25250	20200
	100	0.047±0.058	0.033±0.043	0.013±0.014		2149	3061	7769
	200	1.49±1.47	0.40±0.33	0.044±0.032		68	253	2295
CDV	20	0.16±0.08	0.26±0.22	0.25±0.16	351	2194	1350	1404
	100	0.41±0.18	0.81±0.51	0.49±0.47		856	433	716
	200	0.88±0.55	2.15±1.71	0.74±0.45		399	163	474
GCV	20	2.5±2.8	3.51±2.48	1.8±1.0	461	184	131	256
	100	5.0±3.1	8.1±4.5	3.2±1.9		92	57	144
	200	9.7±2.5	6.4±2.1	7.3±6.2		48	72	63
PFA	20	12±2	13±8	55±21	504	42	39	9
	100	73±25	62±8	78±30		7	8	6
	200	100±9	93±38	128±21		5	5	4

[a] A viral inoculum of 20, 100 and 200 PFU, represents a MOI of 0.001, 0.005 and 0.01, respectively.
[b] Concentrations required to reduce viral cytopathic effect by 50%. Results are means for at least three independent experiments.
[c] Concentration required to reduce cell growth by 50%.
[d] SI, ratio CC_{50} to IC_{50}.

interacts with a step of the viral replication preceding DNA polymerisation.

3.3.2. Time of removal

For CMV423, incubation with infected HEL cells for a period as short as 6 h sufficed to elicit antiviral activity against HCMV, while for GCV under the same conditions, no antiviral activity was detected with exposure times less than 72 h (data not shown).

3.4. Effect on HCMV antigen expression

CMV423 inhibited the expression of viral IE antigen as measured on day 1 (Fig. 4). Inhibition was not sustained on day 2s and 3, while from day 4 or 5 there was again clear inhibition of HCMV

Table 2
Effect of CMV423 on virus yield

Strain	MOI	CMV423		CDV		GCV	
		IC_{90}[a]	IC_{99}[a]	IC_{90}	IC_{99}	IC_{90}	IC_{99}
Davis	0.0009	0.08	0.17	1.4	4.0	<2	6.5
	0.0018	0.09	0.44	1.4	1.6	<2	5.5
CI-4803[b]	0.0009	0.14	0.35	<0.64	1.6	<2	2.0
	0.0018	0.11	13.5	<0.64	1.5	<2	5.6
CI-15[b]	0.00075	0.08	0.8	<0.64	< 0.06	<2	<2
	0.0015	0.17	18	0.64	1.4	<2	2

Virus yield was determined at 8 days post infection. The results are from a single experiment, representative of two independent experiments.
[a] Values are expressed in μM. IC_{90} and IC_{99} values represent the concentration of the compound required to reduce viral yield by 90 or 99%, respectively.
[b] Clinical isolates.

Table 3
Susceptibility profile of HCMV-resistant strains

	Inoculum (PFU)	IC_{50} (μM)			
		CDV	GCV	PFA	CMV 423
Clinical strains[a]					
GCV^r					
LY9990	20	0.57	23.6	42	0.0003
U9070	20	0.49	25	85	0.0018
LY9725	20	0.73	9.85	80.6	0.0051
GCV^r/CDV^r					
DER 521	20	3.23	55	77	0.0181
DER 648	20	6.34	388	–	0.0012
Means for wt clinical strains	20	0.25	1.8	55	0.005
In vitro selected strains					
GCV^R	100	3.90	26.3	60.6	0.16
CDV^R	100	4.44	23.2	59.2	0.13
PFA^R	100	0.28	0.22	340.7	0.12
ACV^R	100	0.63	3.94	116.6	0.18
AD169 (wt)	100	0.28	3.82	61.6	0.07

IC_{50}: concentration required to reduce viral plaque formation (20 FPU) or virus-induced cytopathic effect (100 PFU) by 50%. The results are means for at least two independent experiments. The isolation and characterization of these strains have been previously reported (Snoeck et al., 1996).

[a] Clinical strains recovered from two different immunocompromised patients.

IE antigen expression, days 4–5 corresponding to the beginning of the second replicative cycle (data not shown). DS was a strong inhibitor of IE antigen expression, whereas GCV and CDV did not interfere with HCMV IE antigen expression. All the compounds inhibited E antigen expression (at days 3 and 5) (data not shown).

3.5. Activity in human retinal pigment epithelial (RPE) cells

The activity of CMV423 on HCMV IE, early and late protein expression was measured by immunofluorescence and Western blot analysis. The effect was measured based on the evaluation of IE, early or late protein synthesis. For three different donors tested, an IC_{50} of 0.1 μM was obtained for CMV423, when expression of IE antigen was measured.

The expression of IE and late proteins was significantly blocked by CMV423 at a concentration of 0.1 or 1 μM when cells were infected at a MOI of 0.01 (Fig. 5). Expression of both IE1 and IE2 proteins was inhibited.

3.6. Activity in human bone marrow (BM) cells

The effect of CMV423 on HCMV-infected BM stromal cells was evaluated by measuring the expression of different viral antigens by flow cytometry. When expression of IE antigens was analysed on day 5, using an MOI of 0.025, the mean IC_{50} for six independent experiments was 0.185 μM, with IC_{50} values ranging from 0.0036 to 2.82 μM. Similar results were obtained with two clinical different HCMV isolates.

4. Discussion

CMV423 has emerged from screening tests as the lead compound among a series of tetrahydroindolizine derivatives. We have demonstrated that CMV423 in the virus plaque and CPE reduction assays on fibroblasts is a very potent

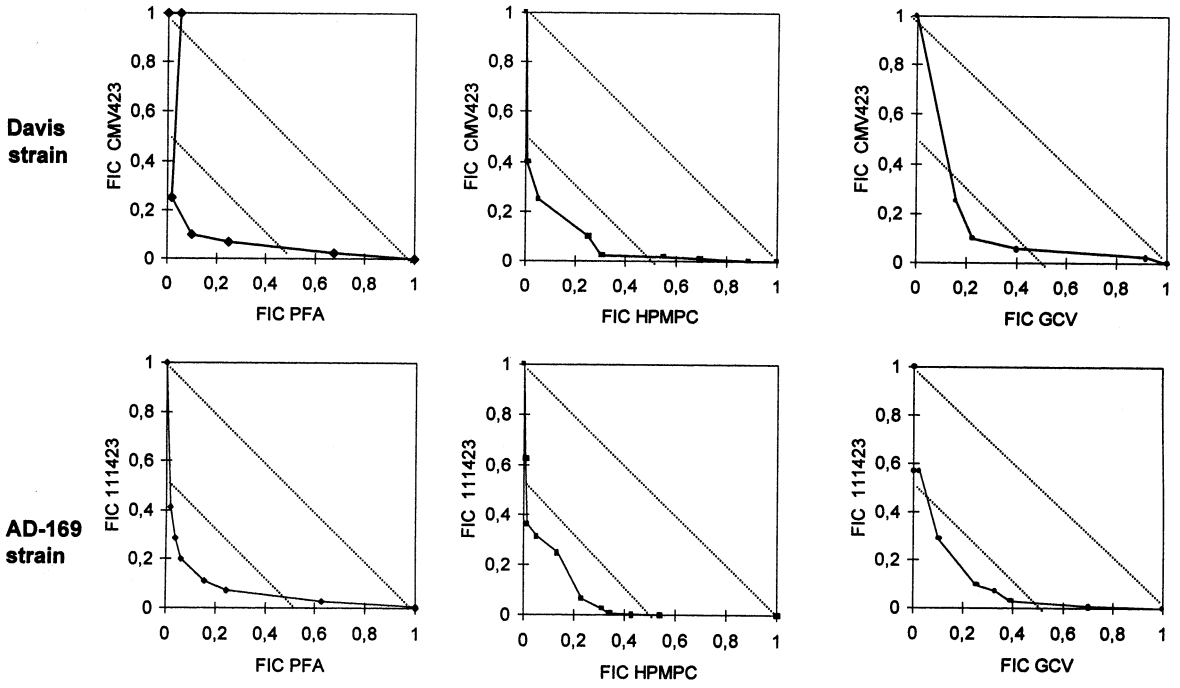


Fig. 2. Isobologram representation of the inhibitory effects on Davis and AD-169 strains-induced CPE in HEL cells of combinations of CMV423 with PFA, GCV or CDV. FIC, fractional inhibitory concentration. FIC_x, IC_{50} of compound 'X' in combination/IC_{50} of compound alone. FIC_y, IC_{50} of compound 'Y' in combination/IC_{50} of compound alone. FIC index (FIC of the compounds combined): FIC_x+FIC_y. The broken lines represent the unit lines for FIC equal to 1 and 0.5. The results are from a single experiment, representative of two independent experiments.

inhibitor of both laboratory HCMV strains and clinical isolates. Toxicity, as determined with growing fibroblasts is relatively low, so that CMV423, attains a higher in vitro SI than the established anti-HCMV drugs (GCV, CDV or PFA) (Table 1). However, the in vitro experiments seem to indicate that the anti-HCMV activity of CMV423 is more dependent on the viral inoculum used than any of the other anti-HCMV drugs. This effect was less pronounced for the clinical isolates.

CMV423 has significant activity against HHV-6 but not other human herpesviruses. Virus yield reduction assays showed that complete inhibition of virus production (IC_{90} and IC_{99}) could not be reached or only at very high concentrations. Time of addition experiments, confirmed by analysis of HCMV IE antigen expression by flow cytometry, clearly point to the α phase of the replicative cycle as the target site for CMV423. Preliminary investigations using a reporter system under the control of the HCMV IE promotor showed that CMV 423 at least partially inhibits the IE HCMV promoter in a dose-dependent manner, while a structurally related drug, inactive in the plaque reduction assay, was unable to do so (data not shown).

CMV 423 demonstrated potent activity against all the drug-resistant HCMV strains (GCV^r, CDV^r, PFA^r) tested. Combination experiments of CMV423 with either GCV, CDV or PFA demonstrated strong synergistic antiviral activity without significantly increased toxicity (Fig. 2).

Finally, the anti-HCMV activity of CMV423 has been confirmed in two clinically relevant ex vivo models, bone marrow microenvironment and retinal epithelial cells (Lagneaux et al., 1996a,b; Miceli et al., 1989).

It is of interest that no virus-drug resistance for CMV423 could be detected even after 24 months of passage in cell culture. This may not be

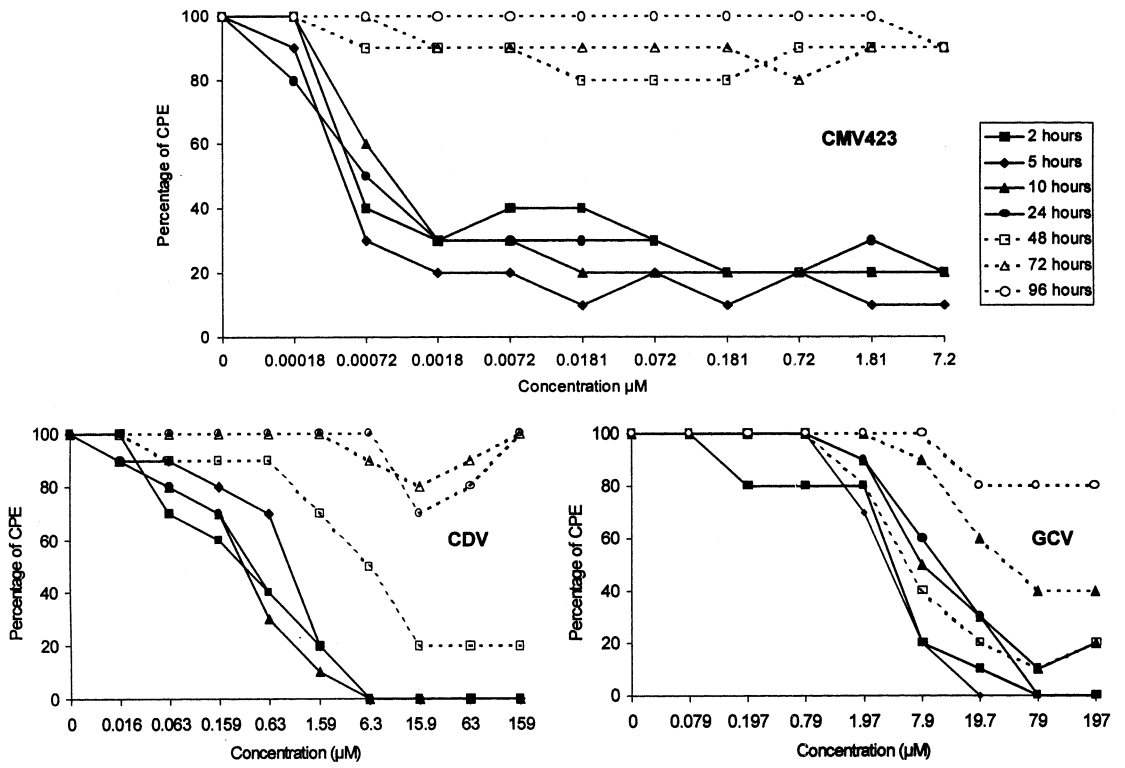


Fig. 3. Time of addition experiments performed using CPE reduction assays and comparing CMV423 to reference drug CDV and GCV. The results are from a single experiment, representative of five independent experiments.

surprising in light of earlier studies with compounds that reportedly inhibit HCMV at an early step in replication. (Jacobson et al., 1999; Boulware et al., 2001).

While the data presented here are consistent with an effect of CMV423 on HCMV IE protein expression and function, more work needs to be done to understand this phenomenon. The inhibition of IE could prove to be beneficial at more than one level. There is now compelling evidence that the synthesis of IE proteins of HCMV, which begins soon after virus penetration into target cells, may by itself accounts for pathophysiological phenomena (Lagneaux et al., 1996b; Zhou et al., 1999). IE proteins 1 and 2 (IE1 and IE2) accumulate at early times after infection. Both IE1 and IE2 are driven by the same major IE promoter (MIEP)-regulatory region and are potent transactivators, not only of early viral promoters but also of cellular promoters (Tanaka et al., 1999). This may, in turn, switch on the synthesis of a series of proteins, which, from the early phase of infection, may lead to the induction of host reactions. This has been demonstrated in an in vitro model of HCMV myelosuppression, based on culturing primary stromal cells, that mimicks the in vivo situation (Lagneaux et al., 1996a): the generation of myeloid progenitors discontinues following HCMV infection of stromal cells, and the supernatant from HCMV-infected stromal cells does not allow growth and differentiation of bone marrow progenitors.

The effect of HCMV on stromal cells results in deprivation of essential growth factors, such as G-CSF and GM-CSF, and must be consequential to viral replication, since anti-HCMV drugs like GCV and CDV reversed the effect. In contrast, the release of inhibitory factors, such as TNF-α, TGF-β and MIP-1α, was observed as early as 24 h after infection depending on the presence of an

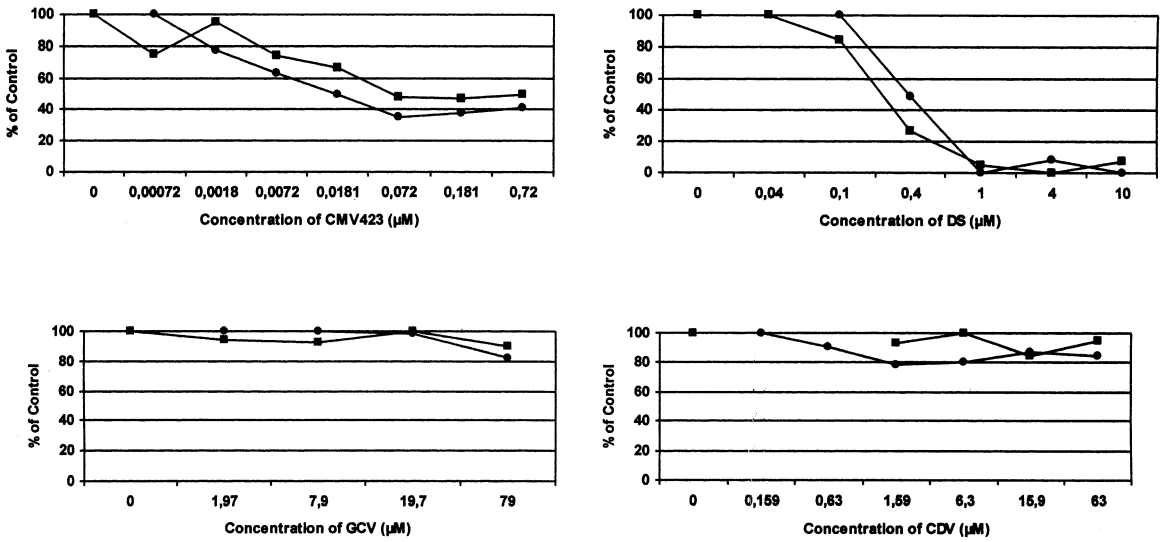


Fig. 4. Inhibition of IE antigen expression on day 1 measured by flow cytometry. The percentage is relative to the number of cells expressing IE antigens in the untreated controls. DS, dextran sulfate; GCV, ganciclovir; CDV, cidofovir. The two lines represent the results of two independent experiments. The percentage of cells expressing IE antigens in the untreated controls was 6.84 (-●-●-) and 16.34 (-■-■-).

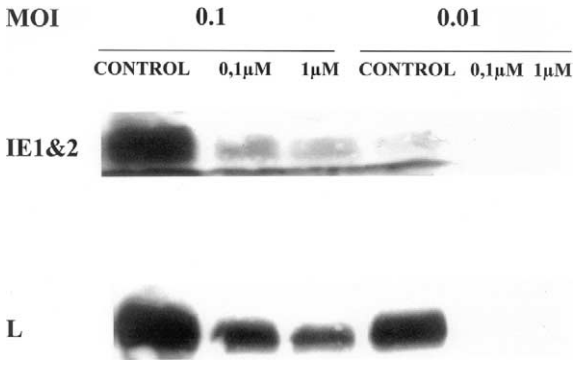


Fig. 5. Western Blotting demonstrating the inhibition of both IE 1 and 2 and late protein expression after treatment with, respectively, 0.1 and 1 µM of CMV423. The experiment was performed at two different MOIs, 0.1 and 0.01, showing the influence of the viral inoculum on the antiviral activity.

active HCMV genome and was not influenced by drugs inhibiting the DNA polymerase. HCMV is also able to induce the concomitant release of inflammatory cytokines (Il-6 and LIF), as early as 2 h post-infection with UV-inactivated HCMV, suggesting that this release occurs independently from productive infection (Lagneaux et al., 1996a,b).

Il-8 production is also increased when THP-1 cells, a monocytic cell line, are infected by HCMV (Murayama et al., 1997). Similar observations were made in 75 allogeneic bone marow transplant patients where different inflammatory cytokines were measured (Humar et al., 1999). In addition, the binding of HCMV to human monocytes has been recently shown to initiate a signal transduction pathway that leads to activation of the monocytes (Yurochko and Huang, 1999). Not only IE production but also late protein production could interfere with host mechanisms of defence as demonstrated recently by Penfold et al. (1999). Thus, molecules acting early in the viral replicative cycle, i.e. at the time of IE antigen production, may be effective in blocking the virus-induced pathophysiological phenomena and could be of particular importance in patients such as BMT recipients.

Tetrahydroindolizine derivatives, as exemplified by CMV 423, may fulfill the requirements needed for a new generation of anti-HCMV agents, by acting at an early phase of viral replication. Their exact mechanism of action is under investigation and may lead to the identification of new mole-

cular targets that are important for viral replication and virus-induced disease.

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