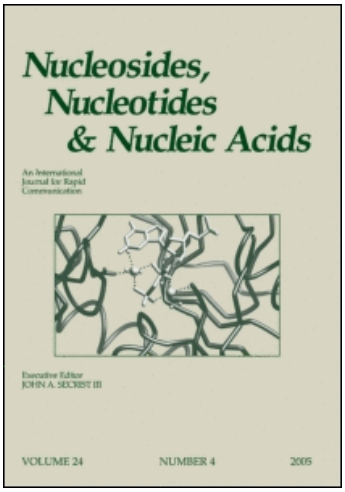

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Efficacy of Methylenecyclopropane Analogs of Nucleosides Against Herpesvirus Replication In Vitro[#]

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ABSTRACT

We have reported previously that purine methylenecyclopropane analogs are potent agents against cytomegaloviruses. In an attempt to extend the activity of these compounds, the 2-amino-6-cyclopropylaminopurine analog, QYL-1064, was selected for further study by modifying the purine 6 substituent. A total of 22 analogs were tested against herpes simplex virus types 1 and 2 (HSV-1, HSV-2), varicella zoster virus (VZV), human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV), Epstein-Barr virus (EBV), human herpesvirus type 6 (HHV-6) and human herpesvirus type 8 (HHV-8). Ten of the analogs had activity against at least one of the viruses tested. One compound had moderate activity against HSV-1 and six had activity against VZV. All but one compound was active against HCMV with a mean EC_{50} of $2.1 \pm 0.6\,\mu M$, compared with a mean EC_{50} of $3.9 \pm 0.8\,\mu M$ for ganciclovir. Of special interest was the fact that eight of the ten compounds were active against both HHV-6A and HHV-6B with

[#]In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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mean EC_{50} values of $6.0 \pm 5.2\,\mu M$ and $<2.4 \pm 1.5\,\mu M$, respectively. Only two compounds had activity against EBV, whereas all but one compound was active against HHV-8 with a mean EC_{50} of $3.1 \pm 1.7\,\mu M$. These results indicate that members of this series of methylenecyclopropane analogs are highly active against HCMV, HHV-6, and HHV-8 but are less active against HSV, VZV, and EBV.

Key Words: Nucleoside analogs; Herpesvirus; Antiviral; In Vitro.

INTRODUCTION

The family Herpesviridae is classified into three subfamilies based on genomic organization, tissue tropism, and other biological properties.[1] Members of the subfamily Alphaherpesvirinae include herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and varicella zoster virus (VZV). They have a variable host range, short reproductive cycle, spread rapidly in culture, and establish latent infections primarily in sensory ganglia.[2] In the United States, the most common cause of sporadic life-threatening encephalitis is HSV-1, with mortality rates reaching 70% if left untreated and most survivors experience severe neurologic sequelae.[3] Other clinical manifestations of HSV-1 infection includes neonatal herpes[4] and oral herpes.[5] HSV-2 is the primary causative agent in neonatal herpes[4] and infections of the genital tract.[3,5] VZV is the agent of chickenpox (varicella) and can reactivate years later to produce shingles (zoster) and postherpetic neuralgia.[6]

Members of the subfamily Betaherpesvirinae include human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV), and human herpesvirus type 6 (HHV-6). They have a restricted host range, long reproductive cycle, and a slow infection rate in culture. These viruses maintain a latent form in mononuclear leukocytes, secretory glands, kidneys, and other various tissues.[2] HCMV infections occur in 1 to 2% of all live births and are the leading cause of congenital virus infections.[3] HCMV infects approximately 40 to 80% of the U.S. population with the fetus, neonate, and immunocompromised individual being the most vulnerable to severe complications.[7] Clinical manifestations of HCMV include pneumonitis, hepatitis, retinitis, colitis, increased frequency of graft rejection and opportunistic infections after organ transplant in immunocompromised patients.[8] The immunocompetent individual rarely exhibits symptoms.[7,9] HHV-6 variants A and B (categorized by restriction endonuclease maps) are lymphotropic herpesviruses and share several properties with CMV, such as sequence homology and the ability to establish latent infections in the host.[2] No clear disease has yet been connected with HHV-6A, but HHV-6B is a causative agent in exanthem subitum.[3]

Members of the subfamily Gammaherpesvirinae include two genera: the lymphocryptoviruses (γ-1 herpesvirus, Epstein-Barr virus (EBV)) and the rhadinoviruses (γ-2 herpesvirus, human herpesvirus type 8 (HHV-8)).[1] EBV is a B-cell lymphotropic agent that can transform cells in vitro and is the causative agent of infectious mononucleosis and B cell lymphomas. HHV-8, the etiologic agent of Kaposi's sarcoma[10,11] replicates in mononuclear cells and is associated with primary effusion lymphoma and some forms of multicentric Castleman's disease.[1,10–12]

Currently there are only a few licensed antivirals available for treatment of herpesvirus infections. These include the acyclic guanosine analogs acyclovir

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(ACV), ganciclovir (GCV), and their oral prodrugs, an acyclic nucleotide analog cidofovir (CDV), a pyrophosphate analog, foscarnet (PFA),[13] and an antisense phosphorothioate oligonucleotide, fomivirsen.[14] ACV is the most widely used antiherpesvirus drug available at this time and is used for the treatment of HSV and VZV infections.[15] It is active against EBV in vitro but has little antiviral activity against HHV-6 or HHV-8 replication.[16] GCV, CDV, and PFA have been used to treat HCMV, HHV-6, and HHV-8 infections but prolonged use is problematic due to low oral bioavailability, poor penetration of drug into the eye of retinitis patients,[17] and severe side effects due to toxicity if used over an extended period of time.[16] Fomivirsen, used to treat CMV retinitis in AIDS patients, inhibits replication of HCMV and is active against clinical isolates and GCV drug resistant CMV.[14]

We have reported previously that purine methylenecyclopropane analogs are potent agents against cytomegaloviruses.[7,9,18–21] The 2-amino-6-cyclopropylamino-purine (QYL-1064) was selected for further studies because its activity in vivo was equal to or better than GCV.[22] This XCC series of analogs is an extension of the previous QYL series of methylenecyclopropane analogs, and were synthesized by modifying the purine 6 substituent in QYL-1064.[18,23] All compounds in the XCC series are *Z*-isomers and *S*-(+)-enantiomers with a purine base. The 6 substituent varies according to different compounds in the series (Fig. 1). The purpose of the following studies was to determine the activity of these nucleoside analogs against the various herpesviruses. While we have evaluated a total of 22 compounds in this series, only the ten with antiviral activity against at least one herpesvirus are included in this communication.

MATERIALS AND METHODS

Compounds and Antibodies

The XCC series of methylenecyclopropane analogs were provided through the Antiviral Research Branch, NIAID, NIH. The synthesis of these compounds was reported previously[18,22] and the structures are presented in Fig. 1. They were obtained in powder form and reconstituted in 10% dimethyl sulfoxide (DMSO) at 10 mg/mL, then diluted 1:10 in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 μ/mL penicillin and 25 μg/mL gentamicin to yield a final drug concentration of 1 mg/mL. ACV, CDV, and GCV were purchased from the University of Alabama Hospital Pharmacy and prepared in minimal essential media (MEM) containing 2% FBS, 2 mM L-glutamine, penicillin and gentamicin and stored at 4°C. Primary antibodies for flow cytometry were purchased from Chemicon (Temecula, Calif.) and secondary antibodies from Jackson ImmunoResearch (West Grove, Pa.).

Cell Cultures

Human foreskin fibroblast (HFF) cells and mouse embryo fibroblast (MEF) cells were prepared as primary cultures as reported previously[7,9,24,25] and used in

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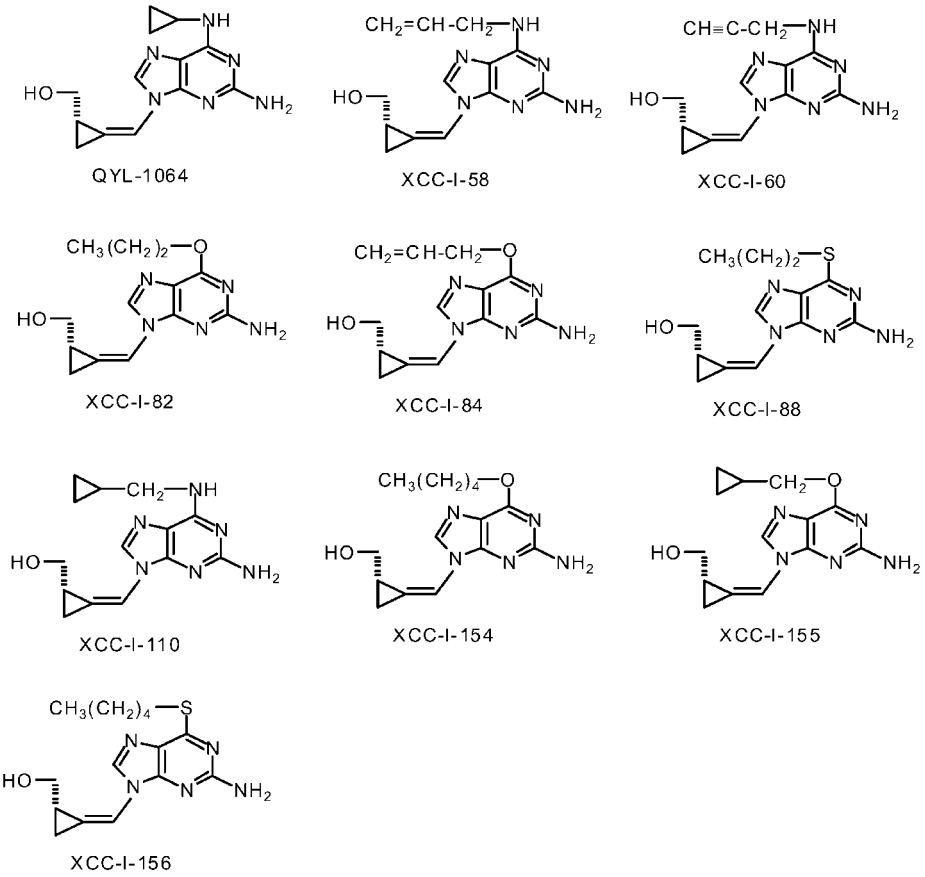

Figure 1. Structure of QYL-1064 and XCC series.

the HCMV, MCMV, HSV-1, HSV-2, and VZV assays. These cells were propagated in MEM containing 10% FBS, L-glutamine, penicillin and gentamicin. Daudi cells (American Type Culture Collection, Manassas, Va.), the human T cell lymphoblastoid line, HSB-2 cells, and body cavity-based lymphoma (BCBL-1) cells (NIH AIDS Research and Reference Program, Rockville, Md.) were split 1:5 every 3–4 days and used in the EBV, HHV-6A, and HHV-8 assays, respectively. These cells were propagated in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, and standard concentrations of antibiotics. Cord blood lymphocyte (CBL) cells were isolated from fresh heparinized umbilical cord blood obtained from the University of Alabama Hospital and prepared as described previously.[24] CBLs used in the HHV-6B assays were propagated in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin and gentamicin, streptomycin, fungizone, Interleukin-2 (Sigma, St. Louis, Mo.) and Phaseolus Vulagaris agglutinin protein.

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Virus Pools

HSV-1, HSV-2, HCMV, MCMV, and VZV. Viruses were propagated using standard virological techniques, as reported previously.[7,25]

EBV. P3HR-1 cells were used to propagate EBV. The cells were incubated for two weeks at 34°C. After incubation, cells were spun down using a Sorvall GSA rotor at 6000 rpm for 15 min. The supernatant was collected and spun down for 90 min at 12000 rpm. The virus pellet was resuspended in 1/100 of the original volume.

HHV-6A_{GS} and HHV-6$B_{Z\text{-}29}$. HHV-6A_{GS} was propagated in HSB-2 cells which were maintained by passaging every 3–4 days. Stock titers of virus was obtained by monitoring percentage of infected cells by immunofluorescence assays (IFA) followed by collection, centrifugation, and freezing. HHV-6$B_{Z\text{-}29}$ was propagated in CBLs by incubation for 10 days followed by monitoring, collection, centrifugation, and freezing.

HHV-8. BCBL-1 cells are a continuous cell line that is latently infected with HHV-8. Lytic HHV-8 infection was induced by the addition of 100 ng/mL Phorbol 12-myristate 13-acetate (TPA).

Determination of Antiviral Drug Cytotoxicity and Cell Proliferation for Adherent Cell Lines (HFF, MEF)

A neutral red uptake assay was performed with HFF or MEF cells as previously described.[20,24] The concentration of drug that reduced cell viability by 50% (CC_{50}) was calculated using MacSynergy II software.[26] A cell proliferation assay was performed with HFF cells as previously reported.[20,24] Inhibition of cell proliferation (IC_{50}) values were calculated using MacSynergy II software.

Determination of Antiviral Drug Cytotoxicity and Cell Proliferation for Non-adherent Cell Lines (HSB-2, CBL, Daudi, BCBL-1)

Cell Cytotoxicity Assay. Serial 5-fold dilutions of drug starting at 50 μg/mL were prepared in media and added to 1×10^6 cells. Controls were prepared by incubating 1×10^6 cells in drug-free media. After incubation of 3–6 days depending on the assay, 200 μL was transferred to a 96 well plate in duplicate, 20 μL of MTS added, the plate wrapped in foil and incubated at 37°C for 4 h. MTS was bioreduced by dehydrogenase enzymes found in metabolically active cells into an aqueous soluble formazan. The quantity of formazan product was measured at 490 nm absorbance and was directly proportional to the number of living cells in culture. Drug concentration was plotted against the optical density of each sample and CC_{50} values were calculated using MacSynergy II software.

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Cell Proliferation Assay. Serial 5-fold dilutions of drug starting at 50 μg/mL were prepared in media and added to 1×10^6 cells. Controls were prepared by incubating 1×10^6 cells in drug-free media. After an incubation period of 3–4 days, the number of cells for each sample was determined using hemacytometer slides (HSB-2 and Daudi cell lines only). Drug concentration was plotted against the total concentration of cells for each sample and IC_{50} values were calculated using MacSynergy II software.

Determination of Antiviral Drug Efficacy

Cytopathic Effect Inhibition Assay for HSV and VZV. Low passage HFF cells were seeded into 96 well tissue culture plates at 2.5×10^5 cells/mL in MEM supplemented with 10% FBS. After 24 h media was removed from the first row of cells and 125 μL of experimental drug added to triplicate wells. ACV was used as a positive control. The drug in the first row of wells was diluted serially 1:5 throughout the remaining wells. After a 1 h incubation, 100 μL of the appropriate virus concentration was added to each well, excluding cell control wells, which received 100 μL of MEM. For HSV-1 and HSV-2, the virus concentration utilized was 1000 pfu per well, and for VZV 2500 pfu per well. The plates were incubated at 37°C for three days for HSV-1 and HSV-2, and 10 days for VZV. After incubation, media was aspirated and cells stained with a 0.5% crystal violet solution in ethanol and formaldehyde for 4 h. The stain was removed and plates rinsed using tap water until all excess stain was removed. The plates were allowed to dry for 24 h and read on a BioTek Plate Reader at 620 nm. By comparing drug treated with untreated wells, EC_{50} values were calculated using the MacSynergy II linear regression program.[25]

Plaque Reduction Assay for HCMV, MCMV, and VZV. HFF or MEF cells were placed into 6 well or 12 well plates and incubated at 37°C. Two days (HFF) or one day (MEF) later, drug was serially diluted 1:5 in MEM with 2% FBS starting at 100 μg/mL. ACV or GCV was used as positive controls. Virus was diluted in MEM containing 10% FBS to contain 20–30 plaques per well. The media was aspirated and 0.2 mL of virus added to each well in triplicate with 0.2 mL of media added to drug toxicity wells. The plates were incubated for 1 h with shaking every 15 min. and the appropriate drug concentration added to each well. After incubation for 7 days for MCMV, 8 days for HCMV and 10 days for VZV, cells were stained for 6 h with 2 mL of 5.0% neutral red/PBS solution. The stain was aspirated, and plaques counted using a stereomicroscope at 10× magnification. By comparing drug treated with untreated wells, EC_{50} values were calculated using the MacSynergy II software program.[26]

Determination of Antiviral Drug Efficacy Against HHV-6. Serial 5-fold dilutions of test compounds or CDV were prepared in media starting at 50 μg/mL. To determine antiviral efficacy 1×10^6 cells were incubated for 1 h with sufficient virus to infect approximately 35% of the cells. After infection, the appropriate dilution

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of drug was added and cells incubated for 4 to 6 days at 37°C. Negative controls were prepared by incubating 1×10^6 cells in drug-free media for the designated period and virus controls prepared by incubating 1×10^6 cells for 1 h with sufficient virus to infect 35% of the cells followed by incubation in drug-free media for the designated period. After incubation, cells were rinsed with PBS and permeabilized overnight in methanol at −80°C for use in flow cytometric assays (FCA).

Determination of Antiviral Drug Efficacy Against HHV-8. Serial 5-fold dilutions of test compounds or CDV were prepared in media starting at 50 μg/mL. To determine antiviral efficacy, 1×10^6 TPA induced BCBL-1 cells were incubated with the appropriate dilution of drug for five days with 2 mL fresh media added on day two of incubation. TPA induced and uninduced controls were prepared by incubating 1×10^6 TPA induced and uninduced BCBL-1 cells in drug-free media. After incubation, cells were rinsed with PBS and permeabilized overnight in methanol at −80°C for FCA.

Flow Cytometric Assay for HHV-6 and HHV-8. Methods for staining cells have been reported previously.[16] After staining, samples were fixed in 2% paraformaldehyde in PBS and analyzed using a Becton-Dickenson FacsCalibur instrument (Becton-Dickenson, Franklin Lakes, N.J.). Flow cytometry data were analyzed using the WinMDI 2.7 data analysis program (Scripps Research Institute, La Jolla, Calif.) and the EC_{50} value was extrapolated from the plot of drug concentration vs. percentage of antigen positive cells.[24]

Determination of Antiviral Drug Efficacy Against EBV. Serial 5-fold dilutions of test compounds or ACV were prepared in media starting at 50 μg/mL. To determine antiviral efficacy 1×10^6 Daudi cells were incubated for 1 h with sufficient EBV P3HR-1 to infect 10% of the cells. After infection, appropriate dilutions of drug were added and cells incubated for three days at 37°C. Virus free controls were prepared by incubating 1×10^6 Daudi cells in drug-free media for three days at 37°C. Virus controls were prepared by incubating 1×10^6 Daudi cells with sufficient EBV P3HR-1 for 1 h to infect 10% of the cells, followed by incubation in drug-free media for three days at 37°C. After incubation, hemacytometer slides were used to determine the concentration of cells for each sample. Cells were rinsed thoroughly with PBS and for each dilution of drug, 4×10^5 cells were added to three duplicate wells of a 96 well plate and allowed to dry. These plates were used for Enzyme-linked immunosorbent assay (ELISA).

ELISA. Cells were fixed in 95% ethanol/5% acetic acid for 20 min. at room temperature and then incubated at 37°C with a monoclonal antibody to EBV viral capsid antigen (Chemicon, Temecula, Calif.) for 1 h, followed by an incubation with horseradish peroxidase labeled goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, Ala.) for thirty min. Plates were rinsed thoroughly with PBS containing 0.005% Tween 20 between incubations. Substrate containing o-phenylenediamine dihydrochloride, citrate buffer (pH 5.0) and hydrogen peroxide was added to each well to initiate the colorimetric reaction. Plates were covered and gently shaken at room temperature for ten minutes. The reaction was stopped by the

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addition of 3N sulfuric acid. Plates were immediately read on a microplate reader (Bio-Tek Instruments) at 492 nm. The EC_{50} value for each drug was extrapolated from the plot of drug concentration vs. average OD_{492} for each concentration of drug.[24]

RESULTS

Activity of Methylenecyclopropane Analogs Against HSV-1, HSV-2, or VZV. To determine the activity of this series of analogs against alphaherpesvirus replication, the parent compound, QYL-1064 and analogs were tested against a representative strain of HSV-1, HSV-2, and VZV. Their activity compared with the positive control, ACV, is summarized in Table 1. The EC_{50} for ACV ranged from 5–7 μM for the three viruses. The parent QYL-1064 had little activity against HSV, but had an EC_{50} of 7 μM against VZV. Only one of the analogs, XCC-I-155, was active against HSV-1, but it was not active against HSV-2. Five of the compounds had activity against VZV, four of which were comparable to ACV. Since only one analog, XCC-I-155, was active against HSV-1 and VZV, there was little correlation between the three alphaviruses in their susceptibility to these compounds.

Activity Against HCMV, MCMV, HHV-6A, and HHV-6B. The next series of experiments were designed to determine the in vitro activity of these analogs against 4 members of the betaherpesviruses group and the results are summarized in Table 2. The positive control for HCMV and MCMV was GCV. The parent compound QYL-1064 and all of the analogs except one had activity against HCMV that was as good as, or better than GCV. In addition, all the compounds had very good activity against MCMV, a surrogate virus used for animal studies. The positive control for HHV-6A and HHV-6B was CDV and both viruses were inhibited by about

Table 1. Activity of methylenecyclopropane analogs against HSV-1, HSV-2, and VZV.

	EC_{50} (μM)[a]		
Compound	HSV-1	HSV-2	VZV
ACV	7.24 ± 2.4	5.5 ± 1.6	2.1 ± 3.3
QYL-1064	63.5	>73.4	7.0
XCC-I-58	227	>367	>367
XCC-I-60	>370	>370	>74
XCC-I-82	42.5	62.8	0.5
XCC-I-84	>366	>366	1.1
XCC-I-88	>343	65.9	>69
XCC-I-110	>349	>349	10.1
XCC-I-154	>329.6	>329.6	>65.9
XCC-I-155	6.3	171.6	0.5
XCC-I-156	49.2	310.9	2.3

[a]These results are presented as means ± standard deviation of several assays.

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Table 2. Activity of methylenecyclopropane analogs against HCMV, MCMV, HHV-6A, and HHV-6B.

	EC_{50} (μM)[a]			
Compound	HCMV	MCMV	HHV-6A	HHV-6B
CDV	NT[b]	NT[b]	3.1 ± 1.2	1.0 ± 1.0
GCV	3.9 ± 0.8	5.2 ± 0.5	NT[b]	NT[b]
QYL-1064	1.9	0.1	3.3 ± 2.7	1.6 ± 0.7
XCC-I-58	2.8	0.3	8.3 ± 3.9	3.6 ± 1.4
XCC-I-60	2.6	0.4	100.1 ± 2.4	5.4 ± 0.6
XCC-I-82	2.0	<0.1	1.1 ± 1.2	1.0 ± 0.2
XCC-I-84	2.1	0.3	3.8 ± 3.3	<1.1 ± 1.4
XCC-I-88	3.0	1.4	9.6 ± 6.0	3.0 ± 2.7
XCC-I-110	52.0	1.7	107.8 ± 51	90 ± 43.3
XCC-I-154	1.4	1.4	2.3 ± 2.3	1.2 ± 0.08
XCC-I-155	1.7	0.3	3.1 ± 1.0	1.2 ± 0.5
XCC-I-156	1.5	1.4	16.8 ± 15.6	3.4 ± 0.2

[a]These results are presented as means ± standard deviation of several assays.
[b]NT = Not tested.

2 μM. In general, most of the analogs had activity against both types of HHV-6 at levels of 3–10 μM and there was good agreement between them and HCMV. Overall, these compounds were more efficacious against HHV-6B than HHV-6A.

Activity Against EBV and HHV-8. We next determined the activity of this series of compounds against two members of the gammaherpesviruses, EBV and HHV-8, and the results are summarized in Table 3. The positive control, ACV, for EBV had an EC_{50} of 6.2 μM and similar results were obtained for QYL-1064. Only one other compound in this series, XCC-I-110, had appreciable activity. In contrast, all the compounds had activity against HHV-8 ranging from 1–6 μM and all were more active than the positive control, CDV. Therefore, with the exception of QYL-1064, there was essentially no correlation in activity of these analogs between the two gammaherpesviruses EBV and HHV-8.

Toxicity of Methylenecyclopropane Analogs. Toxicity of these compounds in HFF and MEF cells was determined by two methods that represent differing types of toxicity. The first was a cytotoxicity assay (CC_{50}) that was determined by uptake of a vital dye, neutral red, to assess direct cytotoxicity or cell killing. The second method measured inhibition of cell proliferation (IC_{50}) and indicates the effect a compound has on cell growth. All of the analogs were essentially non-cytotoxic for both HFF and MEF cells and did not inhibit cell proliferation at concentrations that were at least ten times their EC_{50} (Table 4). The CC_{50} and IC_{50} was also determined for the lymphoblastic cells, HSB-2, CBL, Daudi, and BCBL-1 cells. As seen above in fibroblast cultures the methylenecyclopropane analogs were generally not toxic to lymphoblastic cells whether determined by direct cytotoxicity or inhibition of cell proliferation (Table 5).

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Table 3. Activity of methylenecyclopropane analogs against EBV and HHV-8.

	EC_{50} (μM)	
Compound	EBV	HHV-8
ACV[b]	6.2 ± 3.4	NT[a]
CDV[b]	NT[a]	12.8 ± 13.8
QYL-1064	6.2	1.5 ± 0.7
XCC-I-58	>183.7	4.05 ± 0.8
XCC-I-60	>185.1	6.45 ± 1.2
XCC-I-82	>181.6	1.1 ± 0.2
XCC-I-84	>183.0	2.85 ± 0.35
XCC-I-88	>171.6	3.7 ± 1.4
XCC-I-110	0.6	>174.6
XCC-I-154	23.4	1.2 ± 0.07
XCC-I-155	29.2	2.6 ± 0.7
XCC-I-156	39.8	4.2 ± 0.4

[a]NT = Not tested.
[b]These results are presented as means ± standard deviation of several assays.

Table 4. Toxicity of methylenecyclopropane analogs in HFF and MEF cells.

	CC_{50} (μM)[a]		IC_{50} (μM)[b]
Compound	HFF	MEF	HFF
ACV	> 444	> 444	> 444
GCV	> 359	> 359	> 359
QYL-1064	> 367	> 36.7	213
XCC-I-58	> 367	> 367	> 367
XCC-I-60	> 370	> 370	306
XCC-I-82	> 363	363	69.4
XCC-I-84	> 366	> 366	220
XCC-I-88	> 343	> 343	283
XCC-I-110	> 349	279	> 349
XCC-I-154	178	224	41.9
XCC-I-155	> 348	278	112
XCC-I-156	> 313	156	215

[a]Cell cytotoxicity determined by neutral red uptake.
[b]Cell proliferation assay.

DISCUSSION

Effective therapeutic agents, such as GCV, PFA, and CDV, have been utilized to combat the manifestations of serious chronic herpesvirus infections; however, their use has been limited due to adverse side effects and the selection of resistant strains. It is necessary therefore to continue searching for new, more active and less toxic

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Table 5. Toxicity of methylenecyclopropane analogs in HSB-2, CBL, Daudi, and BCBL-1 cells.

	CC_{50} (μM)				IC_{50} (μM)	
Compound	HSB-2[b]	CBL[b]	Daudi	BCBL-1[b]	HSB-2[b]	Daudi
ACV	NT[a]	NT[a]	>222	NT[a]	NT[a]	>222
CDV	>150 ± 23	>132 ± 28	NT[a]	>154 ± 20	>159	NT[a]
QYL-1064	26 ± 2	>178 ± 7	176	110 ± 25	>156 ± 4	13
XCC-I-58	>184	>184	>184	>184	>184	>184
XCC-I-60	162 ± 32	>185	>185	>185	>185	>185
XCC-I-82	26 ± 2	>162 ± 28	>182	>182	131 ± 72	>182
XCC-I-84	46 ± 38	>165 ± 25	>183	118 ± 2	163 ± 28	154
XCC-I-88	61 ± 41	>148 ± 33	>172	148 ± 33	83 ± 61	>172
XCC-I-110	>175	>175	>175	>175	>175	>175
XCC-I-154	>96 ± 97	>99 ± 93	>165	109 ± 26	>165	> 165
XCC-I-155	54 ± 29	138 ± 28	112	130 ± 18	>174	> 174
XCC-I-156	>156	>156	>156	>156	>156	>156

[a]NT = Not tested.
[b]These results are presented as means ± standard deviation of duplicate assay.

compounds to treat serious herpesvirus infections. A new series of nucleoside analogs was synthesized where a methylenecyclopropane group replaced the ribofuranose moiety and served as the parent compound.[20,21] QYL-1064 is the *S*-(+) enantiomer of the original racemic 2-aminopurine analog QYL-769 which was reported to be a potent inhibitor of replication of HCMV.[22] It also exhibited excellent antiviral activity that was equivalent or superior to GCV in SCID mice infected with HCMV.[22] The racemic 2-aminopurine analog, QYL-769 also had excellent activity against MCMV, rat CMV, rhesus monkey CMV, and guinea pig CMV that was comparable to GCV.[7] QYL-769 was then tested in MCMV infected mice and demonstrated significant activity when administered orally.[7] Thus, the *R* and *S* enantiomer of this racemic analog was synthesized in an attempt to extend the activity of these compounds.

The present study examined 22 methylenecyclopropane analogs substituted at the 6 purine position, of which ten had in vitro activity against at least one of the herpesviruses. Only one compound was active against HSV-1 and VZV, however all but one were found to be active against HCMV with activity similar to or better than GCV and were also active against MCMV and HHV-6B. Eight of the ten analogs tested in HHV-6A were found to be active and comparable to CDV.

Indirect evidence indicates that methylenecyclopropane nucleoside analogs are activated by phosphorylation,[18] and phosphorylated intermediates may be important in the mechanism of action of both *Z*- and *E*- methylenecyclopropane analogs of nucleosides.[19] Lack of enantioselectivity may suggest that a cellular enzyme with poor enantioselectivity such as deoxycytidine (dC) kinase may be involved. We have reported a strict *S* enantioselectivity in HCMV and MCMV which would indicate that an enzyme other than dC kinase is responsible for phosphorylation.[18] In CMV assays UL97 may be responsible for phosphorylation of

methylenecyclopropanes although in a different way than GCV.[27] Studies with a series of racemic methylenecyclopropane analogs including the pyrimidine derivatives have shown that neither thymidine kinase type 1 or 2, nor dC kinase are involved in phosphorylation (J. Balzarini, unpublished results). It has been suggested that HHV-6 lacks its own phosphorylating enzymes and that phosphorylation is dependent on the cell type used.[28] In our studies essentially all the analogs were active using either HSB-2 or CBL cells. We have observed unusually complex patterns of enantioselectivity in this series of purine methylenecyclopropane analogs which suggest that different phosphorylation enzymes may be involved in their mechanism of action.[18]

Of special interest was the fact that these compounds were active against HHV-8 but were inactive against EBV. Although genomic similarities are shared between these two viruses, they have been categorized into two different genera. Nuclear proteins responsible for episome maintenance and virus replication are related between the viruses and the genes encoding proteins that function to inhibit B cell receptor signaling and maintain virus latency are located at similar genomic locations.[1] There are several cellular homologues which the HHV-8 genome encodes that are not found in EBV, such as viral interleukin 6 and viral cyclin D.[1] It is unlikely that these differences are responsible for why these methylenecyclopropane analogs are active against HHV-8 but not against EBV. Since the mechanism of antiviral activity of these compounds appears to be related to their phosphorylation by viral or cellular kinases with subsequent inhibition of the viral polymerase by the corresponding triphosphate, the differential activity seen between the two gammaherpesviruses is probably due to differences in phosphorylation and/or inhibition of polymerase activity.

In a previous study using (*R*)-(−)- and (*S*)-(+)-synadenol (QYL-587A and QYL-788, respectively), both enantiomers were equipotent against HCMV and VZV but the (*S*)-(+) enantiomer was more effective against HSV-1, HSV-2 and MCMV.[18,29] Interestingly, the (*R*)-(−) enantiomer showed greater antiviral activity against EBV and HIV-1 than the *S*-enantiomer,[18,29] however activity was not determined for HHV-8. In the current study the XCC series are all (*S*)-(+) enantiomers which could help explain why they were inactive against EBV.

In addition to efficacy, cellular toxicity plays a crucial role in determining a compound's potential as an antiviral agent. Neutral red uptake assays in HFF and MEF cells indicated no toxicity of all ten of the active compounds. Cell proliferation assays indicated moderate or no toxicity of most of the compounds in HFF, MEF or lymphoblastic cells. The compound XCC-I-82 was the most efficacious compound against all herpesviruses tested and had activity comparable to ACV, CDV and GCV. These results support the potential use of these methylenecyclopropane analogs as antiviral agents and warrant the need for future evaluation of these and similar compounds.

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