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Potent, selective and cell-mediated inhibition of human herpesvirus 6 at an early stage of viral replication by the non-nucleoside compound CMV423

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Abstract

CMV423 (2-chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine-1-carboxamide) is a new antiviral agent with potent and selective *in vitro* activity against the β-herpesvirus human cytomegalovirus (HCMV), but not against α- or γ-herpesviruses. Here we report that its activity also extends to human herpesvirus 6 (HHV-6) and 7 (HHV-7). When compared *in vitro* to ganciclovir and foscarnet (the standard drugs recommended for treatment of HHV-6 infections), CMV423 showed a superior selectivity, due to its high activity (antiviral IC_{50}: 53 nM) and low cytotoxicity (CC_{50}: 144 μM), both in continuous cell lines and in CBLCs infected with HHV-6. From mechanistic experiments at the level of viral mRNA and protein expression, we learned that CMV423 targets an event following viral entry but preceding viral DNA replication. Its antiviral action was dependent on the cell line used, implying involvement of a cellular component. When compared to a panel of known protein kinase inhibitors, CMV423 was found to share anti-HHV-6 characteristics with herbimycin A, which affects tyrosine kinase activity through heat shock protein 90 (Hsp90) inhibition. We demonstrated that high concentrations of CMV423 have an inhibitory effect on the total cellular protein tyrosine kinase activity, and that CMV423 and herbimycin A, when combined, act synergistically against HHV-6. The activities of cyclin-dependent kinases, protein kinases A and C, and the HHV-6-encoded pU69 kinase were not affected. We, therefore, conclude that CMV423 exerts its activity against HHV-6 through inhibition of a cellular process that is critical at early stages of viral replication and that may affect protein tyrosine kinase activity.

Keywords: Human herpesvirus 6; β-Herpesvirus; Antiviral; Non-nucleoside; Protein tyrosine kinase; Herbimycin A

1. Introduction

HHV-6 is a ubiquitous lympho- and neurotropic beta-herpesvirus, with a seropositivity of ∼80% in adults [1]. It is closely related to human herpesvirus 5 (HCMV) and human herpesvirus 7 (HHV-7). HHV-6 clinical isolates fall into two variants, A and B, which differ in antigenic properties and in DNA sequence, with an overall identity of 88% at the nucleotide level [2]. Primary infection with HHV-6B occurs early in life and is the main cause of exanthema subitum, a childhood disease characterized by high fever and a mild skin rash, occasionally complicated by seizures and encephalitis [3]. During episodes of immune suppression, as in transplant recipients or HIV-infected individuals, reactivation of latent HHV-6 may occur and results in a wide range of clinical symptoms such as febrile illness, encephalitis, pneumonitis, hepatitis, graft failure and bone marrow suppression [4]. HHV-6 has also been suggested as a cofactor in AIDS progression [5]

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Abbreviations: ACV, acyclovir; CBLCs, cord blood lymphocytes; CDV, cidofovir; CHX, cycloheximide; CPE, cytopathic effect; GCV, ganciclovir; HCMV, human cytomegalovirus; HHV-6, human herpesvirus 6; p.i., post infection; PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase; RT-PCR, reverse transcriptase-polymerase chain reaction.

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and has been associated with certain neoplastic disorders [6], and with the pathogenesis of multiple sclerosis [7].

The drugs indicated for the treatment of HHV-6 infections are the same as those used in HCMV therapy or prophylaxis and consist of the nucleoside analogs ganciclovir and acyclovir, the nucleotide analogue cidofovir and the pyrophosphate analogue foscarnet, all targeting the catalytic site of the viral DNA polymerase [8]. Whereas foscarnet shows high and consistent activity against HHV-6, its use in patients is limited by its nephrotoxicity after long-term use. Ganciclovir has good *in vitro* activity against HHV-6 based on the selective phosphorylation to its monophosphate metabolite by the HHV-6-encoded pU69 kinase (although the phosphorylation efficiency is 10-fold lower when compared to the HCMV homologous kinase pUL97) [9]. Only a few small-scale studies have been published on the clinical efficacy of ganciclovir against HHV-6 [4,10], but as with foscarnet, prolonged use of ganciclovir is associated with considerable adverse side effects (particularly neutropenia). Acyclovir is considered safer but has only moderate activity against HHV-6 [10]. In addition, long-term use of these anti-herpes drugs, as in immunocompromised patients, may lead to the emergence of drug-resistant virus mutants [11]. Therefore, new antiherpetic compounds with favorable safety profiles and directed against targets other than the viral DNA polymerase catalytic site should be developed.

New such compounds, directed against herpesvirus-encoded proteins have recently been identified and include non-nucleoside inhibitors of the viral DNA polymerase, terminase, protease and primase [12], or the HCMV pUL97 protein kinase. Besides, antiherpetic compounds may be targeted at cellular proteins with a crucial function in virus replication, such as cyclin-dependent kinases [13], mitogen-activated protein kinases (MAPKs) [14,15] and PTKs [16,17]. Except for herbimycin A, these new approaches have not yet been explored for their potential application in HHV-6 therapy.

Here we report on the anti-HHV-6 activity of the novel non-nucleoside compound CMV423, which was recently found to exhibit excellent activity against the β-herpesvirus HCMV but not against α- or γ-herpesviruses [18]. Evaluation of the effects of this compound on HHV-6 mRNA and protein expression has led us to the conclusion that CMV423 targets an early event in the HHV-6 replication cycle. Moreover, our data suggest that CMV423 exerts its anti-HHV-6 effect through an inhibition of a cellular process involving PTK activity.

2. Materials and methods

2.1. Compounds

2-Chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine-1-carboxamide (CMV423) (Fig. 1) was synthesized at Rhône-Poulenc Rorer (now Aventis Pharma). The compound was found to be soluble in RPMI-1640 medium containing 10% fetal calf serum (FCS) at concentrations of at least 1 mM. The following reference compounds were used: foscarnet (phosphonoformic acid, PFA, Foscavir®, from AstraZeneca Pharmaceuticals), ACV (Zovirax®, from GlaxoSmithKline), GCV (Cymevene®, from F. Hoffmann-La Roche), CDV (Vistide®) and adefovir (PMEA) (both from Gilead Sciences), cycloheximide (CHX) and Dactinomycin (both from Sigma–Aldrich), herbimycin A, genistein, leflunomide, PP2, Gö6976, staurosporine, H-89, roscovitine and SB202190 (all from Calbiochem) and U0126 (Promega).

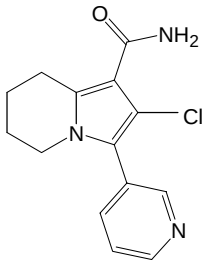

Fig. 1. Structure of CMV423.

2.2. Cells and virus

The human T-lymphoblast cell lines HSB-2 (ATCC, Manassas, VA) and Molt-3 (Advanced Biotechnologies (ABI)) were used to propagate strain GS of HHV-6A (kindly provided by Dr. R. Gallo when at NIH) and strain Z29 of HHV-6B (ABI), respectively. Human T-lymphoblast SupT-1 cells (ATCC) were used for the propagation of HHV-7 and for their ability to support the growth of both HHV-6 strains. Cells were grown in RPMI-1640 medium, supplemented with 10% FCS, 2 mM L-glutamine and 0.1% sodium bicarbonate (all from Invitrogen). Human CBLCs were isolated by density gradient centrifugation and cultured in growth medium, supplemented with 10 μg/mL phytohemagglutinin-M (PHA) and 20 U/mL interleukin-2 (IL-2, both from Roche Molecular Biochemicals). After 2 days of stimulation, cells were infected with HHV-6 and further cultured in medium containing 0.2 mg/mL PHA and 2 U/mL IL-2. Cell cultures were incubated at 37° in a CO_2-controlled incubator. Cytosolic thymidine kinase-deficient human osteosarcoma 143B cells (ATCC) were grown in Eagle's minimal essential medium, containing 10% FCS, 2 mM L-glutamine, 0.75% sodium bicarbonate and 1 mM sodium pyruvate (all from Invitrogen). HHV-6 stocks were prepared by 10-fold concentrating HHV-6-infected cells showing extensive CPE in RPMI-1640 medium containing 20% FCS. Cell-free HHV-6 stocks and recombinant vaccinia virus stocks were prepared as described before [9]. All virus stocks were kept at −80°. Titers were determined according to the method of Reed

and Muench [19] and expressed as 50% cell culture infectious dose ($CCID_{50}$).

2.3. Cytotoxicity assays and antiviral studies

Toxicity of the compounds was determined by cell counting after 10–12 days (with intermediate subcultivation every 3–4 days) and expressed as CC_{50} (the compound concentration that causes a 50% inhibition of cell proliferation). Antiviral assays were performed as follows: cultures were infected with HHV-6 (100 $CCID_{50}$ per 10^6 cells); after 90 min adsorption at 37°, unadsorbed virus was removed by centrifugation and cells were resuspended in RPMI-1640 medium supplemented with 10% FCS, gentamycin (20 μg/mL) and amphotericin B (2.5 μg/mL) to a density of 0.8×10^6 cells/mL. Then, 4–5-fold serial dilutions of the compounds were added. In RT-PCR and immunofluorescence experiments, compounds were added 30 min prior to infection, and kept with the cells during virus adsorption and subsequent incubation. Cultures were subcultivated every 3–4 days by 2-fold dilution with medium containing fresh compound. The CPE was scored 10–12 days p.i., when virus growth reached its maximum. Antiviral activity was expressed as IC_{50} (the compound concentration that produced 50% inhibition of virus replication).

2.4. Slot-blot detection of viral DNA

Viral DNA was quantified using a slot-blot DNA hybridization technique as described earlier [20]. Briefly, total DNA extracted from infected cultures was blotted on a nylon membrane (Amersham Biosciences) and hybridized with a digoxigenin-labeled DNA probe from the U67 region of HHV-6. Viral DNA band intensities were determined by densitometric scanning based upon chemiluminescence detection.

2.5. RT-PCR analysis

Total RNA was extracted from the cells at 16 and 48 hr after infection using the RNeasy column system (Qiagen). DNA contamination was eliminated by digestion with 1 U/μL DNase (Roche Molecular Biochemicals). First strand cDNA synthesis from 2 μg of total RNA was carried out using 0.5 μg of oligo-$dT_{(15)}$ primer (Promega), 0.25 mM of each dNTP, 125 U human placenta ribonuclease inhibitor (HPRI, Amersham Biosciences) and 3 U RAV-2 reverse transcriptase (Amersham Biosciences) in a final reaction volume of 50 μL. PCR was done in the presence of 1.5 mM $MgCl_2$ (2.5 mM for U41 and U100), 0.1 mM dNTPs (Invitrogen), 0.5 μM of each primer (Invitrogen) and 1 U/50 μL Taq polymerase (SphaeroQ). PCR was performed as follows: 2 min initial denaturation at 94°, 26–40 thermal cycles of 30 s at 94°, 45 s annealing at 55° (U41, U94), 60° (U16, U69, U100, U86), or 63° (U12) and 1 min elongation step at 72°, followed by a final elongation at 72° for 5 min. All primers were derived from Genbank sequences, except for U41 and U94 [21]. The absence of contaminating DNA was certified for every set of samples by PCR using HHV-6 U69 primers. Because of the large difference in abundance between human β-actin mRNA and HHV-6 mRNAs, both PCRs were run separately but repeated to ensure reproducibility. PFA was used to discern late HHV-6 transcripts because of its strong and selective inhibitory effects on HHV-6 DNA replication. Dactinomycin and CHX, inhibitors of RNA and protein synthesis, respectively, allowed discrimination between immediate-early and early HHV-6 transcripts.

2.6. Immunofluorescence assays

Cells were spotted on glass slides, allowed to dry, fixed in 1% formaldehyde, and permeabilized for 5 min using 0.1% Tween-20. Primary antibodies used were a rabbit polyclonal raised against the HHV-6 immediate-early protein pU90 (kind gift from Dr. Y. Inoue, Centers for Disease Control, Atlanta, GA), and mouse monoclonals against the early HHV-6 protein p38/41 (Chemicon MAB8532) and the late glycoproteins gp60/110 (Chemicon MAB8537) and gp110 (Chemicon MAB8530). Cells were subsequently stained with donkey anti-rabbit or rabbit anti-mouse FITC-conjugated secondary antibody (DAKO) and finally mounted in glycerol containing 1 mg/mL *p*-phenylenediamine (Sigma–Aldrich). The cell preparations were examined under a confocal Zeiss LSM410 microscope using Ar excitation at 488 nm.

2.7. Protein tyrosine kinase assay

HSB-2 cells were prepared at a density of 1×10^6 cells/mL, and incubated with herbimycin A (1 μM) or CMV423 (1, 10 and 100 μM) for 24 hr. Cells were then washed in PBS, resuspended in lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, 1 mM Na_3VO_4, 5 mM β-mercaptoethanol, 25 mM β-glycerophosphate) and further kept on ice. After sonication, cell lysis was confirmed by trypan blue staining. The cell lysates were then centrifuged at 23,000 *g* for 15 min, and the protein concentration was adjusted to 1 mg/mL for every sample. In a separate set of experiments, cells were not pretreated and the compounds were added only during the enzyme activity assay. Herbimycin A and CMV423 were then added to the reaction buffer in the same concentrations as mentioned above. PTK activity in the samples was measured using an ELISA assay (Oncogene Research Products), according to the Manufacturer's protocol.

2.8. Serine/threonine kinase assays

HSB-2 cells were plated at a density of 0.5×10^6 cells/mL and left untreated or incubated with CMV423

(0.01–100 μM) or staurosporine (20 nM) for 24–72 hr. Cell lysates were prepared by sonication in a buffer containing 50 mM Tris–HCl, 50 mM β-mercaptoethanol, 10 mM EGTA, 5 mM EDTA, 1 mM PMSF and 10 mM benzamidine, pH 7.5, and subsequently centrifuged at 23,000 *g* for 60 min. The protein concentration for all extracts was adjusted to 400 μg/mL. PKA and PKC phosphorylation of an immobilized pseudosubstrate was measured using the same colorimetric protein kinase assay kit (Oncogene Research Products), but under different assay conditions and according to the Manufacturer's recommendations.

2.9. HHV-6 pU69 kinase assay

HPLC analysis of GCV metabolism using a recombinant vaccinia virus, expressing the pU69 kinase of HHV-6A(GS), was performed as described earlier [9]. Briefly, 143B TK$^-$ cells were infected with the recombinant vaccinia virus at 1 plaque forming unit per cell. Cells were then kept in the presence of 50 μM [8-^{3}H]GCV (Moravek Biochemicals) (specific activity: 20 Ci/mol) and 0.1, 1 or 10 μM CMV423 for 24 hr. Cell extracts, made in 66% methanol in water, were centrifuged for 5 min at 23,000 *g* and analyzed on a Partisphere SAX anion exchange column (Whatman). The radioactivity of the fractions containing the phosphorylated metabolites of [8-^{3}H]GCV (mono-, di- and triphosphates) was determined by liquid scintillation counting.

2.10. Flow cytometric cell cycle analysis

Exponentially growing Sup-T1 cells (plated at a density of 0.25×10^6 cells/mL) were exposed to CMV423 or adefovir. After 48, 72 and 96 hr, propidium iodide staining of cellular DNA was performed using the CycleTEST^{TM} PLUS DNA Reagent Kit (Becton Dickinson). The DNA content of the cells was assessed by flow cytometry on a FACScalibur flow cytometer equipped with CellQuest software (Becton Dickinson).

3. Results

3.1. Antiviral activity of CMV423 against HHV-6 and HHV-7

The antiviral activity of CMV423 against the A and B variants of HHV-6 was determined both in human T-lymphoblast cell lines and in CBLCs. The antiherpetic drugs foscarnet (phosphonoformic acid, PFA), ACV, GCV and CDV were used as reference compounds. The inhibitory effect on virus growth was determined by microscopic estimation of the CPE (data not shown) and by quantitation of viral DNA replication at 10–12 days p.i. (Table 1). The 50% antivirally effective concentrations (IC_{50}) obtained by both techniques were in the same range. CMV423 was found to be a potent inhibitor of HHV-6A(GS) replication in HSB-2 cells (IC_{50}: 0.030 μM as obtained by CPE assay, and 0.053 μM as obtained by viral DNA quantitation). Within a concentration range of 0.001–0.40 μM, the antiviral effect of CMV423 was concentration-dependent, with 95% inhibition of viral DNA replication at a concentration of 0.37 μM. The IC_{50} values for the reference compounds are in accordance with data reported elsewhere [8,22]. The IC_{50} values for CMV423 and foscarnet, obtained in CBLCs, were in the same range (Table 2), whereas ACV, GCV and CDV were at least 5-fold more active in these primary cells.

CMV423 produced cytotoxicity (expressed as CC_{50} or the compound concentration effecting a 50% inhibition of cell proliferation) in HSB-2 cells at a concentration of 144 μM, resulting in a remarkably high selectivity index of 2717, as compared to 78 for PFA, 3.7 for ACV, <1.6 for GCV and 2.9 for CDV (Table 1). Whereas foscarnet, ACV, GCV and CDV were able to completely block HHV-6A replication at a concentration 1.5- to 4-fold their IC_{50} value, CMV423 reached 95% inhibition at 0.37 μM, however, at concentrations ≥5 μM (about 100-fold the IC_{50}), the antiviral effect of CMV423 actually decreased. Moreover, in contrast to the reference drugs, the activity of CMV423 was highly dependent on the viral inoculum: at high

Table 1
Antiviral activity and cytotoxicity of CMV423 and a panel of nucleoside analogues in HHV-6-infected continuous cell lines[a]

	HHV-6A(GS) in HSB-2 cells[b]			HHV-6B(Z29) in Molt-3 cells[b]		
	IC_{50} (μM)	CC_{50} (μM)	SI[c]	IC_{50} (μM)	CC_{50} (μM)	SI[c]
CMV423	0.053 ± 0.02	144 ± 15	2717	>100	91.4 ± 45	–
PFA	16.0 ± 10	1250 ± 57	78	25.4 ± 7.8	1016 ± 274	40
ACV	180 ± 37	668 ± 77	3.7	185 ± 63	258 ± 47	1.4
GCV	31.9 ± 6.5	<50	<1.6	68.6 ± 30	<50	<0.7
CDV	9.01 ± 1.5	25.9 ± 4.0	2.9	9.76 ± 3.9	55.6 ± 42	5.7

[a] Antiviral activity (based on viral DNA quantitation) and cytotoxicity (based on cell counting) were determined at 12 days p.i. Values are means ± SD for at least three experiments.
[b] HSB-2, Molt-3: human T-lymphoblastoma cell lines.
[c] SI, selectivity index (ratio of CC_{50} to IC_{50}).

Table 2
Antiviral activity and cytotoxicity of CMV423 and a panel of nucleoside analogues in HHV-6A(GS)-infected CBLCs[a]

	Antiviral activity IC_{50} (μM)[b]	Cytotoxicity in CBLCs	
		CC_{50} (μM)[c]	SI[d]
CMV423	0.017 ± 0.006	>30	>1765
PFA	9.5 ± 4.8	647 ± 128	68
ACV	10 ± 5.3	277 ± 56	28
GCV	5.8 ± 3.3	100 ± 6.2	17
CDV	0.56 ± 0.34	102 ± 13	182

[a] Antiviral activity and cytotoxicity were determined at 12 days p.i. Values are means ± SD for at least three experiments.
[b] IC_{50} as determined by DNA hybridization.
[c] CC_{50} as determined by automatic cell counting.
[d] SI, selectivity index (ratio of CC_{50} to IC_{50}).

multiplicities of infection (MOI), the inhibitory activity against HHV-6A was significantly lower (Fig. 2). Surprisingly, CMV423 was devoid of any antiviral effect in HHV-6B-infected Molt-3 cells at concentrations up to 100 μM, whereas HHV-6B clearly showed sensitivity to CMV423 when propagated in Sup-T1 cells (IC_{50}: 0.058 μM, SI: 4070). These observations suggest that, unlike the reference compounds PFA, ACV, GCV and CDV, CMV423 may exert its anti-HHV-6 effect by cell-dependent processes.

The antiviral activity of CMV423 against the HKR strain of HHV-7 was evaluated in Sup-T1 cells using CD4 receptor downregulation as a parameter for HHV-7 replication [23]. The IC_{50} for HHV-7 was 14.3 nM, and the CC_{50} in Sup-T1 cells was 236 μM, thus resulting in a selectivity index of 16,500.

3.2. Effect on HHV-6 gene transcription

In order to assess at which stage CMV423 inhibits the HHV-6 replication cycle, its effect on the transcription of selected HHV-6 immediate-early, early and late genes was studied, using semi-quantitative RT-PCR analysis. Transcripts were selected as representatives of their kinetic class and irrespective of any specific role in HHV-6 replication. HSB-2 cells were infected with cell-free inocula of HHV-6A and kept in the presence of 10 μM CMV423. The RNA synthesis inhibitor dactinomycin (80 μM), the protein synthesis inhibitor CHX (200 μM) and the viral DNA polymerase inhibitor PFA (1 mM), were included to temporally categorize each transcript. RNA extracts were made at 16 and 48 hr post infection (within the first viral replication cycle), and RT-PCR amplification of HHV-6 transcripts and human β-actin mRNA was performed on the same cDNA sample. Since no immediate-early (IE), early (E) or late (L) mRNAs were present in the dactinomycin-treated samples, we concluded that the mRNA in the samples was newly formed. Based on their decreased levels following incubation with dactinomycin, CHX or foscarnet, the selected HHV-6 transcripts were classified as IE (U86, U89 and U94), E (U16, U69, U41 and U12), or L (U100). CMV423 clearly had no inhibitory effect on the transcription of the IE genes U86 (Fig. 3), U89 and U94 (data not shown). In contrast, inhibition of two early transcripts (U69 and U41) was observed, indicating that CMV423 exerts its action in an early stage of the viral replication cycle and prior to DNA synthesis, since no effect of PFA was seen on the transcription of these genes. U100 appeared as the only late gene in our panel as its expression was strongly decreased upon PFA treatment; its expression level was clearly reduced following incubation with CMV423 (Fig. 3). No inhibition of HHV-6B gene expression in Molt-3 cells could be observed at either stage (data not shown).

3.3. Effect on HHV-6 protein expression

The effect of CMV423 on HHV-6A protein expression was evaluated using an indirect immunofluorescence assay for IE, E and L HHV-6 proteins. HSB-2 cells were pretreated with CMV423 (5 μM), PFA (1 mM) or CHX

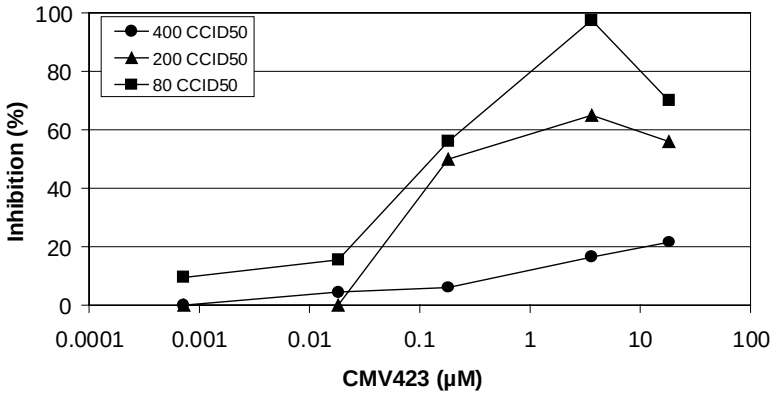

Fig. 2. Antiviral effect of CMV423 against HHV-6 at different multiplicities of infection. HSB-2 cells were infected with HHV-6A(GS) at an MOI of 80, 200 and 400 $CCID_{50}$ per 10^6 cells and incubated with CMV423 for 10 days. The percentage inhibition of virus growth was determined by quantitation of viral DNA in infected cultures using a Southern blot assay.

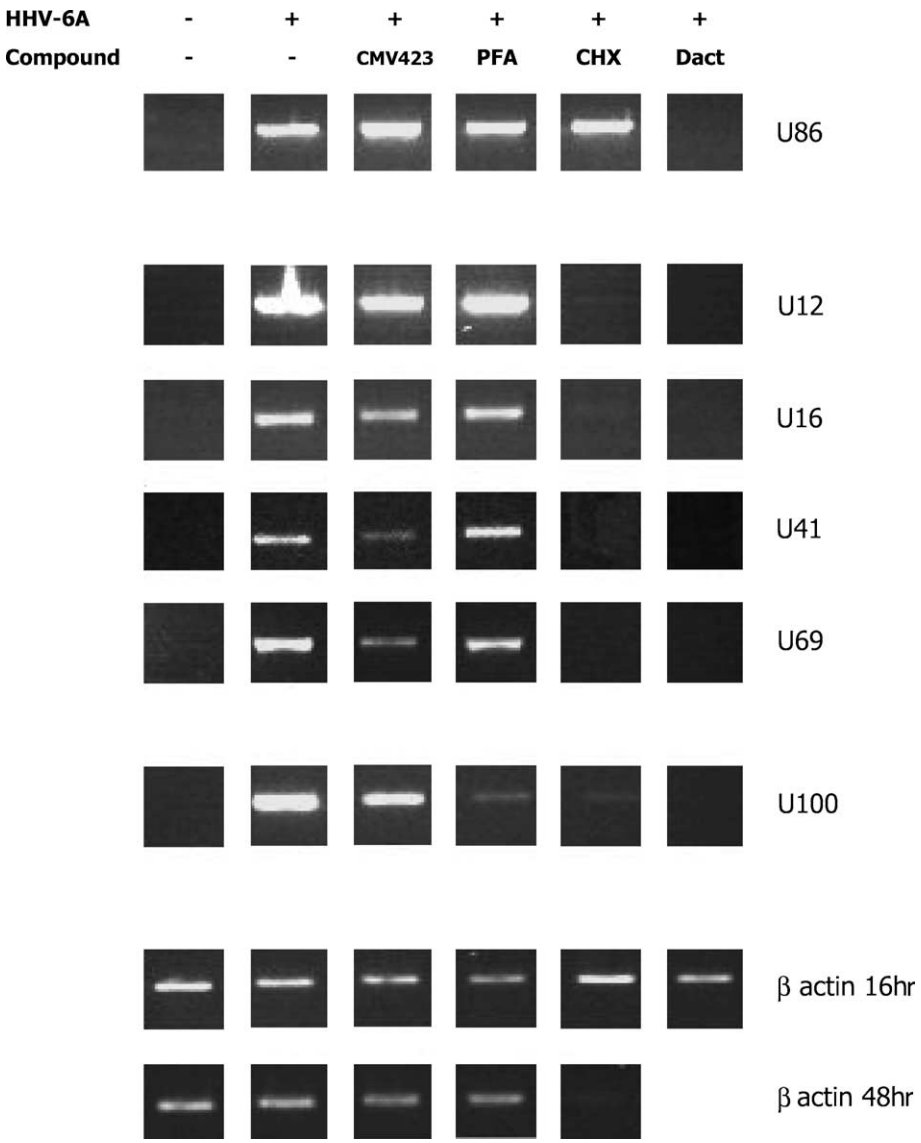

Fig. 3. Ethidium bromide stained agarose gel showing the result of RT-PCR analysis for a panel of HHV-6 mRNA transcripts after a 16 hr (immediate-early and early transcripts) or 48 hr (late transcripts) CMV423 treatment (10 μM). Based on the inhibition of their expression by the reference compounds dactinomycin (Dact), CHX, or foscarnet (PFA), the genes were classified as immediate-early (U86), early (U12, U16, U41 and U69) and late (U100). Human β-actin was included as a control.

(25 μM), infected with cell-free HHV-6A virus and incubated with the compounds for 1–4 days. Immunofluorescent analysis after 24 hr showed strong expression of the IE pU90 protein, which was completely inhibited by CHX (Fig. 4, panel 1C), whereas CMV423 had no influence on the expression of this protein (Fig. 4, panel 1B). The expression of the early nuclear protein p38/41 by CMV423 was not completely inhibited, but clearly reduced after 24 hr (Fig. 4, panel 2B) and 96 hr (Fig. 4, panel 3B). The HHV-6 late proteins gp60/110 (Fig. 4, panel 4C) and gp110 (data not shown) were not detected in the presence of PFA. A marked effect of CMV423 on the expression of these HHV-6 proteins was observed after 4 days (Fig. 4, panel 4B); after 24 hr, expression of HHV-6 late proteins was relatively low, making an evaluation of the effect of CMV423 difficult.

3.4. Effect on HHV-6 DNA polymerase activity

To investigate the possibility that CMV423 might act as a non-nucleoside DNA polymerase inhibitor, we used an HHV-6 DNA polymerase assay based on *in vitro* transcription/translation for the production of the pU38 catalytic subunit of the enzyme [24]. The enzyme activity was evaluated by measuring the incorporation of [^{3}H]dGTP (1 μM) into activated calf thymus DNA in the presence of serial dilutions of the compound for 40 min at 37°. In contrast to the reference compounds forscarnet and GCV triphosphate (50% inhibitory concentration: 6.02 and 2.53 μM, respectively), CMV423 was shown not to inhibit HHV-6 DNA polymerase activity within a concentration range of 0.001–10 μM (Fig. 5).

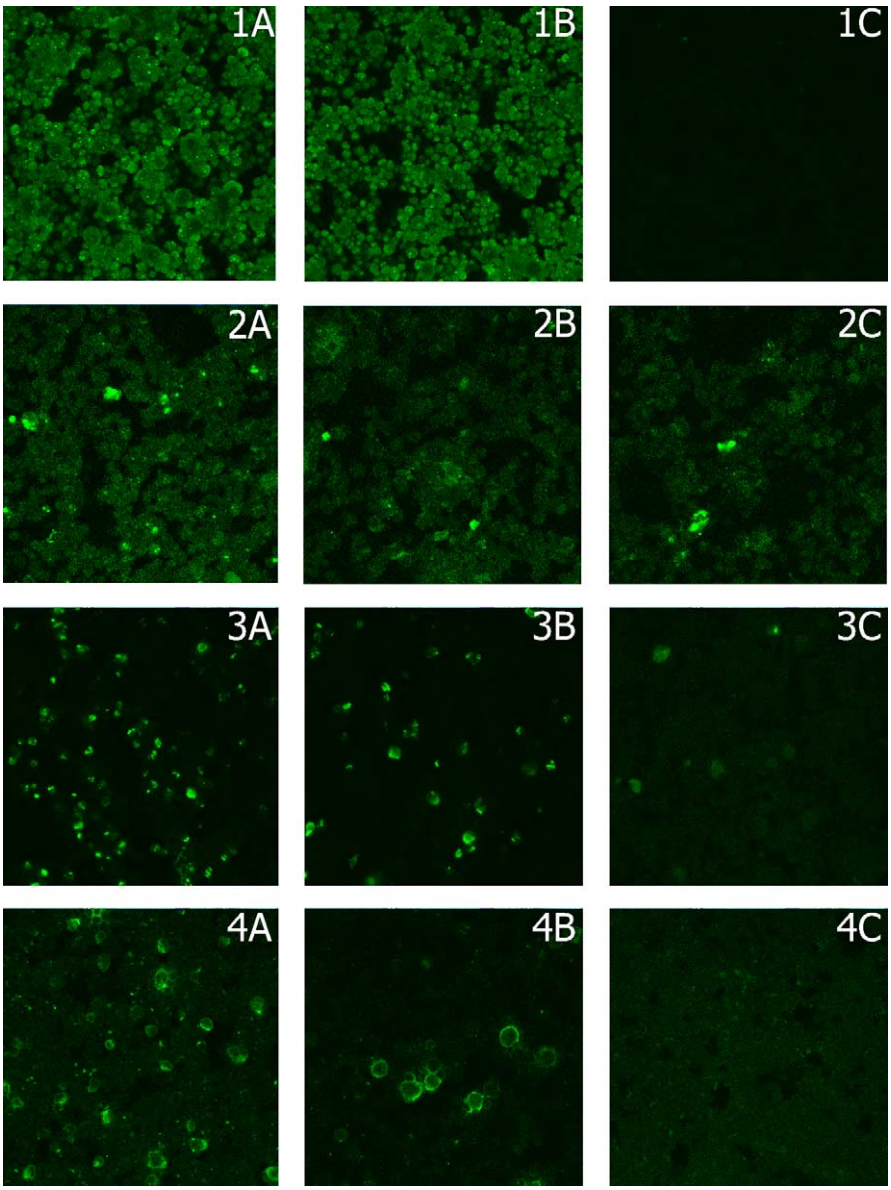

Fig. 4. Immunofluorescence analysis of the effect of CMV423 on HHV-6A protein expression. HSB-2 cells were infected with HHV-6A(GS) and left untreated (panels A), or incubated with CMV423 (panels B) or the reference compounds CHX (panel 1C) and foscarnet (panels 2C, 3C and 4C). An indirect immunofluorescent assay was used to determine the effect on the expression of the immediate-early protein pU90 (panels 1A, 1B and 1C) at 24 hr p.i.. The early antigen p38/41 was visualized both at 24 hr p.i. (panels 2A, 2B and 2C) and 96 hr (panels 3A, 3B and 3C), and the late antigen gp60/110 at 96 hr p.i. (panels 4A, 4B and 4C). Magnification: 40×.

3.5. Comparison with inhibitors of cellular protein kinases

The anti-HHV-6 activity of a panel of non-nucleoside compounds, reported to have antiviral activity against other herpesviruses, was compared to that of CMV423 in HHV-6A-infected HSB-2 cells and HHV-6B-infected Molt-3 cells, and using the CPE and DNA hybridization assays as described above (Table 3). Except for herbimycin A, none of the compounds was found able to significantly suppress HHV-6 replication at subtoxic concentrations. Interestingly, the anti-HHV-6 activity of herbimycin A showed similarities with that of CMV423, i.e. good activity in HHV-6A-infected HSB-2 cells (IC_{50}: 32.2 nM), no activity in HHV-6B-infected Molt-3 cells, MOI-dependent activity and incomplete inhibition of virus replication even at high compound concentrations. Next, the possibility of a synergistic antiviral activity between CMV423 and herbimycin A was examined. We, therefore, monitored the IC_{50} value of CMV423 when combined with increasing concentrations of herbimycin A in HHV-6A-infected HSB-2 cells. In parallel, a similar experiment with CMV423 and PFA was performed. The isobolograms in Fig. 6 show that with both combination regimens, the IC_{50} of CMV423 was found to be significantly reduced. However, whereas the combination of PFA and CMV423 resulted in a weak synergism, herbimycin A and CMV423

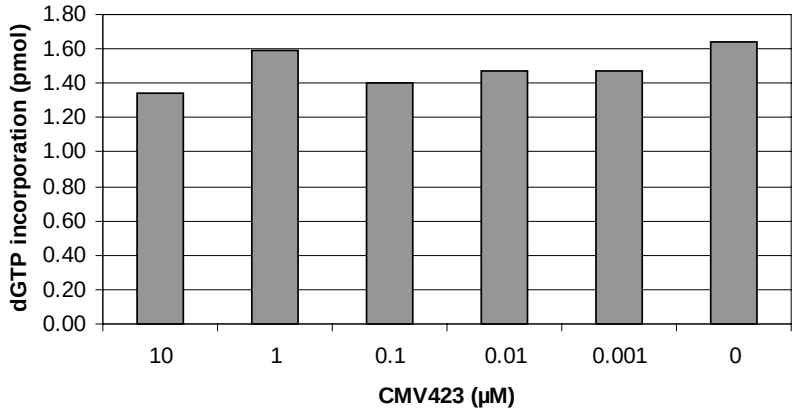

Fig. 5. Effect of CMV423 on HHV-6 DNA polymerase activity. The assay was performed using the catalytic subunit of the enzyme, measuring incorporation of [^{3}H]dGTP into activated calf thymus DNA.

interacted in a strongly synergistic manner, reinforcing each other's anti-HHV-6 activity.

3.6. Inhibition of cellular protein tyrosine kinase activity

To further investigate the possible resemblance between the modes of action of CMV423 and herbimycin A, we compared both compounds for their direct or indirect inhibitory activity against cellular PTK. Using a non-radioactive PTK assay, we determined the effect of CMV423 and herbimycin A on the total cellular tyrosine kinase activity in crude HSB-2 cell extracts (Fig. 7). Both the direct inhibitory effects (by adding the compounds during the kinase reaction only) and the indirect effects (by pre-incubation of the cells with the compounds prior to protein extraction) were examined. The results shown were obtained from three independent experiments. A marked decrease in PTK activity was seen after pre-treatment with herbimycin A at 1 μM (80% inhibition), and, to a lesser extent, CMV423 at 100 μM (44% inhibition). For CMV423, the inhibition appeared to be concentration-dependent, although the inhibition at 1 and 10 μM (5 and 25%, respectively) was less pronounced. The direct inhibitory effects on PTK activity of both herbimycin A and CMV423 were minor.

3.7. Effect on cellular serine/threonine kinase activity

To ascertain the specificity of the observed tyrosine kinase inhibition, we evaluated the influence of CMV423 on serine/threonine kinase activity in crude HSB-2 cell lysates. The broad-spectrum serine/threonine protein kinase inhibitor staurosporine was included as a control. Again, the compounds were added during preincubation of the cells for 24–72 hr, or during the enzyme assay only. No concentration-dependent inhibition by CMV423 of either PKA (Fig. 8, panel A) or PKC (Fig. 8, panel B) activity were observed in a concentration range of 0.1–100 μM, irrespective of preincubation; staurosporine (20 nM) inhibited PKA for 20–50%, and resulted in >90% inhibition of PKC activity.

Table 3
Anti-HHV-6 activity of a selection of kinase inhibitors

Compound	Target	IC_{50} (nM)[a]		Reported activity against (with references in brackets)
		HHV-6A (HSB-2 cells)	HHV-6B (Molt-3 cells)	
H-89	PKA	NA[b]	NA	HSV-1 [32]
Staurosporine	Ser/Thr kinases	NA	NA	
Gö6976	PKC, HCMV pUL97	NA	NA	HCMV [33,34]
Herbimycin A	Hsp90	32.2 ± 25.9	NA	HSV [17], HHV-6 [16]
Leflunomide	PTKs	NA	NA	HSV-1 [35], HCMV [36]
Genistein	PTKs	NA	NA	HSV [37]
PP-2	Src PTKs	NA	NA	
SB-202190	p38 MAPK	NA	NA	HCMV [14]
U0126	MKK1/2	NA	NA	HCMV [15]
Roscovitine	cdk-2	NA	NA	HSV [38], HCMV [39]
CMV423	?	53 ± 20	NA	HCMV [18]

[a] IC_{50} values as determined by DNA hybridization.
[b] NA: not active at subtoxic concentrations.

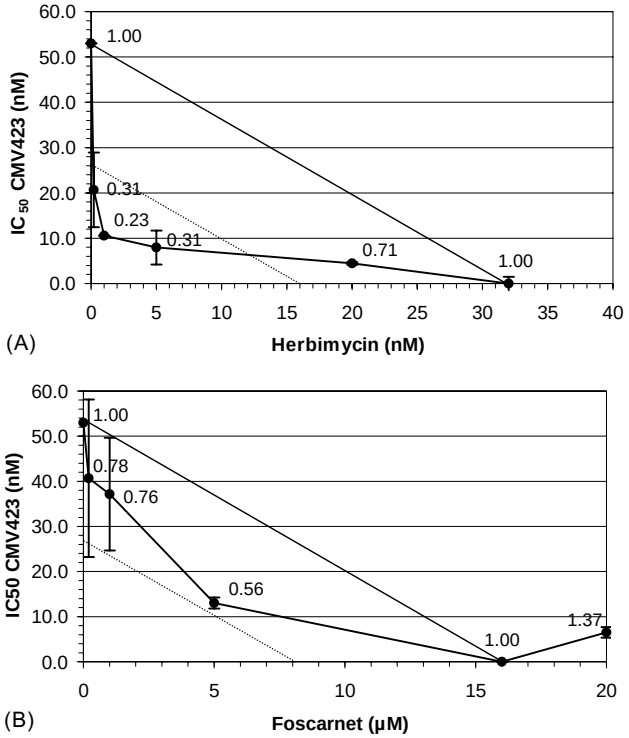

Fig. 6. Isobologram representation of the combined inhibitory effects of herbimycin A (A) or foscarnet (B) and CMV423 on HHV-6A growth in HSB-2 cells. The FIC (fractional inhibitory concentration) index is indicated for every combination of concentrations and is defined as follows: FIC index: [(IC_{50} of drug A in combination)/(IC_{50} of drug A alone)] + [(IC_{50} of drug B in combination)/(IC_{50} of drug B alone)]. A FIC index between 0.5 and 0.9 is suggestive of synergism, whereas a FIC index <0.5 indicates significant synergism. Values are the means ± SD for two independent experiments.

3.8. Effect on HHV-6 pU69 activity

The HHV-6 U69 gene encodes a functional serine/threonine kinase, which is responsible for the conversion of GCV to its monophosphorylated metabolite [25]. Using an assay that was developed to measure [8-^{3}H]GCV phosphorylation by pU69 (expressed by a recombinant vaccinia virus) [9], we evaluated the effect of CMV423 on the activity of this enzyme. 143B TK$^-$ human osteosarcoma cells were infected with a recombinant vaccinia virus, expressing pU69 from HHV-6A, and incubated with 50 μM GCV and 0.1, 1 or 10 μM CMV423 for 24 hr. No effect on the total [8-^{3}H]GCV phosphate level was observed after CMV423 treatment (82, 78, 80 and 78 pmol/10^7 cells for 0, 0.1, 1 and 10 μM CMV423, respectively).

3.9. Effect of CMV423 on cell cycle progression

In order to evaluate the effect of CMV423 on kinases involved in cell cycling, cell cycle analysis was performed on Sup-T1 and Molt-3 cells, incubated with CMV423. The HSB-2 cell line could not be used for this purpose, since it was found to be of heterogeneous ploidy (data not shown). The reference compound adefovir, included as a control, caused a strong S phase arrest, as expected [26]. No major arrest at any phase of the cell cycle could be observed after incubation with CMV423 for 48, 72 or 96 hr, neither at 1 nor 100 μM (data not shown).

4. Discussion

CMV423 is the prototype compound of a new class of antiviral molecules, the tetrahydroindolizine derivatives. It was developed through lead optimization of a precursor that was selected from antiviral testing programs for its activity against HCMV. Snoeck *et al*. recently reported on its anti-HCMV activity [18]: excellent activity and selectivity were obtained both against laboratory HCMV strains

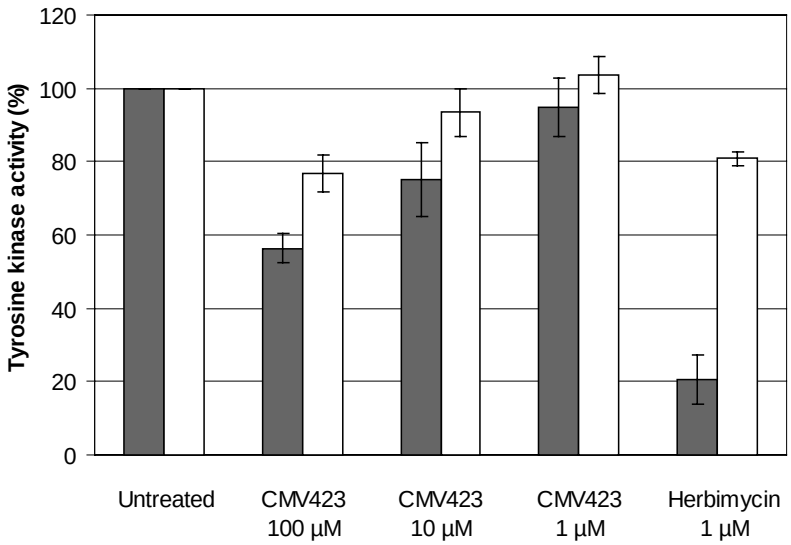

Fig. 7. Analysis of PTK activity in crude HSB-2 cell lysates in the presence of CMV423 and herbimycin A. White bars: compounds were added to the cells only during the assay. Dark bars: cells were preincubated with the compounds for 24 hr. Untreated cells represent 100% activity. Results are means ± SD for three independent experiments.

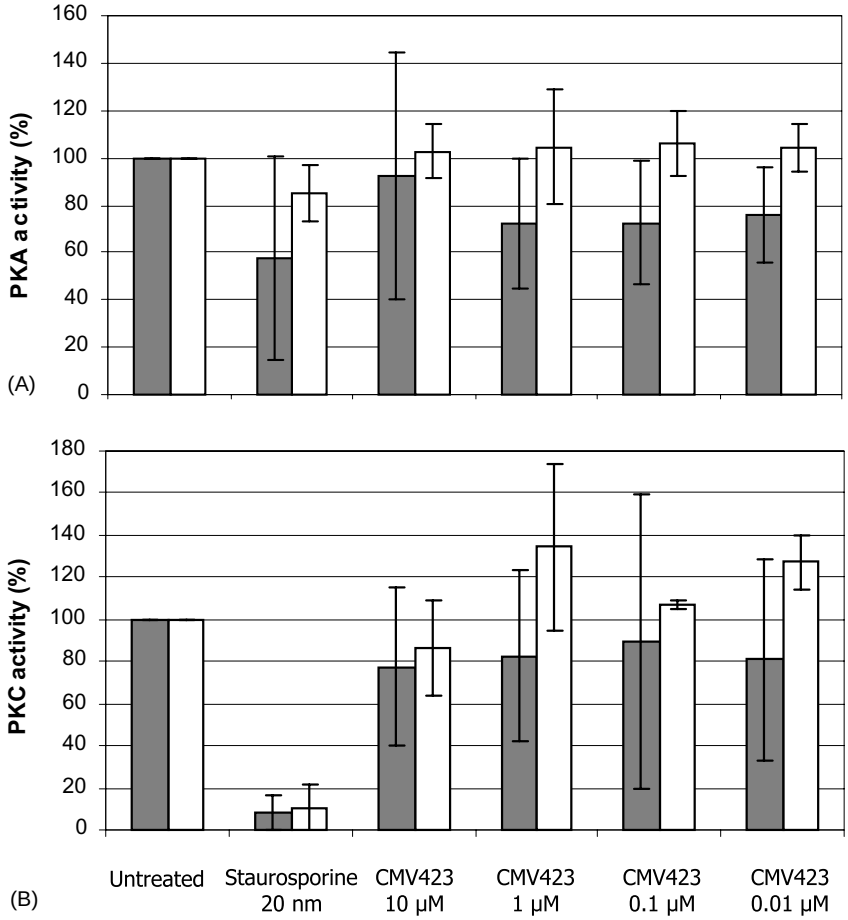

Fig. 8. Analysis of PKA (panel A) and PKC (panel B) activity in crude HSB-2 cell lysates in the presence of CMV423 and staurosporin. White bars: compounds were added to the cells only during the assay. Dark bars: cells were preincubated with the compounds for 24 hr. Untreated cells represent 100% activity. Results are means ± SD for two independent experiments.

and clinical isolates *in vitro*, as well as in two *ex vivo* models. Here we report on the anti-HHV-6 and anti-HHV-7 activity of CMV423. The activity spectrum of CMV423 thus extends to all known human β-herpesviruses.

As for HCMV, we found excellent activity of CMV423 against HHV-6 and HHV-7, both in continuous cell lines (HSB-2, Sup-T1) and in CBLCs. Compared to the reference compounds acyclovir, ganciclovir, cidofovir and foscarnet, all targeting the viral DNA polymerase, CMV423 showed a markedly superior selectivity. Its mode of action clearly differed from that of the classical anti-herpetic drugs, as supported by mechanistic studies, where the influence of CMV423 on the expression of viral immediate-early, early and late mRNA transcripts and proteins was investigated. Although the degree of inhibition of transcription varied for the individual early genes, these studies demonstrated that CMV423 targets an early event in the viral replicative cycle that takes place prior to or at viral DNA replication, yet after immediate-early protein synthesis. Our finding that the HHV-6 DNA polymerase does not serve as a target for CMV423 is indirectly supported by the observation that HCMV strains showing resistance to ganciclovir, CDV or PFA were still sensitive to CMV423 [18].

An early blockade of the herpesvirus replication cycle has also been reported for a variety of non-nucleoside compounds that are known to be inhibitors of cellular protein kinases. The p38 MAPK inhibitor SB-202190 and the MKK1/2 inhibitor U0126 have both been reported to inhibit HCMV at a stage prior to viral DNA synthesis [14,15]; roscovitine blocks the transcription of viral immediate-early and early genes through an inhibition of cellular cyclin-dependent kinases [27]; and an inhibition at both early and late stages of HSV-1 replication has been reported for the tyrphostin PTK inhibitor AG17 [17]. Several observations in our antiviral experiments suggested that the antiviral target for CMV423 also is not strictly of viral origin, but involves a cellular component, associated with HHV-6 replication. Notably, CMV423 exerts no activity against HHV-6B in Molt-3 cells, whereas HHV-6B was susceptible to CMV423 when evaluated in the Sup-T1 cell line. Furthermore, the antiviral efficacy of CMV423 proved highly dependent on the viral inoculum.

We, therefore, hypothesized that CMV423 acts through an inhibition of cellular protein kinases that interact strongly with viral replication processes, and that may be upregulated during HHV-6 infection. Indeed, in addition

to the fact that HHV-6 is known to induce host cell protein synthesis [28], a more specific alteration of gene expression after HHV-6 infection has been described for MAPK family members (MAPK4, MAPK11) as well as for several proteins involved in PTK signaling, such as RYK and SSI-1 [29]. To investigate this possibility, we performed a comparative antiviral study with several compounds that are known to be inhibitors of protein kinases, and that have been reported to demonstrate antiviral activity against other herpesviruses (see Table 3 for references). Interestingly, none of the compounds was able to inhibit HHV-6, except for herbimycin A, which affects PTK activity indirectly, by binding Hsp90 and destabilizing its client proteins, a.o. PTKs [30]. This compound was active against HHV-6A (in HSB-2 cells) at nanomolar concentrations, as reported earlier [16]. In contrast, HHV-6B (grown in Molt-3 cells) was not inhibited at subtoxic concentrations. This striking observation, similar to that made for CMV423, prompted us to investigate the direct and indirect PTK inhibiting properties of CMV423. A marked inhibition of total cellular PTK activity was indeed observed after pre-incubation of the cells with the compound at concentrations of 10–100 μM, which is 200–2000-fold higher than the antiviral IC_{50} in cell culture. A possible explanation for this discrepancy may be that the inhibition by CMV423 involves one or a limited number of PTKs (consistent with its high selectivity index in antiviral assays), whereas under our experimental conditions, the global cellular PTK activity was quantified. A mode of action involving PTK inhibition agrees with the antiviral drug combination experiments, in which the synergistic effect obtained in the combination of CMV423 with herbimycin A was found to be much more pronounced than the synergism between CMV423 and foscarnet.

Although none of the other kinase inhibitors from Table 3 was found to be active against HHV-6, the effect of CMV423 on the activity of certain kinases was evaluated. The serine/threonine kinases PKA and C were not found to be inhibited by CMV423, and neither was the virally encoded pU69 kinase, the HHV-6 homolog of the HCMV UL97-encoded kinase which (auto)phosphorylates mainly serine and threonine residues [25]. Herpes simplex virus and HCMV are inhibited by compounds, targeting cyclin-dependent kinases, more specifically cdk-1, -2 and -5 [13]. Inhibition of cdk-2 would result in a cell cycle arrest at the G_1 phase [31]; however, when evaluating the effect of CMV423 on cell cycle progression, no cell cycle arrest was observed. This is in agreement with the observed low cytotoxicity of CMV423.

In conclusion, CMV423 undoubtedly is a powerful lead of an entirely new class of anti-herpesvirus compounds, with an activity spectrum confined to the human β-herpesvirus subfamily. Its mode of action is clearly distinct from that of the compounds licensed so far for the treatment of HCMV (and, by extension, HHV-6). We have provided evidence that CMV423 inhibits (a subset of) cellular PTKs, yet at high concentrations. More detailed studies would be required to define its target more specifically, and to demonstrate the link with its highly specific anti-HHV-6 activity. Our data justify further investigation into the role of cellular processes affecting PTK activity in the HHV-6 replication cycle and their exploitation for anti-HHV-6 therapy.

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