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Effective treatment of murine cytomegalovirus infections with methylenecyclopropane analogues of nucleosides

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Abstract

A number of new nucleoside analogues with a Z- or E-methylenecyclopropane structure exhibited significant activity against human and murine cytomegaloviruses (HCMV, MCMV) in tissue culture that was generally comparable to, or greater than, 9-[(1-3-dihydroxy-2-propoxy)methyl]guanine (ganciclovir, GCV). Several of these analogues were chosen for further evaluation of therapeutic efficacy utilizing a MCMV infection. Intraperitoneal (i.p) inoculation of 3-week-old Balb/c mice with 2.0×10^5 plaque forming units (pfu) of MCMV results in an acute, lethal infection with rapid virus replication in visceral and glandular tissue, thus, making it an ideal model for identifying compounds that have potential for use in humans. Synadenol (QYL-284A) and synguanol (QYL-438) were administered i.p. once daily for 5 days initiated 6, 24, or 48 h post-viral infection. Significant protection was demonstrated at 50 and 16.7 mg/kg compared to placebo, with efficacy comparable to GCV. When delivered orally once or twice daily at 100 mg/kg per day, QYL-438 was active, but less effective than GCV. In addition, 2-amino-6-methoxypurine analogue (QYL-941) was active at 60 mg/kg administered orally twice daily, comparable to GCV, while it's prodrug (QYL-972) was as effective as GCV at 40 mg/kg when delivered twice daily for 5 days. Additionally, analogue 2-amino-6-cyclopropylaminopurine (QYL-769) was found to be highly efficacious when given orally twice daily for 5 days. Mortality of 0% and 13% was observed at 60 and 20 mg/kg, respectively, which was similar to GCV. Oral treatment with QYL-769 or GCV reduced virus replication in target organs, but neither resulted in complete clearance of MCMV. These data indicate that these new analogues have activity comparable to GCV when given orally to mice and should be evaluated further to assess their potential for use in humans.

Keywords: MCMV; Methylenecyclopropane analogues; Phosphoroalaninate prodrugs; Ganciclovir; Oral therapy; Pathogenesis; Mice

1. Introduction

Human cytomegalovirus (HCMV) is a prevalent virus with an extensive distribution and is the

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most significant cause of congenital viral infections, affecting 1% of all live births (Tsutsui et al., 1995). It is a prototype β-herpesvirus which replicates slowly and presents distinctive cytoplasmic and nuclear inclusions, and rarely produces symptoms in immunocompetent individuals (Kern, 1990; Mocarski, 1993). However, in the immunocompromised patient, the virus replicates and spreads throughout the host due to lack of cellular immunity. Therefore, an efficient immune system is necessary to control this virus (Schilt, 1987; Reddehase et al., 1994; Sinzger and Jahn, 1996). HCMV infects 40–80% of the US population with antibody commonly evident by adolescence (Gershon et al., 1997). In cases where clinical manifestations become apparent, they are often severe in the fetus, neonate, and immunocompromised patient (Kern, 1990; Gershon et al., 1997). Several disorders can result, including a mononucleosis-like syndrome, interstitial pneumonia, and various congenital infections affecting the nervous system, such as retardation, hearing loss, and microcephaly (Overall et al., 1976; Kern, 1988; Mercer et al., 1988). The urgency to discover clinically effective treatments for CMV has escalated due to the increasing incidence of immunodeficiencies that result from AIDS, cancer chemotherapy, and organ transplantation. It is estimated that 90–100% of AIDS patients test positive for CMV and often develop severe disease, such as retinitis or colitis (Alford and Britt, 1993; Reddehase et al., 1994).

HCMV is highly species specific and does not replicate or cause disease in animals. Therefore, a non-human CMV with biology similar to HCMV is needed in order to study the virus in animal models (Kern, 1990; Alford and Britt, 1993). Murine cytomegalovirus (MCMV) has proven to be an excellent surrogate model for evaluation of compounds for antiviral activity due to its similar structure, gene products, and target organs as HCMV (Manning et al., 1992; Rawlinson et al., 1996; Kern, 1999). In addition, MCMV is similar to HCMV in the development of acute and chronic infections, latency, and reactivation (Mercer et al., 1988). Intraperitoneal inoculation of BALB/c mice with 2.0×10^5 plaque forming units (pfu) of MCMV results in an acute infection, while 1.0×10^5 pfu causes a chronic infection. Both result in rapid virus replication in the lung, liver, spleen, kidney, intestine, salivary gland, and other visceral and glandular tissue (Kern, 1997). In the lethal infection, animals die approximately 5–7 days post-viral inoculation due to uncontrolled disease with massive tissue destruction (Jordan and Pomeroy, 1991; Kern, 1997). The validity of this model has been demonstrated previously, such as the confirmation that ganciclovir (GCV) is highly efficacious in both the MCMV mouse model and HCMV in humans (Shepp et al., 1985; Kern, 1991). Therefore, this model has proven to be ideal for rapid identification of potential antiviral compounds.

A number of antiviral drugs have been found to be effective against HCMV, such as cidofovir (CDV), foscarnet (PFA), and GCV (Neyts et al., 1992; Gershon et al., 1997). As previously stated, GCV has been shown to significantly reduce mortality when administered either orally or intraperitoneally to MCMV infected mice (Freitas et al., 1985; Kern, 1988, 1991, 1999; Smee et al., 1992). Although GCV is also highly effective in humans, relapses are common once treatment is terminated, thus, long term drug therapy must be maintained in order to sustain antiviral activity (Kern, 1990; De Castro et al., 1991]. In addition, major side effects such as neutropenia and thrombocytopenia may occur as well as the development of resistant isolates (Drew et al., 1991; Neyts et al., 1992, 1993). It has been estimated that approximately 7.6% of patients will produce HCMV resistance to GCV after 3 months of treatment (Drew et al., 1991). In addition, the phosphonyl nucleotide analogue, (*S*)-1-(3-hydroxy-2-phosphonyl methoxypropyl)cytosine (CDV) is a highly potent inhibitor of HCMV in vitro and in vivo (Kern, 1991; Neyts et al., 1992) and has been shown to be more potent in suppressing virus replication in tissues of mice compared to GCV (Smee et al., 1992). However, although CDV is a highly efficacious compound, its use is restricted to treatment of CMV retinitis in AIDS patients due to kidney toxicity (Lalezari, 1997; Safrin et al., 1997). Therefore, current therapeutic interventions are not optimal and improved anti-CMV agents are needed. Recently, nucleoside analogues with a Z- or E-methylenecyclopropane moiety have been evaluated for antivi-

ral activity. A number of these compounds have demonstrated in vitro activity against HCMV and MCMV, that was comparable to, or greater than, GCV (Qiu et al., 1998a,b). The purpose of the following studies was to further evaluate the antiviral activity of these compounds compared to GCV in tissue culture and to determine the therapeutic efficacy in BALB/c mice with acute MCMV infections.

2. Materials and methods

2.1. Experimental infection

Groups of 15 3-week-old female BALB/c mice, weighing approximately 10–12 g, obtained from Charles River Breeding Laboratories, Raleigh, NC, were inoculated intraperitoneally with 2.0×10^5 pfu of MCMV, strain Smith, in a 0.1 ml volume.

2.2. Media, virus strains, and cell cultures

The media utilized was Earle's minimal essential medium (MEM) (Mediatech Herndon, VA) with Earle's balanced salt solution supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 25 μg/ml gentamycin, and 2mM L-glutamine.

For in vitro studies, the MCMV virus used was strain Smith, and strain AD169 for HCMV. The rat CMV (RCMV), strain Prescott, was obtained from the American Type Culture Collection (Rockville, MD) and guinea pig CMV (GPCMV) was provided by Dr William Britt of the University of Alabama at Birmingham. Rhesus CMV (RhCMV) was provided by Dr Peter Barry of the University of California at Davis. The virus pool of MCMV was prepared in mouse salivary gland by inoculating 21 day old female BALB/c mice i.p. with a non-lethal concentration of MCMV. The salivary glands were removed on day 12 and prepared as 10% homogenates (wt./vol.) in MEM. The virus pool titered 3.8×10^7 pfu/ml when assayed on mouse embryo fibroblast (MEF) cells.

MEF cells were prepared from mouse embryos (aged 14–16 days) as previously reported (Kern et al., 1973). Human foreskins were obtained from the University of Alabama at Birmingham Hospital and the cells (HFF) were prepared in a manner similar to that for MEF cells and were used in the HCMV and RhCMV assays. Rat embryos (aged 17 days) and guinea pig embryos (aged 40 days) were used for preparation of cells and virus pools for use in the RCMV and GPCMV assays, respectively, utilizing procedures similar to those described for MEF cells.

2.3. In vitro evaluation

The efficacy of these nucleoside analogues against HCMV and MCMV was determined by plaque reduction assays in HFF and MEF cells, respectively, and toxicity in HFF cells was determined by neutral red uptake as previously reported (Qiu et al., 1998a). Toxicity in MEF cells was performed in plaque reduction assays by visual inspection. In addition, 2-amino-6-cyclopropylamino analogue (QYL-769) and synguanol (QYL-438) were further evaluated against MCMV, RCMV, GPCMC, and RhCMV in plaque reduction assays. In general, these assays were performed once due to a limited drug supply. However, when possible, two assays were performed and the mean and standard deviations (S.D.) were calculated. A computer program was used to calculate the effective concentration 50% (EC_{50}) and the cytotoxic concentration 50% (CC_{50}).

2.4. Antiviral drugs

The methylenecyclopropane analogues and prodrugs are as follows: synadenol (QYL-284A), QYL-438, QYL-769, prodrug of 2-amino-6-methyoxypurine analogue (QYL-972), and 2-amino-6-methoxypurine analogue (QYL-941). The synthesis of these compounds were reported previously (Qiu et al., 1998a,b) and the structures are presented in Fig. 1. For in vitro evaluation, the compounds were prepared in DMSO at 10 mg/ml and diluted 1:10 in MEM containing 2% FBS to reach a final stock concentration of 1000 μg/ml. For the in vivo studies, drugs were prepared in 0.4% carboxymethyl cellulose (CMC),

due to solubility difficulties, and gently sonicated. GCV (Hoffmann-La Roche, Nutley, NJ) was purchased from the University of Alabama at Birmingham Hospital Pharmacy and prepared in sterile water. Placebo controls for the test analogues and GCV were 0.4% CMC and sterile water, respectively.

2.5. Drug treatment

QYL-284A and QYL-438 were administered i.p. once daily for 5 days initiated 6, 24, or 48 h post-infection at 50, 16.7, or 5.6 mg/kg. Each of these compounds was also delivered orally at 100, 60, or 20 mg/kg once daily or 50 mg/kg twice daily for 5 days beginning 24h post-viral inoculation. QYL-941 was administered at 60, 20, 6.7, or 2.2 mg/kg, QYL-972 at 40 or 10 mg/kg, and QYL-769 at 60 or 20 mg/kg twice daily for 5 days. These compounds were given orally twice daily for 5 days beginning 24 h post-infection. For all studies, the necessary placebo and GCV controls were administered on the same treatment schedule as the experimental drugs. Where appropriate, uninfected mice were treated with drug to determine toxicity, and all animals were monitored daily for 21 days. Mortality was the definitive parameter for toxicity, however, the occurrence of physical signs such as ruffled fur and hunching were noted if observed.

2.6. Pathogenesis of infection with QYL-769

To determine the effect of drug treatment on viral replication in target tissue, QYL-769, GCV, or placebo were administered orally to mice at 20 mg/kg twice daily for 5 days in 0.2 ml doses beginning 24 h post-viral inoculation. On days 1–7, 10, and 14, three animals from each group were sacrificed and lung, liver, spleen, kidney, salivary glands, adrenal glands, and pancreas were removed aseptically. Like tissues from the three animals in each group were pooled, homogenized, and prepared as previously reported (Kelsey et al., 1976).

2.7. Statistical evaluation

In order to determine therapeutic efficacy of the compounds, animals treated with the experimental compounds or GCV were compared to placebo-treated animals. Final mortality rates and

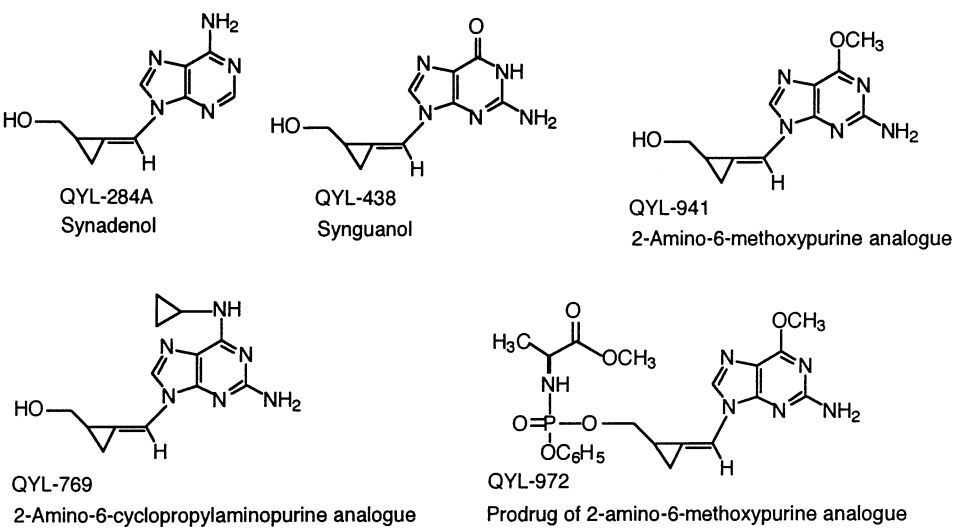

Fig. 1. Methylenecyclopropane analogues of nucleosides.

Table 1
Activity of methylenecyclopropane analogues against human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV) in vitro

Drug name	EC_{50} (μM)[a]		CC_{50} (μM)	
	HCMV	MCMV	HFF dells[b]	MEF cells[c]
Synadenol				
QYL-284A	1.3	2.1	>460	>460
Synguanol				
QYL-438	1.2	0.30	>429	>429
2-amino-6-cyclopropylamino analogue				
QYL-769	2.4	0.37	327	294
Prodrug of 2-amino-6-methoxypurine analogue				
QYL-972	4.5	0.24	33.3	126
2-amino-6-methoxypurine analogue				
QYL-941	5.3	0.4	>404	261
GCV	2.3	4.7	>392	>39.2

[a] Plaque reduction assay.
[b] Neutral red uptake assay.
[c] Visual inspection.

mean day of death (MDD) values were evaluated using Fisher's exact test and the Mann–Whitney/Wilcoxon rank-sum test, respectively. A *P*-value of 0.05 or less was considered significant.

3. Results

3.1. Efficacy in vitro

QYL-284A, QYL-438, QYL-769, QYL-941, and QYL-972 demonstrated activity comparable to, or greater than, GCV in plaque reduction assays against HCMV and MCMV (Table 1). QYL-284A and QYL-438 were slightly more effective against HCMV than GCV, while QYL-972 and QYL-941 were slightly less effective. All five compounds were more active than GCV against MCMV. Toxicity in HFF cells was calculated using neutral red uptake assays and was comparable to GCV, with the exception of QYL-972, which was found to be more toxic than GCV. In MEF cells, QYL-284A and QYL-438 demonstrated toxicity similar to GCV, while QYL-769, QYL-972, and QYL-941 were moderately toxic. In addition, QYL-438 and QYL-769 were further evaluated against additional non-human CMV strains and both had good efficacy against RCMV and RhCMV, and except for QYL-438, good activity against GPCMV (Table 2).

3.2. Efficacy of intraperitoneal QYL-284A, QYL-438, and GCV in vivo

Animals were inoculated with MCMV and treated i.p. once daily for 5 days at 50, 16.7, or

Table 2
Efficacy of synguanol (QYL-438) and 2-amino-6-cyclopropylamino analogue (QYL-769) against various non-human cytomegalovirus (CMV) strains in vitro

CMV strain	EC_{50} (μM)[a]		
	QYL-769[b]	QYL-438[b]	GCV
Murine CMV	0.37	0.30	4.7 ± 0.39
Rat CMV	10.7 ± 0.037	4.7 ± 3.0	57.2 ± 32.1
Guinea Pig CMV	7.7 ± 0.74	54.5 ± 0.86	217.0 ± 33.3
Rhesus CMV	2.2	11.6 ± 0.02	23.9 ± 10.6

[a] Plaque reduction assay.
[b] See Table 1 for compound names.

Table 3
Effect of Treatment with synadenol (QYL-284A) or ganciclovir (GCV) on the mortality of mice inoculated with murine cytomegalovirus (MCMV)

Treatment[a]	Mortality				
	Number	Percent	*P*-value	MDD[b]	*P*-value
Placebo–0.4% CMC	15/15	100	–	4.1	–
QYL-284A					
50.0 mg/kg+6 h	5/15	33	<0.001	4.6	NS[c]
50.0 mg/kg+24 h	7/15	47	<0.01	2.6	<0.01
50.0 mg/kg+48 h	13/15	87	NS	7.1	<0.001
50.0 mg/kg−Tox.[d]	0/10	0	–	–	–
16.7 mg/kg+6 h	11/15	73	NS	4.7	NS
16.7 mg/kg+24 h	9/15	60	<0.05	3.3	NS
16.7 mg/kg+48 h	10/15	67	<0.05	5.1	NS
16.7 mg/kg−Tox.	1/10	10	–	2.0	–
5.6 mg/kg+6 h	13/15	87	NS	5.6	<0.01
5.6 mg/kg+24 h	15/15	100	NS	5.2	NS
5.6 mg/kg+48 h	15/15	100	NS	6.6	<0.001
5.6 mg/kg−Tox.	0/10	0	–	–	–
Placebo–PBS	15/15	100	–	4.9	–
GCV					
50.0 mg/kg+6 h	0/15	0	<0.001	–	–
50.0 mg/kg+24 h	3/15	20	<0.001	2.7	<0.05
50.0 mg/kg+48 h	7/15	47	<0.01	8.6	<0.001
16.7 mg/kg+6 h	1/15	7	<0.001	6.0	NS
16.7 mg/kg+24 h	3/15	20	<0.001	3.0	<0.05
16.7 mg/kg+48 h	2/15	13	<0.001	12.0	NS
5.6 mg/kg+6 h	9/15	60	<0.05	2.8	<0.001
5.6 mg/kg+24 h	6/15	40	<0.001	5.3	NS
5.6 mg/kg+48 h	9/15	60	<0.05	5.2	NS

[a] Animals were treated i.p. once daily for 5 days beginning at the times and the concentrations listed above. QYL-284A was prepared in 0.4% carboxymethyl cellulose and GCV was prepared in sterile water.
[b] MDD, mean day of death.
[c] NS, not statistically significant when compared to the appropriate placebo control.
[d] Uninfected animals treated as stated above.

5.6 mg/kg beginning 6, 24, or 48 h post-viral inoculation (Tables 3 and 4). Significant protection was demonstrated with QYL-284A and QYL-438 at 50 and 16.7 mg/kg when compared to the placebo control ($P < 0.001$–0.05), with comparable efficacy to GCV. In contrast, GCV was efficacious at 5.6 mg/kg while the test compounds exhibited no significant activity at this concentration. Neither compound appeared to be toxic at the highest concentration tested, 50 mg/kg.

3.3. Efficacy of oral QYL-284A, QYL-438, QYL-941, QYL-972, and GCV in vivo

In the initial study, animals were treated orally with QYL-284A or QYL-438 at 60 or 20 mg/kg once daily for 5 days beginning 24 h post-viral inoculation (Table 5). No significant protection was demonstrated with either QYL-284A or QYL-438. In comparison, GCV was efficacious at both 60 and 20 mg/kg ($P < 0.05$). Slight toxicity

(10%) was observed with QYL-284A at 60 and 20 mg/kg.

In a second study, animals were treated orally with 100 mg/kg once daily or 50 mg/kg twice daily for 5 days with QYL-284A or QYL-438 (Table 6). In this study, protection was demonstrated with QYL-438 at 100 mg/kg given once daily ($P < 0.01$) but not with 50 mg/kg given twice daily. QYL-284A was ineffective at both concentrations. The positive control, GCV, again exhibited significant efficacy with either 100 mg/kg once daily or 50 mg/kg twice daily ($P < 0.01$).

In the next experiment, QYL-941 was evaluated in animals treated orally with 60, 20, 6.7, or 2.2 mg/kg twice daily for 5 days (Table 7). Significant protection was demonstrated only at 60 mg/kg ($P < 0.0001$) compared to the placebo control. The results were comparable with GCV at 60 mg/kg ($P < 0.0001$), but GCV was also active at 20 mg/kg ($P < 0.0001$).

The analogue QYL-972 was administered orally twice daily for 5 days at 40 and 10 mg/kg and exhibited significant efficacy at 40 mg/kg, comparable to GCV ($P < 0.0001$), Table 8.

Table 4
Effect of treatment with synguanol (QYL-438) or ganciclovir (GCV) on the mortality of mice inoculated with murine cytomegalovirus (MCMV)

Treatment[a]	Mortality				
	Number	Percent	*P*-value	MDD[b]	*P*-value
Placebo–0.4% CMC	13/15	87	–	5.2	–
QYL-438					
50.0 mg/kg+6 h	2/15	13	<0.001	3.0	NS[c]
50.0 mg/kg+24 h	1/15	7	<0.001	4.0	NS
50.0 mg/kg+48 h	6/15	40	<0.05	5.7	NS
50.0 mg/kg−Tox.[d]	0/10	0	–	–	–
16.7 mg/kg+6 h	1/15	7	<0.001	5.0	NS
16.7 mg/kg+24 h	2/15	13	<0.001	13.5	<0.05
16.7 mg/kg+48 h	13/15	87	NS	6.4	<0.01
16.7 mg/kg−Tox.	0/10	0	–	–	–
5.6 mg/kg+6 h	10/15	67	NS	5.2	NS
5.6 mg/kg+24 h	9/15	60	NS	5.1	NS
5.6 mg/kg+48 h	15/15	100	NS	4.7	NS
5.6 mg/kg−Tox.	0/10	0	–	–	–
Placebo–PBS	14/15	93	–	5.1	–
GCV					
50.0 mg/kg+6 h	0/15	0	<0.001	–	–
50.0 mg/kg+24 h	0/15	0	<0.001	–	–
50.0 mg/kg+48 h	2/15	13	<0.001	5.0	NS
16.7 mg/kg+6 h	3/15	20	<0.001	9.3	NS
16.7 mg/kg+24 h	1/15	7	<0.001	6.0	NS
16.7 mg/kg+48 h	9/15	60	NS	5.2	NS
5.6 mg/kg+6 h	2/15	13	<0.001	7.0	NS
5.6 mg/kg+24 h	3/15	20	<0.001	4.3	NS
5.6 mg/kg+48 h	13/15	87	NS	6.2	<0.05

[a] QYL-438 was prepared in 0.4% carboxymethyl cellulose and GCV was prepared in sterile water and were delivered i.p. in 0.2 ml doses once daily for 5 days initiated at the times indicated above.
[b] MDD, mean day of death.
[c] NS, not statistically significant when compared to the appropriate placebo control.
[d] Uninfected animals treated as stated above.

Table 5
Effect of oral treatment with synadenol (QYL-284A), synguanol (QYL-438), or ganciclovir (GCV) on the mortality of BALB/c mice inoculated with murine cytomegalovirus (MCMV)

Treatment[a]	Mortality				
	Number	Percent	*P*-value	MDD[b]	*P*-value
Placebo–0.4% CMC	10/14	71	–	3.8	–
QYL-284					
60 mg/kg	5/14	36	0.13	4.0	NS[c]
20 mg/kg	12/14	86	NS	4.2	NS
60 mg/kg−Tox.[d]	1/10	10	–	6.0	–
20 mg/kg−Tox.	1/9	11	–	9.0	–
QYL-438					
60 mg/kg	4/13	31	0.08	3.3	NS
20 mg/kg	7/14	50	NS	3.4	NS
60 mg/kg−Tox.	0/9	0	–	–	–
20 mg/kg−Tox.	0/9	0	–	–	–
Placebo–PBS	14/15	93	–	4.4	–
GCV					
60 mg/kg	6/13	46	<0.05	3.7	NS
20 mg/kg	7/14	50	<0.05	4.4	NS

[a] QYL-284A and QYL-438 were prepared in 0.4% carboxymethyl cellulose and GCV in sterile water and were delivered p.o. in 0.1 ml doses once daily for 5 days post inoculation.
[b] MDD, mean day of death.
[c] NS, not statistically significant when compared to the appropriate placebo control.
[d] Uninfected animals treated as stated above.

3.4. Efficacy of oral QYL-769 and GCV in vivo

Animals were inoculated with MCMV i.p. and treated orally with 60 or 20 mg/kg twice daily for 5 days (Table 9). Both QYL-769 and GCV were found to be highly efficacious and yielded similar results. Mortality values of 0% at 60 mg/kg and 13% at 20 mg/kg were observed for both drugs ($P < 0.0001$).

3.5. Efficacy of QYL-769 on the pathogenesis of MCMV infection

The optimal method of evaluating drug efficacy is to determine the effect of therapy on viral replication in various target organs. Because of the good oral activity observed with QYL-769 in reducing mortality, we next performed a study to determine the effect of this drug on the pathogenesis of MCMV. Animals were treated orally with 20 mg/kg twice daily for 5 days beginning 24 h after infection and the lung, liver, kidney, spleen, adrenal gland, and salivary gland were removed on days 1–7, 10, and 14 for quantitation of viral replication. The results in Fig. 2 indicate that QYL-769 successfully reduced replication in the target organs when compared to the placebo control, although this is less evident in the lung, but did not result in complete clearance of MCMV from these organs. Similar results were seen for GCV.

4. Discussion

Considerable progress has been made in attempt to definitively diagnose and treat HCMV infections and understand their epidemiology. Although GCV is specifically effective against HCMV, there is still a need to improve drug efficacy while simultaneously reducing toxic side effects. In addition, the development of resistance

to GCV necessitates the search for new anti-CMV agents.

The investigation of new nucleoside analogues as antiviral agents has stimulated much interest during the past few decades. It has been previously reported (Hayashi et al., 1988) that unsaturated acyclic nucleoside analogues, where the ribofuranose moiety was replaced by a rigid allenic residue (adenallene and cytallene), display potent anti-HIV activity. The latter agents served as a structural basis for development of 'second generation' antivirals where the allenic grouping was substituted with a methylenecyclopropane moiety. This change resulted in a much broader spectrum of antiviral activity, i.e. several types of herpesviruses (Qiu et al., 1998a,b). In addition, the report (Daluge et al., 1997) that the 6-cyclopropylamino analogue is a more potent anti-HIV agent than the parent compound, carbovir, motivated a design of a similar methylenecyclopropane derivative (QYL-769) as an antiviral agent (Qiu et al., 1998b). Antiviral properties of lipophilic phosphate prodrugs of methylenecyclopropane analogues are also reported on (Zemlicka et al., 1998). One of such compounds, QYL-972, has now been investigated in detail as an anti-CMV agent in in vitro and in vivo assays.

Evaluation in vitro of the methylenecyclopropane analogues indicated that the purine Z-isomers were significantly more efficacious than the pyrimidine counterparts and exhibited moderate to high activity against HCMV below toxic levels (Qiu et al., 1998a,b). Particularly potent were the compounds QYL-284A and QYL-438 (Qiu et al., 1998a). These results warranted the current investigation which presents the efficacy of several methylenecyclopropane analogues against HCMV, MCMV, and other non-human

Table 6
Effect of oral treatment with synadenol (QYL-284A), synguanol (QYL-438), or ganciclovir (GCV) on the mortality of BALB/c mice inoculated with murine cytomegalovirus (MCMV)

Treatment[a]	Mortality				
	Number	Percent	*P*-value	MDD[b]	*P*-value
Placebo–0.4% CMC	12/14	86	–	4.0	–
QYL-284A					
100 mg/kg	9/15	60	NS[c]	3.4	NS
50 mg/kg	7/14	50	NS	5.6	NS
100 mg/kg−Tox.[d]	0/10	0	–	–	–
50 mg/kg−Tox.	0/10	0	–	–	–
QYL-438					
100 mg/kg+24 h	3/15	20	<0.01	3.3	NS
50 mg/kg+24 h	7/14	50	NS	2.4	NS
100 mg/kg−Tox.	0/10	0	–	–	–
50 mg/kg−Tox.	0/10	0	–	–	–
Placebo–water	14/14	100	–	4.0	–
GCV					
100 mg/kg	7/15	47	<0.01	5.4	NS
50 mg/kg	7/15	47	<0.01	3.6	NS

[a] QYL-284A and QYL-438 were prepared in 0.4% carboxymethyl cellulose and GCV was prepared in sterile water and both were delivered p.o. in 0.1 ml doses. All drugs at 100 mg/kg were administered once daily and at 50 mg/kg twice daily for 5 days initiated 24 h post-viral inoculation.
[b] MDD, mean day of death.
[c] NS, not statistically significant when compared to the appropriate placebo control.
[d] Uninfected animals treated as stated above.

Table 7
Effect of oral treatment with 2-amino-6-methoxypurine analogue (QYL-941) or ganciclovir (GCV) on the mortality of Balb/c mice inoculated with murine cytomegalovirus (MCMV)

Treatment[a]	Mortality				
	Number	Percent	*P*-value	MDD[b]	*P*-value
Placebo–0.4% CMC	15/15	100	–	5.0	–
QYL-941					
60 mg/kg	1/16	6	<0.0001	5.0	NS[c]
20 mg/kg	12/16	81	NS	5.0	<0.05
6.7 mg/kg	15/16	94	NS	5.0	<0.05
2.2 mg/kg	16/16	100	NS	4.0	NS
60 mg/kg−Tox.[d]	0/10	0	–	–	–
20 mg/kg−Tox.	0/10	0	–	–	–
Placebo–water	14/15	93	–	5.0	–
GCV					
60 mg/kg	1/15	0	<0.0001	18.0	NS
20 mg/kg	1/15	7	<0.0001	7.0	NS
6.7 mg/kg	13/15	80	NS	6.0	<0.001
2.2 mg/kg	15/15	100	NS	5.0	NS

[a] QYL-941 was prepared in 0.4% carboxymethyl cellulose and GCV in sterile water. Both drugs were delivered p.o. in 0.1 ml doses. Animals were treated twice daily for five days beginning 24 h post-viral inoculation.
[b] MDD, mean day of death.
[c] NS, not statistically significant when compared to the appropriate placebo control.
[d] Uninfected animals treated as stated above.

Table 8
Effect of oral treatment with prodrug of 2-amino-6-methyoxypurine analogue (QYL-972) or ganciclovir (GCV) on the mortality of BALB/c mice inoculated with murine cytomegalovirus (MCMV)

Treatment[a]	Mortality				
	Number	Percent	*P*-value	MDD[b]	*P*-value
Placebo–0.4% CMC	13/15	87	–	6	–
QYL-972					
40 mg/kg	1/15	7	<0.0001	6	NS[c]
40 mg/kg−Tox.[d]	0/10	0	–	–	–
10 mg/kg	8/15	53	NS	5	NS
10 mg/kg−Tox.	0/10	0	–	–	–
Placebo–water	14/15	93	–	6	–
GCV					
40 mg/kg	2/15	13	<0.0001	10	NS
10 mg/kg	7/15	47	<0.05	7	NS

[a] QYL-972 was prepared in 0.4% carboxymethyl cellulose (CMC) due to solubility difficulties and GCV in sterile water. Both drugs were delivered p.o. in 0.1 ml doses. Animals were treated twice daily for 5 days beginning 24 h post-viral inoculation.
[b] MDD, mean day of death.
[c] NS, not statistically significant when compared to the appropriate placebo control.
[d] Uninfected animals treated as stated above.

CMVs including RCMV, GPCMV, and RhCMV in plaque reduction assays in comparison to GCV. In these studies, QYL-284A and QYL-438 were found to be more effective than GCV, QYL-769 was similar to GCV, while QYL-941 and QYL-972 were less effective than GCV. All five compounds were found to be more effective than GCV against MCMV in vitro. It was also found that QYL-284A, QYL-438, and QYL-941 were comparable to GCV in level of toxicity, while QYL-769 was only slightly more toxic. In contrast, QYL-972 was found to be highly toxic in HFF cells. QYL-438 and QYL-769 were further evaluated for efficacy and were found to be consistently more effective than GCV as well.

The in vitro results clearly demonstrate the significant potential of these analogues as antiviral agents, however, tissue culture alone cannot be used to predict a drug's activity in humans. Although the animal models do not identically mimic clinical disease seen in humans, they are essential in providing an insight into the therapeutic efficacy, toxicity, and possible treatment regimes. The optimal modality of drug administration for HCMV infections is oral, however, potential antivirals are often initially evaluated by the intraperitoneal route to obtain preliminary data regarding activity because it closely mimics intravenous administration.

The studies indicate that QYL-284A given i.p. was comparable to GCV at 50 mg/kg but less effective at 16.7 mg/kg. QYL-438 was slightly more effective than QYL-284A, demonstrating activity equivalent to GCV at 50 and 16.7 mg/kg. When these compounds were delivered orally they exhibited no significant activity, with the exception of QYL-438 at 100 mg/kg given orally once daily. QYL-941 was as effective as GCV at the high dose of 60 mg/kg but the activity dramatically decreased when drug was delivered at 20 mg/kg and less. QYL-972 was also comparable to GCV at 40 mg/kg orally. The 2-amino-6-cyclopropylamino analogue, QYL-769, was highly effective orally at both concentrations administered with only 13% mortality at 20 mg/kg, equivalent to GCV. It should be pointed out, however, that GCV is poorly absorbed in humans compared to mice. It may be, therefore, that QYL-769 may be better absorbed in man than GCV. This compound demonstrated the most significant potential

Table 9
Effect of oral treatment with analogue 2-amino-6-cyclopropylaminopurine (QYL-769) or ganciclovir (GCV) on the mortality of BALB/c mice inoculated with murine cytomegalovirus (MCMV)

Treatment[a]	Mortality				
	Number	Percent	*P*-value	MDD[b]	*P*-value
Placebo–0.4% CMC	15/15	100	–	4.8	–
QYL-769					
60 mg/kg	0/15	0	<0.0001	–	–
20 mg/kg	2/15	13	<0.0001	7.0	NS[c]
60 mg/kg – Tox.[d]	0/15	0	–	–	–
20 mg/kg – Tox.	0/10	0	–	–	–
Placebo–water	15/15	100	–	5.2	–
GCV					
60 mg/kg	0/15	0	<0.0001	–	–
20 mg/kg	2/15	13	<0.0001	6.5	<0.05

[a] QYL-769 was prepared in 0.4% carboxymethyl cellulose and GCV was prepared in sterile water. Both drugs were delivered p.o. in 0.2 ml doses. Animals were treated twice daily for 5 days beginning 24 h post viral inoculation.
[b] MDD, mean day of death.
[c] NS, not statistically significant when compared to the appropriate placebo control.
[d] Uninfected animals treated as stated above.

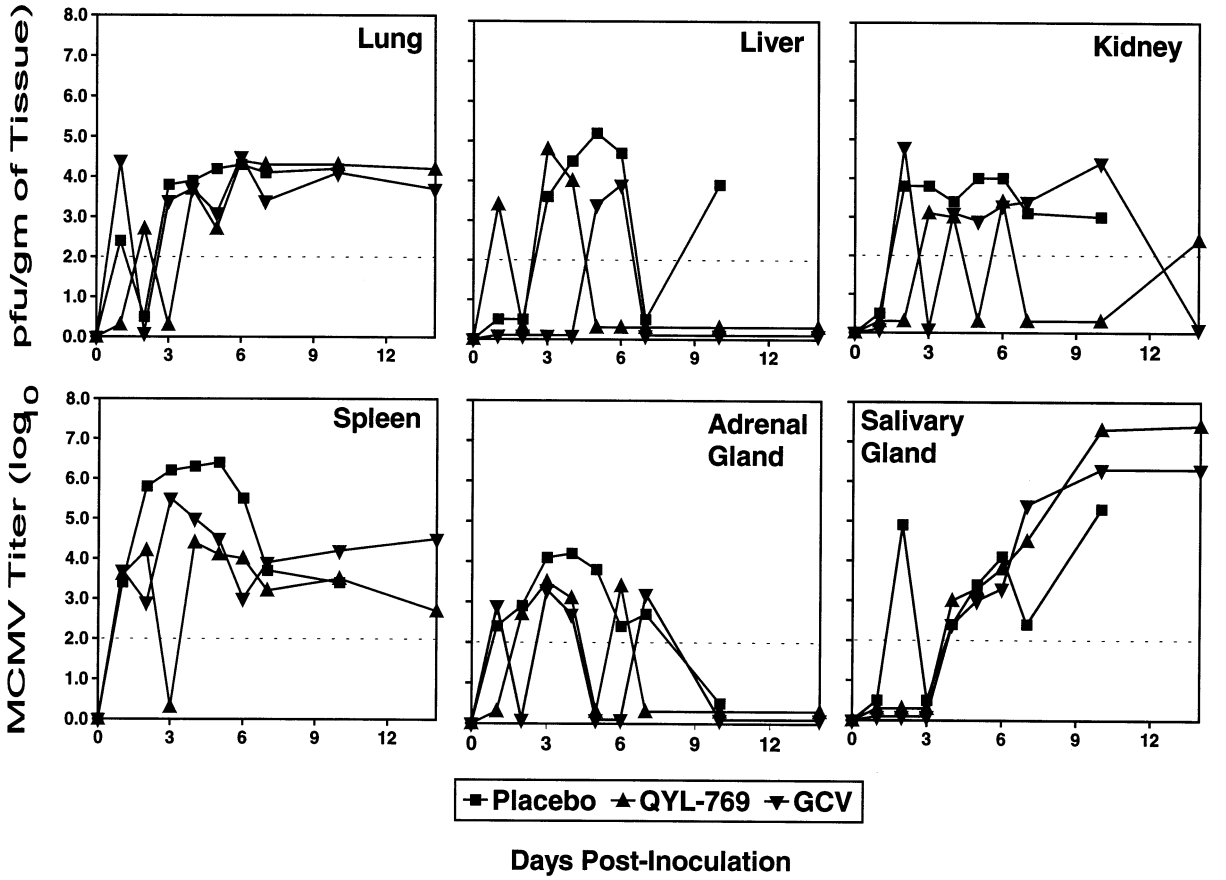

Fig. 2. Effect of treatment with Qyl-769 on the pathogenesis of MCMV infection in BALB/c mice.

in vivo, therefore, it was further evaluated in a pathogenesis study, which allowed a more detailed analysis of the effect of treatment on virus replication in target tissues. The results suggested that GCV and QYL-769 were comparable in reducing MCMV titer in organs, however, virus was not cleared from the body with either agent, thus indicating that MCMV replication in tissues can continue after termination of treatment. The results do not reflect the high activity exhibited in the efficacy study with QYL-769 or GCV, which may have been due to the limited 5 day treatment schedule. The incorporation of a longer treatment regimen may be required in a future pathogenesis study to more clearly define these results. In addition, the toxicity data in vivo were very encouraging, presenting no mortality with any of the analogues tested, with the exception of 284A, which demonstrated slight toxicity when administered orally at 16.7 mg/kg. QYL-972, which was found to be more toxic than GCV in HFF cells and moderately toxic in MEF cells in tissue culture, exhibited no toxicity in the treated animals.

In conclusion, these results support the utility of the MCMV model in simulating HCMV disease and determining the efficacy of potential antiviral agents. The tissue culture data, in addition to in vivo results, provide convincing evidence for the CMV activity of these methylenecyclopropane analogues. Efficacy was

comparable to, or better than, GCV, the current drug of choice, thus suggesting the need for future research into these and similar compounds. It should also be stressed that compounds QYL-284A, QYL-438, QYL-769, and QYL-941 are racemic mixtures. Prodrug QYL-972, is then a mixture of four diastereoisomers. It is then likely that the appropriate enantiomeric (diastereoisomeric) forms will have improved activity and hopefully reduced toxicity.

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