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Inhibition of herpesvirus replication by a series of 4-oxo-dihydroquinolines with viral polymerase activity

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Abstract

Herpesviruses cause a wide variety of human diseases ranging from cold sores and genital herpes to encephalitis, congenital infections and lymphoproliferative diseases. These opportunistic viruses cause major problems in immunocompromised individuals such as transplant recipients, cancer patients, and HIV-infected persons. The current treatment of these infections is not optimal and there is a need for more active, less toxic compounds that might be used in place of or in addition to current therapies. We have evaluated a new series of 4-oxo-dihydroquinolines, which have a different mechanism of action than nucleosides and have activity against multiple herpesviruses. Of the four new compounds evaluated, two (PHA-529311 and PHA-570886) had greater activity than the parent, PHA-183792, against several herpesviruses and one (PHA-568561) was as effective as the parent. A fourth, PHA-243672, was considerably less effective. They had greater efficacy against cytomegalovirus (CMV) than the other herpesviruses tested and also had activity against acyclovir-resistant herpes simplex virus and varicella-zoster virus isolates and ganciclovir or foscarnet-resistant CMV isolates. These results confirm the broad-spectrum efficacy of these compounds against multiple herpesviruses and suggest that members of this class may have a potential role for treatment of a variety of herpesvirus infections.

Keywords: Herpesvirus; Antiviral; 4-Oxo-dihydroquinolines; Non-nucleosides; Polymerase

1. Introduction

Infections with human herpesviruses, including those caused by herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) are common and usually self-limiting in otherwise healthy individuals. However, each of these viruses and the emerging human herpesviruses (HHV) 6, 7, and 8 can cause severe disease in the immunocompromised patient and collectively remain a significant health problem. For example, in HIV-infected individuals and in transplant recipients, current management of virus-induced disease is complex and often limited (de Jong et al., 1998a; Cohen-Stuart et al., 1998). The nucleoside antivirals, ganciclovir (GCV) and acyclovir (ACV), the nucleotide cidofovir (CDV), the pyrophosphate analog foscarnet (PFA), and the antisense agent formivirsen have been approved for treatment of certain herpetic infections but are less than ideal due to their associated toxicity, efficacy against only one or two human herpesviruses or route of delivery requirements (de Jong et al., 1998b; Hoffman and Skiest, 2000; Pillay et al., 2000). Additionally, drug resistance may develop during treatment of herpesvirus infections, and thus drug-resistant HSV and VZV (Talarico et al., 1993; Field and Biron, 1994) and CMV (Emery and Griffiths, 2000) strains have been identified. Cross-resistance of herpesviruses to antiviral drugs has also been reported (Pelosi et al., 1998).

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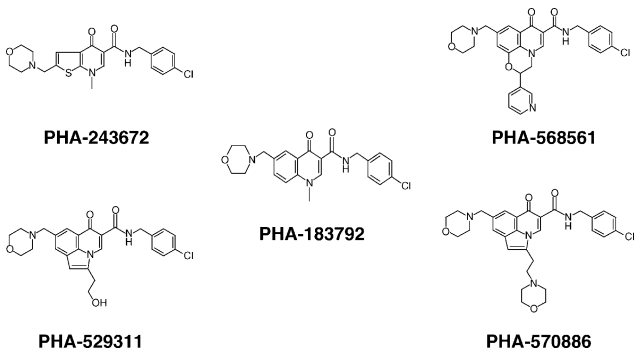

Fig. 1. Structures of dihydroquinolines and thienopyridines.

In order to address the need for new agents with oral activity against wild-type and drug-resistant strains of human herpesviruses, particularly ones with an improved safety profile, an effort was begun initially to identify anti-CMV compounds. Compounds belonging to the naphthalene carboxamide class were found to have anti-CMV DNA polymerase activity (Vaillancourt et al., 2000). Additional modification of the carboxamide template led to discovery of the 4-oxo-dihydroquinolines (4-oxo-DHQ) which were found to inhibit the polymerases of HSV, VZV, and CMV. These compounds also inhibited viral replication in cells and reduced mortality in murine CMV-infected mice (Brideau et al., 2002; Oien et al., 2002). The 4-oxo-DHQ's were also found to inhibit low-passage clinical isolates of CMV and VZV in cell culture (Knechtel et al., 2002). This work has now been extended to include additional chemical modification of the 4-oxo-DHQ, PHA-183792 to yield PHA-243672, PHA-529311, PHA-568561 and PHA-570886 analogs (Fig. 1). The purpose of these studies was to determine the activity of these new dihydroquinoline and thienopyridine analogs against seven members of the herpesvirus group.

2. Materials and methods

2.1. Antiviral compounds

The 4-oxo-dihydroquinoline analogs were synthesized and provided by scientists at Pharmacia, Infectious Disease Research, Kalamazoo, MI. The structures of these compounds are presented in Fig. 1. GCV, CDV, and ACV were purchased from the University of Alabama Hospital Pharmacy and were used as positive controls. PFA was purchased from Sigma and was also used as a positive control for selected resistant HCMV strains. ACV, GCV, CDV, and PFA solutions were all prepared in minimal essential medium (MEM). The 4-oxo-DHQ analogs were prepared in DMSO at 1000 μM, then aliquoted and frozen at −20 °C, until used and no sample was thawed more than twice. On the day of the assay, the analogs were diluted in MEM to the desired concentration(s).

2.2. Cell and virus preparation

Human foreskin fibroblast (HFF) cells were prepared as primary cultures. They were cultured in MEM with 10% fetal bovine serum (FBS) and standard concentrations of antibiotics. These cells were used in propagating HSV-1 and-2, HCMV, and VZV. All antiviral assays for these viruses were also conducted in HFF cells (Williams et al., 2003). Daudi cells (ATCC, Manassas, VA), HSB-2 cells (NIH AIDS Research and Reference Reagent Program, Rockville, MD), and Body-cavity based lymphoma (BCBL-1) cells (NIH AIDS Research and Reference Reagent Program, Rockville, MD) were propagated for use in EBV, HHV-6 and HHV-8 assays (Williams et al., 2003; Kushner et al., 2003). These cells were cultured in RPMI-1640 with 10% FBS and split 1:5 twice a week. Cord blood lymphocyte (CBL) cells were isolated from umbilical cord blood on a Histopaque 1077 (Sigma Cell Culture, St. Louis, MO) gradient and used in HHV-6 assays. They were maintained as mentioned above. P3HR-1 cells were used to propagate EBV and were incubated for 2 weeks at 34 °C (Williams et al., 2003). After incubation, cells were centrifuged using a Sorvall GSA rotor at 6000 rpm for 15 min. The supernatant was collected, centrifuged for 90 min at 12000 rpm and the virus pellet resuspended in 1/100 of the original volume. HHV-6 A, strain GS, was propagated in HSB-2 or SupT-1 cells and HHV-6 B, strain Z-29, was propagated in cord blood lymphocytes (Kushner et al., 2003). All cells were passaged and monitored for percentage of infection by IFA for 3–10 days depending on the cell line. Cells were then centrifuged; pellets were resuspended in media and frozen at −80 °C. Body-cavity based lymphoma cells were treated with TPA to induce production of HHV-8.

2.3. Antiviral assays

2.3.1. Plaque reduction assay for HSV, CMV, and VZV in HFF cells

HFF cells were plated into six-well plates and incubated at 37 °C. Two days later, drug was serially diluted 1:5 in MEM with 2% FBS to obtain six concentrations of drug with a starting concentration of 100 μM. ACV, GCV or PFA were used as a positive control. The virus to be used was diluted in MEM containing 10% FBS to a desired concentration which gave 20–30 plaques per well. The media were aspirated from the wells and 0.2 ml of virus was added to each well in triplicate with 0.2 ml of medium being added to drug toxicity wells. The plates were incubated for 1 h with shaking every 15 min and drug added to each well. After incubating for 3–10 days, cells were stained with 1% crystal violet in 20% MeOH for HSV-1 and-2 or Neutral Red in Dulbecco's phosphate-buffered saline (D-PBS) for HCMV and VZV. The stain was aspirated, cells were washed with D-PBS, and plaques counted using a stereomicroscope. By comparing drug-treated with untreated wells, EC_{50} values were calculated using MacSynergy II software (Prichard and Shipman, 1990).

2.3.2. Immunofluorescence assays (IFA) for EBV

Daudi cells infected with EBV were fixed and incubated with a monoclonal antibody to EBV viral capsid antigen (VCA) gp125 (Chemicon, Temecula, CA), followed by FITC-labeled IgG + IgM secondary antibody (Jackson ImmunoResearch, West Grove, PA) at 37 °C for 1 h intervals. Slides were rinsed thoroughly with PBS between incubations. Cells were counterstained with 0.1% Evans blue dye (Fisher, Fair Lawn, NJ) in PBS for 5 min and coverslips mounted using 50% glycerol in PBS. Cells were viewed at a magnification of 400× using a Nikon fluorescence microscope (Nikon, Melville, NY) and the percentage of positive cells was calculated at each drug concentration. Drug efficacy was determined by plotting drug concentration versus the percentage of positive cells and extrapolating the concentration of drug required to inhibit 50% of virus replication (EC_{50}) (Williams et al., 2003).

2.3.3. Enzyme-linked immunosorbent assay for EBV

ELISA was performed on fixed cells by incubation with a monoclonal antibody to EBV VCA gp125 (Chemicon, Temecula, CA) followed by incubation with horseradish peroxidase labeled goat antimouse IgG1 (Southern Biotechnology, Birmingham, AL). The colorimetric reaction was initiated by addition of o-phenylenediamine dihydrochloride in citrate buffer (pH 5.0) and hydrogen peroxide. The reaction was stopped by the addition of 3N sulfuric acid. The EC_{50} value for each drug was extrapolated from the plot of drug concentration versus average OD_{492} for each concentration of drug (Williams et al., 2003).

2.3.4. In situ hybridization assay for EBV

The Simply Sensitive Horseradish Peroxidase-AEC In Situ Detection System (Enzo Diagnostics, Farmingdale, NY) was used to monitor EBV DNA synthesis in the presence of antiviral compounds and was performed according to the manufacturer's instructions. The EC_{50} value for each drug was extrapolated from the plot of drug concentration versus the percentage of positive cells (Williams et al., 2003).

2.3.5. Determination of efficacy against HHV-6

Serial five-fold dilutions of drug starting at 50 μg/ml were prepared in RPMI 1640 with 10% FBS. CDV was used as a positive control. Samples for determining antiviral efficacy were prepared by incubating 10^6 HSB-2 cells for 1 h with sufficient virus to infect approximately 35% of the cells. After infection, the appropriate concentrations of drug were added, and cells were incubated for 4–6 days at 37 °C. Virus-free controls were prepared by incubating 10^6 cells in drug-free medium for the designated period, and virus controls were prepared by incubating 10^6 cells for 1 h with sufficient virus to infect 35% of cells followed by an incubation in drug-free medium for the designated period (a low-multiplicity, multiple-cycle assay). After incubation, cells were rinsed with PBS and permeabilized overnight in methanol at −80 °C for use in FACS analysis as described below.

2.3.6. Determination of efficacy against HHV-8

Lytic HHV-8 infection was induced in BCBL-1 cells (a continuous cell line latently infected with HHV-8) by the addition of 100 ng of phorbol 12-myristate 13-acetate (TPA) per ml. Serial five-fold dilutions of drug starting at 50 μg/ml were prepared in RPMI 1640. CDV was used as a positive control. Samples used for determining antiviral efficacy were prepared by incubating 10^6 TPA-induced BCBL-1 cells with the appropriate dilution of drug for 5 days, with new media added on day 2 of incubation. TPA-induced and uninduced controls were prepared by incubating 10^6 TPA-induced and-uninduced BCBL-1 cells in drug-free medium. After incubation, cells were rinsed with PBS and permeabilized by overnight storage in methanol at −80 °C for subsequent use in FACS assay.

2.3.7. Flow cytometric assays (FCA)

Cells were rinsed thoroughly with PBS, and a blocking solution containing 5% FBS, 4% normal goat serum and 0.5% dimethylsulfoxide was added. Cells were then incubated with a monoclonal antibody to HHV-6 early nuclear proteins (101 kDa virion, Chemicon, Temecula, CA) or HHV-8 (KS8.1, Bala Chandran, University of Kansas, Kansas City, KS), followed by FITC-labeled IgG + IgM secondary antibody (Jackson ImmunoResearch, West Grove, PA). Cells were washed thoroughly with blocking solution after each incubation. After staining, samples were fixed in 2% paraformaldehyde in PBS and analyzed using a Becton-Dickenson FacsCalibur instrument. Flow cytometry data were analyzed using the WinMDI 2.7 data analysis program and the EC_{50} value was extrapolated from the plot of drug concentration versus percentage of antigen-positive cells (Williams et al., 2003; Kushner et al., 2003).

2.3.8. Polymerase assays

The catalytic form of the HCMV DNA polymerase was expressed as c-terminal histidine-tagged proteins using the baculovirus expression system and purified by standard Ni-affinity chromatography. Compounds were evaluated against the purified DNA polymerase of HCMV using a scintillation proximity assay (SPA) as described previously (Vaillancourt et al., 2000). Briefly, SPA conditions were as follows: 6.4mM HEPES (pH 7.5); 12 mM KCl; 25 mM NaCl; 2 mM CHAPS; 4.5 mM DTT; 5 mM $MgCl_2$; 5% glycerol; 5% DMSO; 46 μg/ml BSA; 10 nM oligo dT annealed to poly(A) homopolymer; and 0.1 μM ^{3}H-dTTP. The reaction was terminated after 12 min by the addition of 200 μg streptavidin-coated SPA beads and 133 nM EDTA (Amersham Pharmacia Biotech, Piscataway, NJ). All polymerase reactions were performed at 27 °C. Viral polymerases were estimated to be > 50% pure after comparing profiles of mock-expressed baculovirus products found to be devoid of polymerase activity (N.L. Oien, personal communication). BSA was added to the polymerases to provide stability during storage. Removal of all extraneous polymerases was demonstrated

by repeating the recombinant baculovirus containing genes other than polymerases (data not shown). Polymerase assays were performed under conditions of first-order kinetics. Inhibition of viral polymerase activity by selected test compounds using dCTP, dATP, and dGTP was also determined (data not shown, N.L. Oien, personal communication).

2.4. Cytotoxicity assays

2.4.1. Adherent cells: (HFF)

For cytotoxicity, cells were seeded into 96-well plates at a concentration of 2.5×10^4 cells/well. After 24 h, media were aspirated and 125 μl of drug was added to the first row and diluted serially 1:5. The plates were incubated for 7 days. The media were aspirated and the cell monolayer was stained with neutral red and incubated for 1 h. The cells were washed and solubilizing solution (50% ETOH 1% glacial acetic acid) was added. The plates were shaken for 15 min on a rotating shaker and the optical densities read at 550 nm. Effects on cell proliferation were determined by seeding cells in six-well plates at a concentration of 2.5×10^4 cells/well. After 24 h, the media were aspirated and drug, serially diluted 1:5, was added to duplicate wells. After incubation for 72 h at 37 °C, cells were trypsinized and counted using a Beckman Coulter Counter. Cellular toxicity (CC_{50}) or inhibition of cell proliferation (IC_{50}) values were calculated by comparing drug-treated wells with untreated wells (Williams et al., 2003; Kushner et al., 2003).

2.4.2. Non-adherent cell lines (BCBL-1, CBL, Daudi, HSB-2, and SupT-1)

1×10^6 cells were added to 12×75 glass round bottom tubes. Experimental compounds were diluted serially 1:5 and appropriate concentrations added to each tube in duplicate. The cells were incubated with compound for 3–6 days depending on the cell type. A Promega CellTiter 96 Aqueous assay (Promega; Madison, WI) was used to determine CC_{50} values. This colorimetric method determines the number of viable cells in comparison with untreated controls. The MTS/PMS solution was prepared by adding PBS to MTS (3-4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfophenyl)-2-H-tetrazolium, inner salt) to give a final concentration of 2 mg/ml. After incubation, 200 μl of sample was transferred to a 96-well plate in duplicate. One ml of PMS (phenazine methosulfate) was added for every 20 ml of MTS solution and sterilized using a 0.2 micron filter. Twenty μl of MTS/PMS was added to each well the plate was wrapped in foil and incubated at 37 °C for 4 h. The MTS is bioreduced by dehydrogenases in metabolically active cells into an aqueous soluble formazan, in which the amount measured is directly proportional to the number of living cells in culture. The drug concentration was plotted against the optical density of each sample and CC_{50} values were calculated using MacSynergy II (Prichard and Shipman, 1990).

3. Results

3.1. Activity against HSV-1, HSV-2, and VZV

The inhibition of 10 wild-type HSV-1 isolates by ACV and the five 4-oxo-DHQ's is shown in Table 1. The mean ± standard deviation for each compound against the 10 isolates is shown at the bottom of each table. Compounds PHA-183792 (mean $EC_{50} = 3.4$ μM), PHA-529311 (mean $EC_{50} = 1.4$ μM) and PHA-570886 (mean $EC_{50} = 1.8$ μM) had activity that was equivalent to or better than ACV (mean $EC_{50} = 3.5$ μM) against these wild-type HSV-1 isolates. Although PHA-243672 was not run against all isolates, it clearly was the least active of the group. The inhibition of wild-type HSV-2 isolates by these compounds followed the same pattern as seen with HSV-1 (Table 2). Again compounds PHA 183792 (mean $EC_{50} = 4.1$ μM), PHA-529311 (mean $EC_{50} = 1.5$ μM), and PHA-570886 (mean $EC_{50} = 2.0$ μM) had activity that was equivalent to or greater than ACV (mean $EC_{50} = 3.4$ μM).

In the next series of experiments, the compounds were evaluated for their activity against ACV-resistant HSV-1 and HSV-2 isolates. All five compounds were active

Table 1
Activity of 4-oxo-DHQ analogs against wild-type HSV-1 isolates

Virus	Mean EC_{50} (μM) ± S.D.[a]					
	ACV	183792	243672	529311	568561	570886
E-377	2.7 ± 1.2	3.5 ± 0.2	9.2	1.6 ± 0.2	11.3 ± 3.4	1.7 ± 0.1
SC16	1.8 ± 0.6	2.5 ± 0.2	4.4	1.6 ± 1.3	7.2 ± 6.6	1.7 ± 0.4
F	2.4 ± 1.6	2.8 ± 0.7	4.6	1.1 ± 0.7	6.7 ± 5.5	1.6 ± 0.5
HL-3	3.3 ± 0.3	2.9 ± 0.8	10.2	1.1 ± 0.4	9.7 ± 1.2	1.9 ± 0.1
HL-34	2.7 ± 1.9	2.7 ± 1.1	NT[b]	1.3 ± 0	7.5 ± 5.8	1.7 ± 0.1
4E	4.7 ± 0.3	2.8 ± 0.9	NT	1.3 ± 0.7	8.7 ± 0.7	2.0 ± 0.6
E-115	3.1 ± 2.5	7.7 ± 7.7	NT	1.7 ± 1.4	>10.5	1.8 ± 1.5
L6178033	6.2 ± 1.2	3.0 ± 0.2	NT	1.0 ± 0.6	4.0 ± 1.2	1.8 ± 0.3
13231	4.2 ± 0.3	3.3 ± 1.3	9.2	2.3 ± 0	10.8 ± 0.1	2.2 ± 0.1
L6010381	3.8 ± 0.3	3.3 ± 0.7	NT	1.1 ± 0.4	5.6 ± 0.3	1.6 ± 0.3
Mean ± S.D.	3.5 ± 1.3	3.4 ± 1.5	7.5 ± 2.8	1.4 ± 0.4	8.2 ± 2.4	1.8 ± 0.2

[a] Values represent the mean ± standard deviation of two assays, except for 243672.
[b] Not tested.

Table 2
Activity of 4-oxo-DHQ analogs against wild-type HSV-2 isolates

Virus	Mean EC_{50} (μM) ± S.D.[a]					
	ACV	183792	243672	529311	568561	570886
MS	4.9 ± 3.2	2.6 ± 0	3.9	1.3 ± 0.4	6.0 ± 3.6	2.0 ± 1.2
X-79	4.0 ± 0.9	4.7 ± 2.3	3.7	1.8 ± 0.2	> 13.2	1.8 ± 0.3
G	5.3 ± 1.9	7.5 ± 4.5	10.6	1.4 ± 1.2	> 16.4	2.1 ± 0.9
Heeter	0.4 ± 0.3	3.5 ± 1.3	6.2	1.3 ± 0.2	9.8 ± 0.5	1.9 ± 0.1
Jensen	1.3 ± 0	5.2 ± 3.3	NT[b]	1.7 ± 0	12.8 ± 6.6	2.1 ± 0.8
13076	3.6 ± 0.9	1.8 ± 0.02	NT	0.5 ± 0.2	5.6 ± 3.2	1.4 ± 0.3
SR	4.4 ± 0.4	2.6 ± 0	3.7	1.3 ± 0.7	10.8 ± 1.7	2.1 ± 0
L6250234	3.3 ± 2.3	6.2 ± 3.7	NT	1.9 ± 0.3	> 16.2	2.7 ± 0.8
L6262351	2.2 ± 0.9	3.2 ± 0.5	NT	1.8 ± 0.2	> 13.0	2.3 ± 0.3
L6010541	1.9 ± 0.7	3.6 ± 0.5	NT	1.9 ± 0.3	16.3 ± 1.5	2.0 ± 0.3
Mean ± S.D.	3.4 ± 1.4	4.1 ± 1.8	5.6 ± 2.9	1.5 ± 0.4	> 12	2.0 ± 0.3

[a] Values represent the mean ± standard deviation of two assays, except for 243672.
[b] Not tested.

Table 3
Activity of 4-oxo-DHQ analogs against ACV-resistant HSV-1 isolates

Virus	Mean EC_{50} (μM) ± S.D.[a]					
	ACV	183792	243672	529311	568561	570886
SC16-S1	> 444	3.5 ± 1.6	3.0	1.1 ± 0.8	5.9 ± 0.9	1.9 ± 0.1
DM2.1	> 444	1.9 ± 1.6	2.1	0.7 ± 0.2	2.2 ± 0.3	1.7 ± 0.1
11893	242 ± 59	5.9 ± 5.2	2.5	1.4 ± 0.5	5.6 ± 2.8	2.1 ± 0.1
11359	> 444	2.9 ± 0.8	NT[b]	1.1 ± 1.0	6.5 ± 5.5	2.0 ± 0.3
PAA^r	32.4 ± 15	0.6 ± 0.2	0.5	0.2 ± 0	0.9 ± 0.1	0.4 ± 0
11360	> 444	2.9 ± 0.8	2.5	0.9 ± 0.7	5.3 ± 6.4	1.3 ± 0.9
L6191321	> 444	2.6 ± 0.7	NT	1.3 ± 1.0	7.1 ± 5.1	2.0 ± 0.1
L5047716	> 444	2.6 ± 1.3	NT	0.8 ± 0.3	4.7 ± 3.2	1.8 ± 0.4
L6183347	19.9 ± 9.8	9.1 ± 2.1	NT	2.0 ± 0.2	14 ± 4.3	1.6 ± 0.5
B-2006	> 444	4.2 ± 1.3	NT	1.2 ± 0.6	12 ± 0.5	2.2 ± 0.4
Mean ± S.D.	> 340	3.6 ± 2.4	2.1 ± 1.0	1.1 ± 0.5	6.4 ± 4.0	1.7 ± 0.5

[a] Values represent the mean ± standard deviation of two assays, except for 243672.
[b] Not tested.

against ACV-resistant HSV-1 isolates with EC_{50} values of 1–6 μM compared with mean EC_{50} values of > 340 μM for ACV (Table 3). The most active compounds were PHA-529311 (mean EC_{50} = 1.1 μM) and PHA-570886 (mean EC_{50} = 1.7 μM). For ACV-resistant HSV-2 isolates the same pattern was observed (Table 4). While all five compounds were active against both ACV-resistant HSV-1 and HSV-2 isolates (mean EC_{50} = > 424 μM), the analogs, PHA-529311 (mean EC_{50} = 1.5 μM) and PHA-570886 (mean EC_{50} = 1.9 μM) were the most active (Table 4).

Table 4
Activity of 4-oxo-DHQ analogs against ACV-resistant HSV-2 isolates

Virus	Mean EC_{50} (μM) ± S.D.[a]					
	ACV	183792	243672	529311	568561	570886
13077	> 337	2.2 ± 0.5	NT[b]	0.9 ± 0.4	4.3 ± 1.3	1.8 ± 0
AG-3	> 444	3.0 ± 1.0	1.8	1.9 ± 0	8.4 ± 1.7	1.8 ± 0.4
11680	355 ± 124	2.9 ± 0.8	3.2	1.7 ± 0.9	7.5 ± 7.7	2.0 ± 0.5
12247	> 444	2.8 ± 1.2	2.5	1.2 ± 0.6	8.0 ± 7.3	1.8 ± 0.6
11572	> 444	2.6 ± 1.0	2.5	1.1 ± 0.7	4.7 ± 3.8	1.8 ± 0.3
13078	> 444	2.3 ± 0.5	NT	1.3 ± 1.0	5.5 ± 4.7	1.7 ± 0.1
11361	> 444	3.7 ± 0.2	2.3	2.9 ± 0.9	> 17.5	2.0 ± 0.5
L6191429	> 444	2.3 ± 0	NT	0.4 ± 0.3	1.6 ± 1.2	1.3 ± 0.6
L6089158	> 444	2.8 ± 0.7	NT	1.4 ± 1.2	7.4 ± 6.9	2.0 ± 0.3
L6191456	> 444	5.5 ± 2.6	NT	2.1 ± 0	> 18	2.4 ± 0.4
Mean ± S.D.	> 424	3.0 ± 1.0	2.5 ± 0.5	1.5 ± 0.7	> 8.3	1.9 ± 0.3

[a] Values represent the mean ± standard deviation of two assays, except for 243672.
[b] Not tested.

Table 5
Activity of 4-oxo-DHQ analogs against ACV-resistant and-sensitive VZV isolates

VZV strain	ACV sensitivity	EC_{50} (μM)					
		ACV	183792	243672	529311	568561	570886
Ellen	Sensitive	16.4	2.3	NT[a]	1.2	4.1	1.2
40[a]	Resistant	> 444	1.3	NT	0.3	3.9	1.1

[a] Not tested.

The inhibition of wild-type and ACV-resistant VZV was also determined for the 4-oxo-DHQ analogs. In these studies, only two VZV isolates, one ACV-sensitive and one ACV-resistant were tested. All five compounds tested were more active (mean EC_{50} = 2.2 μM) than ACV (mean EC_{50} = 16.4 μM) against the wild-type isolate and all were active (mean EC_{50} = 1.6 μM) against the ACV-resistant VZV isolate (Table 5).

3.2. Activity against CMV

A variety of both laboratory and clinical isolates of CMV were inhibited by all five of these compounds and all had activity that was equivalent to or greater than GCV (mean EC_{50} = 8.8 μM) (Table 6). The most active were PHA-529311 (mean EC_{50} = 1.0 μM) and PHA-570886 (mean EC_{50} = 1.3 μM). In other studies, all five compounds were effective in inhibiting the activity of the CMV polymerase, with PHA-568561 (mean EC_{50} = 0.3 μM) and PHA-529311 (mean EC_{50} = 0.3 μM) being the most active (Table 6). In addition, murine CMV and Rhesus CMV were also sensitive to these compounds at concentrations 3–10 fold less than that for GCV. To determine if drug-resistant isolates of CMV were susceptible to the compounds, GCV-and PFA-resistant isolates representing both UL97 and UL54 mutants were tested. Generally, all five compounds retained their activity against

Table 6
Activity of 4-oxo-DHQ analogs against wild-type CMV isolates

Virus strain	Mean EC_{50} (μM) ± S.D.[a]					
	GCV	183792	243672	529311	568561	570886
AD169	3.8 ± 0.1	6.4 ± 0.2	8.1	1.1 ± 0.02	5.8 ± 1.6	1.2 ± 0.1
Davis	3.4 ± 0.6	5.5 ± 2.6	2.0	0.8 ± 0.6	3.9 ± 3.2	1.6 ± 1.0
Towne	3.2 ± 2.6	2.1 ± 0.1	1.2	0.6 ± 0.4	1.7 ± 0.1	0.7 ± 0.4
Toledo	37.2 ± 51.3	7.7 ± 0.2	NT[b]	1.5 ± 0.1	5.8 ± 0.9	1.8 ± 1.2
EC	4.9 ± 0.3	4.7 ± 2.1	NT	1.2 ± 0.4	5.8 ± 2.1	1.5 ± 0.2
Coffman	5.5 ± 1.2	5.0 ± 4.0	6.9	1.3 ± 0.02	5.2 ± 1.2	1.2 ± 0
C8708/17-1-1	3.6 ± 4	3.7 ± 1.3	1.3	0.9 ± 0.02	3.9 ± 1.3	1.4 ± 0.02
Mean ± S.D.	8.8 ± 12.5	5.0 ± 1.8	3.9 ± 3.3	1.0 ± 0.2	4.6 ± 1.5	1.3 ± 0.3
AD169 DNA polymerase	NT	0.7 ± 0.2	1.2 ± 0.5	0.3 ± 0.1	0.3 ± 0.04	0.5 ± 0.1
MCMV	4.3 ± 1.7	1.4 ± 0.3	NT	0.3 ± 0.1	0.8 ± 0.6	0.2 ± 0.02
Rhesus CMV	11.3 ± 5.5	1.0 ± 0.9	NT	0.2 ± 0.02	3.0 ± 3.4	0.1 ± 0.1

[a] Values represent the mean ± standard deviation of two assays, except for 243672.
[b] Not tested.

Table 7
Activity of 4-oxo-DHQ analogs against drug-resistant HCMV isolates

Virus strain	Resistance		Mean EC_{50} (μM) ± S.D.[a]					
		Control		183792	243672	529311	568561	570886
759^{r}D100	GCV	GCV	113 ± 70	4.2 ± 3.3	3.2	1.1 ± 0.02	2.4 ± 1.1	0.7 ± 0.02
C8706/13-1-1	GCV	GCV	13.3 ± 2.7	1.7 ± 0.3	NT[b]	0.4 ± 0.02	1.1 ± 0.1	0.7 ± 0.2
C8914-6	GCV	GCV	134 ± 81	> 11.0	9.7	3.3 ± 1.1	> 8.7	5.4 ± 0.4
C8805/37-1-1	GCV	GCV	38 ± 0	3.0 ± 1.2	1.5	0.7 ± 0.4	3.9 ± 1.2	1.1 ± 0.1
1117^{r}	CDV, GCV	GCV	40.3 ± 5.1	1.5 ± 0	1.1	0.4 ± 0.02	1.5 ± 0.02	1.0 ± 0.04
$GDG^{r}P53$	CDV, GCV	GCV	42.7 ± 39	9.8 ± 2.1	1.6	2.1 ± 0.4	8.8 ± 0.6	3.8 ± 0.5
$PFA^{r}B300$	PFA	PFA	657 ± 633	4.7 ± 2.8	NT	0.6 ± 0.8	4.1 ± 0.4	1.4 ± 0.1
$VR4760^{r}$ (Va1715 Met)[c]	PFA	PFA	373 ± 70	0.9 ± 0.4	1.5	0.4 ± 0.1	1.1 ± 0.4	0.7 ± 0.1
$VR4955^{r}$ (Thr 700 Ala)[c]	PFA	PFA	613 ± 200	1.6 ± 0.1	1.6	0.5 ± 0.2	1.4 ± 0.4	0.9 ± 0.1

[a] Values represent the mean ± standard deviation of two assays, except 243672.
[b] Not tested.
[c] Mutation in UL54.

Table 8
Activity of 4-oxo-DHQ analogs against EBV, HHV-6, and HHV-8

Virus	Mean EC_{50} (μM) ± S.D.[a]						
	ACV	CDV	183792	243672	529311	568561	570886
EBV IFA	3.3 ± 2.7	–	1.1 ± 0.7	3.7 ± 4.6	> 26	0.9 ± 0.6	0.6 ± 0.7
EBV ELISA	3.0 ± 2.7	–	0.9 ± 0.3	1.6 ± 0.6	0.8 ± 0.8	3.4 ± 4.9	0.7 ± 0.7
EBV DNA	3.8 ± 2.2	–	2.6 ± 0.8	9.7 ± 5.8	37 ± 46	> 43	0.9 ± 0.6
HHV-6A (GS)	–	3.5 ± 1.9	> 20.1	> 23.1	> 12	> 18.8	> 17.9
HHV-6B (Z-29)	–	0.6 ± 0.3	> 22	> 23.1	> 20.8	> 18.8	4.6 ± 1.4
HHV-8	–	9.2 ± 4.1	8.7 ± 5.9	> 23.1	1.9 ± 0.1	11.1 ± 6.2	4.1 ± 2.5

[a] Values represent the mean ± standard deviation of two assays.

both GCV-and PFA-resistant mutants. Again, PHA-529311 and PHA-570886 were the most active against the panel of GCV- and PFA-resistant viruses. (Table 7)

3.3. Antiviral activity against EBV, HHV-6, and HHV-8

Antiviral activity against EBV was measured in three different assay systems (Table 8). For the positive control, ACV, activity was equivalent in all three assays. In the EBV IFA assay the activity of the 4-oxo-DHQ's was similar for all the compounds, except for PHA-529311, which was inactive. In the EBV Elisa assays all the compounds had equivalent activity except for PHA-243672. In the EBV DNA hybridization assay, PHA-570886 and PHA-183792 were the most active. In all three EBV assay systems, PHA-570886 was consistently the most active, followed by PHA-183792.

None of this series of compounds had activity against HHV-6A (Table 8) and only PHA-570886 had some moderate activity against HHV-6B. Against HHV-8 only two of the analogs had better activity than the positive control, CDV, two were equivalent, and one was inactive (Table 8).

3.4. Toxicity of 4-oxo-DHQ analogs

In both non-proliferating and proliferating HFF cells the two compounds that had the best antiviral activity, PHA-592311 and PHA-570886, were considerably more toxic than ACV or GCV (Table 9). In proliferating HSB-2 cells, none were toxic at the highest concentrations tested, and only PHA-570886 was toxic at the concentrations tested in proliferating Daudi cells.

4. Discussion

The non-nucleoside 4-hydroxyquinoline derivatives of naphthalene carboxamides have been reported to have activity against most herpesviruses but not other DNA or RNA viruses (Vaillancourt et al., 2000; Oien et al., 2002; Brideau et al., 2002). The best studied of the compounds, PHA-183792, inhibits CMV DNA polymerase and exhibits activity against HSV, VZV, CMV, and provided protection in mice infected with murine CMV (Brideau et al., 2002). In contrast, human DNA polymerases α, δ, and γ were not inhibited. Additional chemical modifications of the oxo-dihydroquinolines produced the derivatives reported here.

In the current studies we compared the activity of PHA-183792 with four additional analogs for their activity against HSV-1, HSV-2, VZV, HCMV, MCMV, EBV, HHV-6, and HHV-8 and for their toxicity in fibroblast and lymphoblastic cells. The compound PHA-183792 along with PHA-529311 and PHA-570886 had activity that was equivalent to or better than ACV against a panel of 10-wild type HSV-1 and 10 wild-type HSV-2 isolates. All four compounds were also active against ACV-resistant HSV-1 and HSV-2 isolates. Similarly, all five compounds were active against ACV-sensitive and ACV-resistant VZV. Since resistance to ACV is correlated with a mutation in the thymidine kinase gene for HSV and VZV (Field and Biron, 1994; Talarico et al., 1993) these polymerase inhibitors may clearly offer a new treatment for ACV-resistant herpesvirus infections.

All five of the 4-oxo-DHQ analogs were more active in cell culture studies than GCV against a panel of laboratory as well as clinical isolates of HCMV. Two surrogate viruses

Table 9
Toxicity of 4-oxo-DHQ analogs

Drug	Neutral red assay HFF cells CC_{50} (μM)[a]	Cell proliferation assay HFF cells IC_{50} (μM)[b]	Cell proliferation assay HSB-2 cells IC_{50} (μM)[b]	Cell proliferation assay Daudi cells IC_{50} (μM)[b]
183792	> 24	44	> 23.5	> 117
243672	> 23	> 46	> 23.1	> 116
529311	15	18	> 20.8	> 104
568561	> 19	23	> 18.8	> 94
570886	13	9	> 17.9	15.9 ± 1.4
ACV	> 444	> 444	–	> 222
GCV	> 392	157	–	157
CDV	> 317	–	> 159	–

[a] Cell cytotoxicity assay.
[b] Inhibition of cell proliferation assay.

used in animal models for HCMV, MCMV and RhCMV were also sensitive to all the analogs. All five compounds were active against GCV-resistant CMV isolates that had mutations in UL97 or in the DNA polymerase (UL54) induced by foscarnet. Additionally, two isolates that were resistant to both GCV and CDV and had mutations in both UL97 and the DNA polymerase were sensitive to the 4-oxo-DHQ analogs. These results further document the specificity of the mechanism of action of these compounds for the conserved domain III of the herpesvirus DNA polymerase (Thomsen et al., 2003). Using an ELISA to measure production of EBV VCA all the compounds were active, whereas more sporadic results were obtained by IFA and a DNA hybridization assay. As reported in an earlier paper, HHV-8 was also susceptible to the analogs (Thomsen et al., 2003).

The two pyrolloquinoline compounds tested (529311 and 570886) were consistently the most active against all the herpesviruses tested with the exception of HHV-6. This lack of activity against HHV-6 has been correlated with the presence of alanine instead of valine at the conserved domain III of the DNA polymerase of HHV-6, as compared to the DNA polymerases from the susceptible herpesviruses (Thomsen et al., 2003).

All five derivatives in the current study were active against drug-resistant strains of HSV-1, HSV-2, HCMV, and VZV. In previous studies, drug resistance to these compounds has been correlated with a point mutation in the conserved domain III resulting in an amino acid change (Thomsen et al., 2003). Although their target is the viral DNA polymerase, the 4-oxo-DHQ analogs retained their activity against the HCMV isolates that had polymerase mutations induced by foscarnet, thus pointing to a non-overlapping resistance profile with other anti-herpesvirus compounds.

All of the 4-oxo-DHQ analogs, particularly PHA-529311 and PHA-575886, appeared to be more toxic in both fibroblast and lymphoblastic cells, than GCV or CDV. The other three analogs had a respectable selective index (> 10) for most of the viruses tested.

These results indicate that all of these compounds effectively inhibit the replication of HSV-1, HSV-2, VZV, CMV, EBV, and HHV-8 in cell culture studies, and that they are active against ACV-, GCV-, and PFA-resistant mutants. The results obtained from these functional virus assays confirm the results reported earlier for inhibition of the various DNA polymerases for each of the herpesviruses studied (Vaillancourt et al., 2000; Brideau et al., 2002; Oien et al., 2002; Thomsen et al., 2003).

The major advantage of this class of compounds is that they exhibit activity against all the herpesviruses tested except for HHV-6 and HHV-7. Additionally, they are active against a variety of drug-resistant mutants. Although they may be more toxic than the currently licensed drugs, they deserve to be evaluated further in animal model studies as well as other pre-clinical, toxicological, and pharmacological studies to determine their potential for use in treating human herpesvirus infections.

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