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A high throughput colorimetric cell proliferation assay for the identification of human cytomegalovirus inhibitors

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Abstract

A colorimetric assay based on the cleavage of the tetrazolium salt WST-1 has been developed for human cytomegalovirus (HCMV) antiviral susceptibility testing and adapted to a microtiter plate format. Optimal conditions were determined and the standard routine assay was calibrated with a viral input of 0.05–0.10 plaque forming unit (p.f.u.)/cell with a density of 2000 cells/well in a 96-well microtiter plate for an incubation period of 7 days. Ganciclovir (9-(2-hydroxy-1(hydroxymethyl) ethyoxymethyl) guanine; DHPG), and cidofovir ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine; HPMPC) were used as positive control test compounds to validate the assay. The effective EC_{50} concentration values obtained with the two antiviral compounds in the present assay were in good agreement with plaque reduction assay results performed in parallel experiments. This method presents the advantage of being easy and rapid to perform, reliable, reproducible, and convenient for use in a high throughput screening capacity.

Keywords: Cytomegalovirus; Cell viability; Colorimetric assay; HPMPC; DHPG

1. Introduction

Human cytomegalovirus is a serious life threatening opportunistic pathogen in immunocompromized individuals such as HIV-1 infected individuals, patients with cancer, and organ transplant recipients (Alford and Britt, 1993). It is also one of the principal causes of congenital malformation and neurological defects in infected newborns (Alford and Britt, 1993).

Ganciclovir (9-(2-hydroxy-1(hydroxymethyl) ethyoxymethyl) guanine; DHPG), foscarnet (phosphonic acid), and cidofovir ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine;

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HPMPC) are the three anti-HCMV compounds currently approved for the treatment of cytomegalovirus retinitis, which is one of the most common complications encountered in patients with AIDS. Serious side-effects are associated with prolonged use of these antiviral agents such as nephrotoxicity and granulocytopenia (Collaborative DHPG Treatment Study Group, 1986; Causey, 1991; Jacobson et al., 1991; Palestine et al., 1991; Jacobson, 1997). The emergence of clinical resistant isolates is also an additional complication (Jacobson et al., 1991; Palestine et al., 1991; Stanat et al., 1991; Chou et al., 1995; Hanson et al., 1995; Jacobson, 1997). There is a possibility of cross-resistance with cidofovir among highly DHPG resistant clinical isolates which are characterized by a mutation in both the viral UL97 and viral DNA polymerase genes (Smith et al., 1997). Thus, there is still a great need for identifying new compounds for the treatment of HCMV infections.

Several in vitro assay systems are available for evaluating molecules associated with anticytomegaloviral activity. These methods can be tedious and time consuming to perform. The plaque reduction assay is the most commonly used assay and is the gold standard for in vitro susceptibility testing of anti-HCMV drug candidates. In the past few years, numerous methodologies have been developed to increase the rapidity of viral quantification and some of them have been adapted for use in large-scale screening programs for anti-herpes agents. The technologies for direct monitoring of viral production involve the use of recombinant HSV and HCMV viruses (Dicker et al., 1995; Hippenmeyer and Dilworth, 1996), nucleic acid hybridisation (Gadler, 1983; Dankner et al., 1990), immunocytochemical staining (Musiani et al., 1988), and in situ enzyme-linked immunosorbent assay (ELISA) (Tatarowicz et al., 1991). In addition, an HSV-1 cytopathic effect assay based on a vital dye uptake has also been reported in the literature (McLaren et al., 1983).

We present here a quantitative colorimetric assay adapted to a microtiter plate format for the determination of HCMV susceptibility to antiviral compounds. In the system described in this report, virus-induced cytopathic effects (CPE) with respect to cell viability and proliferation, are assessed by reduction of the tetrazolium salt WST-1 into a water soluble formazan product which can be directly quantified using a microtiter ELISA reader.

2. Material and methods

2.1. Cells and virus

Primary newborn human fibroblast (Hs68) cells and HCMV strain AD169 were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were passaged in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA), and 2 mM glutamine (Life Technologies). Penicillin and streptomycin (Life Technologies) were added at 500 U/ml and 50 μg/ml final concentrations, respectively. Cells were used for the plaque reduction and CPE assays between passages 13 and 24.

2.2. Reagents

Cell proliferation reagent WST-1 was purchased from Boehringer Mannheim (Laval, Quebec, Canada). DHPG was a gift from Roche Bioscience (Palo Alto, CA, USA) and (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC) was synthesized at BioChem Pharma. Trypsin-EDTA solution was obtained from Life Technologies and crystal violet from Sigma (St. Louis, MO, USA).

2.3. Colorimetric cell proliferation assay

Hs68 cells were seeded at a density of 8×10^5 cells in DMEM/10% FBS in a polystyrene flask culture vessel of 75-cm^2 surface area (Nunc, No.147589, Roskilde, Denmark) and incubated for 18 h at 37°C in 5% CO_2 in order to obtain, in these conditions, 80% confluence. The cell monolayer was then inoculated with HCMV at a multiplicity of infection (MOI) of 0.05–0.1 plaque forming unit (p.f.u.) /cell in a final volume of 3 ml

in DMEM/2% FBS. Viral adsorption was allowed to proceed for 2 h at 37°C with rocking of the plates every 30 min. Infected monolayers were trypsinized, cells were resuspended in DMEM/2% FBS, counted, and cell suspensions adjusted to a density of 2×10^4 cells/ml. An aliquot of 100 μl was added to wells of a 96-well microtiter plate (Nunc, No.167008) containing 100 μl of test compound at the appropriate concentrations. After 7 days of incubation at 37°C in 5% CO_2, the medium was then removed and cell viability, as an indication of the CPE reached, was assessed by the addition of 100 μl of pre-warmed DMEM/2% FBS containing WST-1 cell proliferating reagent diluted 1/40 to each well. After a period of incubation of 2 h at 37°C, the absorbance was measured on a microplate Dynatech MR5000 Microelisa Autoreader (Dynatech, Alexandria, VA, USA) set at a wavelength of 410 nm. DHPG and HPMPC were used as positive controls for the inhibition of HCMV CPE. Stock solutions were prepared in sterile water at 2 and 10 mg/ml, respectively.

The concentration of drug which reduces the viral CPE by 50% (EC_{50}) was calculated using a sigmoidal dose response (variable slope) equation to perform a non-linear regression analysis using the GraphPad Prism software: version 2.0 (GraphPad Software, San Diego, CA, USA):

- A: Optical density obtained with infected cells cultured in the presence of the minimal drug concentration required to obtained the maximal inhibition of CPE (top plateau)
- B: Optical density obtained with infected cells cultured in the absence of drug (bottom plateau)
- Y: Optical density obtained with infected cells cultured in the presence of a specific drug concentration X
- Hill's coefficient: Variable that controls the slope of the curve. Values of 1.15 and 2.58 were obtained for HPMPC and DHPG, respectively.

$$Y = B + (A - B)/1 + 10^{(\log_{EC_{50}} - X)\text{Hill's coefficient}}$$

As a negative control, mock-infected cells were treated with DHPG and HPMPC at the same concentrations used for the experiments with infected cells in order to determine a potential drug-induced CPE. The drugs were tested at a concentration range of 6.1×10^{-4} to 2.5 μg/ml for DHPG and of 9.8×10^{-5} to 0.4 μg/ml for HPMPC. All experiments were performed three times each in triplicate.

2.4. Plaque reduction assay

In a 12-well tissue culture dish (Corning Costar, No.25815, Orneonta, NY, USA), Hs68 cells were plated at a density of 1.5×10^5 cells/well in 2 ml of culture medium and incubated overnight in 5% CO_2 at 37°C. Medium was removed and cells were infected with 0.5 ml of DMEM/2% FBS containing approximately 125 p.f.u./ml of HCMV. After an adsorption at 37°C for 2 h, the inoculate was removed and the monolayer was overlaid with 1 ml of DMEM/2% FBS containing the test compounds at concentrations ranging from 0.1 to 2.0 μg/ml for DHPG and 0.005 to 0.2 μg/ml for HPMPC. After 7 days of incubation, cells were fixed with 1 vol. of a solution of 8% formaldehyde in water for 30 min, then the solution was removed and cell monolayers stained with 2% crystal violet in 20% ethanol for a few seconds. Cells were rinsed with tap water, dried, and monolayer examined for the presence of plaques using an inverted microscope at 40× magnification.

The percentage of plaque reduction was determined for DHPG and HPMPC by comparison of the mean number of plaques (drug-treated cells) with the infected cells and the 50% effective concentration (EC_{50}) was calculated by non-linear regression analysis.

3. Results

3.1. Optimization of the colorimetric cell proliferation assay in a microtiter plate format: effect of cell density, viral input, and incubation period

The degree of HCMV susceptibility to ganciclovir in the colorimetric cell proliferation assay was highly dependent upon several conditions, including the number of Hs68 cells seeded, the

Table 1
Effect of cell density on DHPG-related CPE on mock-infected Hs68 fibroblasts

DHPG (μg/ml)	Cell density[a]					
	1000 cells/well		2000 cells/well		3000 cells/well	
	O.D.	% cell viability[b]	O.D.	% cell viability	O.D.	% cell viability
0	0.569	100	0.605	100	0.667	100
0.156	0.600	105	0.650	108	0.713	105
0.313	0.575	101	0.631	104	0.691	102
0.625	0.561	99	0.649	107	0.707	104
1.250	0.550	97	0.665	110	0.715	105
2.500	0.463	81	0.645	106	0.690	102
5.000	0.300	53	0.710	118	0.676	100

[a] Hs68 cells were cultured at various densities/well in a 96-well microtiter plate in the presence of DHPG at concentrations up to 5 μg/ml. Cell viability was recorded at day 7 post-inoculation as described in Section 2. Experiments were performed in triplicates.
[b] Cell viability of uninfected Hs68 cells obtained in the absence of DHPG was recorded as 100%.

viral input used, and the period of incubation. The effects of these factors on the dynamic range of the assay was investigated.

The assay was calibrated initially for the optimum cell density. Cell viability was investigated over a period of incubation up to 10 days using seeding concentrations of 1000–4000 uninfected cells/well in a 96-well microtiter plate. Results on Hs68 cell viability when cultured at initial densities of 1000, 2000, and 3000 cells/well in the presence of various concentrations of DHPG up to 5 μg/ml are presented in Table 1. The data indicate a drug-related CPE at a density of 1000 cells/well using a concentration of DHPG of 2.5 and 5.0 μg/ml. A cell viability of 53% was recorded with 5 μg/ml of DHPG when a density of 1000 cells/well was used whereas no such effect was seen at higher cell densities (Table 1). Therefore, if conditions having less than 2000 cells/well were used, Hs68 cells were found to be sensitive to DHPG-induced cytotoxicity. At this sub-confluent plating, cells would enter log-phase growth and hence become sensitive to DHPG-induced toxicity. When more than 3000 fibroblasts were added to the well, cells were less susceptible to DHPG-induced CPE, however cells soon became confluent during the period of incubation. The optimum amount of cells to be added was determined to be within the range of 2000–3000 cells/well.

Cultured cells at a density of 3000/well were infected with a viral input ranging from 0.05 to 0.2 p.f.u./cell in the presence of DHPG. The plates were inspected daily for cell confluence and for the extent of CPE reached. Effects of various MOIs and concentrations of DHPG on cell viability after a period of incubation of 7 days are shown in Fig. 1. Measurable CPE was reached for each of the three MOIs tested, when compared to the mock-infected Hs68 cells cultured in the presence of the same drug concentration (100% cell viability) (Fig. 1). CPE was found to be significantly reduced in the presence of 2.5 or 10 μg/ml of DHPG when compared to the infected Hs68 cells incubated in the absence of DHPG (Fig. 1). Interestingly, a greater extent of CPE reduction was observed at 2.5 μg/ml, as compared to 10 μg/ml (Fig. 1) and higher concentrations (data not shown) at all MOI tested in the assay. The fact that a lack of dose response was not noticed at higher concentrations of DHPG suggests a drug related-toxic effect on cultured HCMV-treated cells. Such drug-related CPE was not noticeable for uninfected cells treated at the same DHPG concentrations (Table 1). Increasing the viral MOI from 0.05 to 0.2 resulted in a reduction of cell viability obtained with a specific concentration of DHPG. For example, at 2.5 μg/ml of DHPG, cell viability of 77, 63, and 53% was recorded at MOIs of 0.05, 0.1, and 0.2 p.f.u./cell,

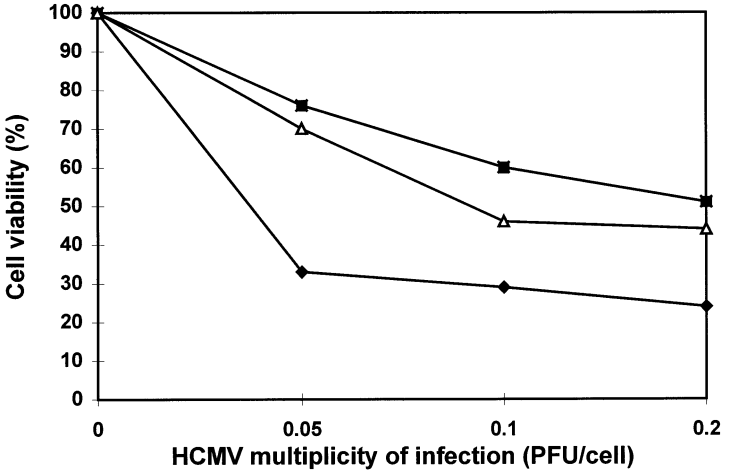

Fig. 1. Effect of variation in viral multiplicity of infection and DHPG concentrations on cell viability after a period of incubation of 7 days. Hs68 cells were infected at an MOI of 0.05, 0.10, or 0.20 p.f.u./cell and seeded at a density of 3000 cells/well in a 96-well microtiter plate containing DHPG at 0 (◆), 2.5 (■), and 10 μg/ml (△). Cell viability of mock-infected Hs68 cells obtained in the presence of a specific concentration of DHPG was recorded as 100%. Experiments were performed in triplicate, the standard variation of the mean not exceeding 3.7, 12.1, and 7.8% for the experimental points obtained using MOIs of 0.05, 0.1, and 0.2, respectively.

respectively (Fig. 1). It has been reported that the efficacy of DHPG is not sensitive to the MOI of HCMV used, at least up to an MOI of 2.0 (Lewis et al., 1994). Therefore, it is unlikely that the reduction in cell viability was due to virus-induced CPE, but more is likely due to a drug-related toxicity.

The effect of the incubation time on DHPG and HPMPC antiviral activity was studied within a range of concentrations up to 16.0 and 1.6 μg/ml, respectively (Fig. 2a,b). The standard routine assay was calibrated using an MOI of 0.05–0.1 p.f.u./cell with a cell density of 2000 cells/well. Cell proliferation reagent WST-1 was added at day 4, 5, 6, and 7 post-infection as described in Section 2. A significant difference of cell viability between the non-infected cells and the HCMV-treated cells incubated in the presence of various concentrations of compounds was observed after 4 days of incubation (Fig. 2a,b). This could be explained in part by a significant level of CPE reached with the MOI used and by the higher proliferative capacity of the uninfected cells. A reduction of viral CPE resulting from the antiviral activity of the two viral DNA polymerase inhibitors, HPMPC and DHPG, was consistently observed after a period of incubation of 6–7 days with no dose response observed above 0.1 and 1.0 μg/ml, respectively at day 7 post-infection (Fig. 2a,b). This illustrates that the maximum reduction of CPE was reached by both compounds despite the fact that the viability of the uninfected cell control was still higher at all time points. The CPE reduction observed with DHPG at 16 μg/ml was not as pronounced as compared with lower drug concentrations which indicates a drug related-toxic effect consistent with the results described above. Cell viability values obtained at day 7 post-infection (Fig. 2a,b) suggest EC_{50} values <1 and ≈ 0.05 μg/ml for DHPG and HPMPC, respectively. The reproducibility of the assay was confirmed by statistical analysis of the EC_{50} values obtained with DHPG and HPMPC. The assays were repeated three times each in triplicate. The overall coefficient of variation between the assays was less than 10%.

The assay was repeated using a slightly higher MOI (0.1 instead of 0.05 p.f.u./cell) with lower drug concentrations to obtain dose-response curves. Measurements for the reduction of virus-induced CPE indicated that dose responses were obtained between 0.08 and 1.25 μg/ml for DHPG,

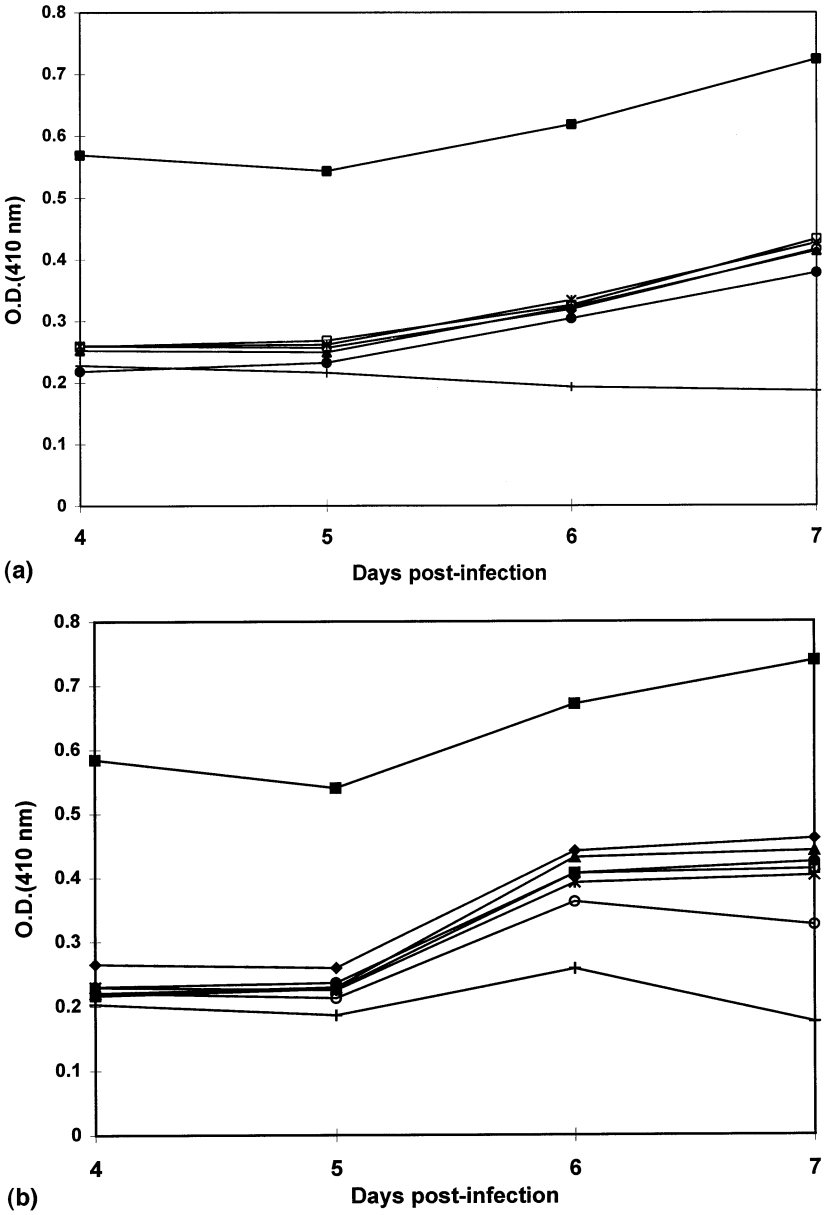

Fig. 2. (a). Effect of DHPG on cell viability over a 7 days incubation period. Hs68 cells were infected at an MOI of 0.05 p.f.u./cell and seeded at a density of 2000 cells/well in a 96-well microtiter plate and cultured in the presence of serial dilutions of DHPG at 0 (|), 1.0 (○), 2.0 (✱), 4.0 (□), 8.0 (▲), and 16 μg/ml (●). Non-infected cells (■) were included as a control for the assessment of the cytopathic effect reached during the course of the infection. Cell viability was measured at day 4, 5, 6, and 7 post-infection. Experiments were performed in triplicate, and the standard variation of the mean was less than 15% for each time point. (b) Effect of HPMPC on cell viability over a 7-day incubation period. Hs68 cells were infected at an MOI of 0.05 p.f.u./cell and seeded at a density of 2000 cells/well in a 96-well microtiter plate and cultured in the presence of serial dilution of HPMPC at 0 (|), 0.05 (○), 0.1 (✱), 0.2 (□), 0.4 (▲), 0.8 (●), and 1.6 μg/ml (◆). Non-infected cells (■) were included as a control for the assessment of the cytopathic effect reached during the course of the infection. Cell viability was measured at day 4, 5, 6, and 7 post-infection. Experiments were performed in triplicate, the standard variation of the mean was less than 15% for each time point.

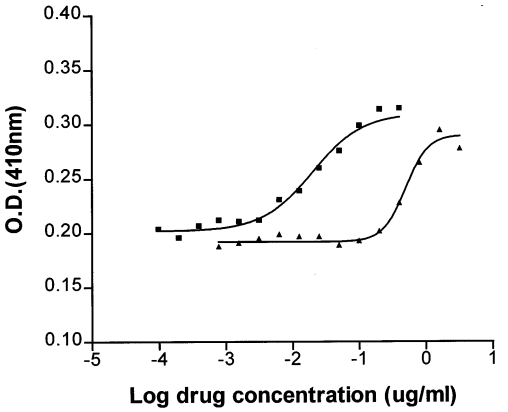

Fig. 3. Antiviral activity of DHPG and HPMPC measured by the colorimetric cell proliferation assay. Hs68 cells were infected at an MOI of 0.1 p.f.u./cell and seeded at a density of 2000 cells/well in a 96-well microtiter plate containing various concentrations of DHPG (▲) or HPMPC (■). Cell viability was determined after a 7-day incubation period. Experiments were performed twice in triplicate, and the standard variation of the mean was less than 15% for each time points.

and between 0.003 and 0.2 μg/ml for HPMPC (Fig. 3). EC_{50}'s of 0.5 and 0.02 μg/ml for DHPG and HPMPC, respectively were determined from these data (Fig. 3, Table 2) and were within the range of values expected from the previous experiment on the effect of time of incubation on the dynamic range of the assay (Fig. 2a,b).

3.2. Comparison of the HCMV susceptibility to DHPG and HPMPC using the plaque reduction and the colorimetric cell proliferation assays

The purpose of this study was to validate the colorimetric assay for antiviral susceptibility testing in a high throughput screening format. Therefore the assay was compared to the plaque reduction assay, which is the gold standard assay used in the HCMV field. HCMV susceptibility to DHPG and HPMPC was determined using the colorimetric cell proliferation assay after a 7-day incubation at a MOI of 0.1 (Fig. 3) and EC_{50}'s obtained using this technique were compared with the values obtained using a standard plaque reduction assay (Table 2). The EC_{50}'s obtained using these two techniques were very similar, suggesting that at least for the two antiviral compounds tested, the colorimetric assay can be predictive of the type of result one would obtain using the standard assay format.

Table 2
Comparison of the antiviral activity of DHPG and HPMPC using the plaque reduction and colorimetric cell proliferation assays

Compound	EC_{50} (μg/ml) for HCMV AD 169 strain	
	Plaque reduction assay	Colorimetric cell proliferation assay
HPMPC	0.03	0.02
DHPG	0.30	0.50

4. Discussion

Several in vitro assays designed to determine herpes virus susceptibilities to potential antiviral agents have been developed in a format suitable for high throughput screening (McLaren et al., 1983; Musiani et al., 1988; Dankner et al., 1990; Dicker et al., 1995; Hippenmeyer and Dilworth, 1996). They usually offer a direct measurement of the amount of virus produced in infected cells. In most cases, the results obtained are comparable to those derived from a plaque reduction assay. These assays, although reliable, present the disadvantage of requiring multiple handling steps which are inconvenient and can add to the overall cost of the assay. The colorimetric cell proliferation assay presented in this study relies on a simple cell viability assessment as an indirect measurement of viral susceptibility to antiviral compounds.

In this study, multiple factors such as cell density, viral input, and time of incubation were investigated for their effects on the dynamic range of the assay. The optimum range of cell density was found to be between 2000 and 3000 cells/well. At densities less than 2000 cells/well, Hs68 cells were found to be susceptible to DHPG-related CPE whereas at higher densities (>2000 cells/well), the cell viability ratio obtained from infected (0.2 p.f.u./cell) Hs68 cells/mock-infected cells was found to be inversely proportional to the

cell density (data not shown). This could be explained by the fact that the rate of cellular replication of uninfected cells in log-phase growth is much higher than the rate of viral replication in the infected cells. Basically, the number of viable cells after 7 days of incubation represents the end-point result of a competition between the cell capacity to proliferate (uninfected cells) and the viral propagation resulting in a cell killing or/and reduction of cell proliferation (infected cells).

Cell viability studies of HCMV-infected Hs68 cells, cultured in the absence of drug, have demonstrated that 25% of the cells, when initially infected at an MOI of 0.2, remained viable after 7 days of incubation (Fig. 1). In order to increase the CPE (cell viability closer to a zero value), higher MOIs or a longer period of incubation could have been used. However, at MOIs higher than 0.2, cells became more susceptible to drug-related CPE (data not shown), as illustrated for MOIs between 0.05 and 0.2 (Fig. 1). Increasing the incubation period resulted in the same phenomenon as described above for experiments using relatively high cell densities.

The overall cell viability of infected cells cultured in the presence of HPMPC and DHPG never reached the level obtained for the uninfected cell control during the 7-day period of incubation. This could be explained in part by the fact that initially infected cells, even those cultured in the presence of DHPG, are condemned to die. A higher degree of toxicity was observed with increasing concentrations of DHPG on Hs68-infected cells (Fig. 1) when compared to non-infected cells at the same density (Table 1). This is independent of the overwhelming CPE observed in a lytic infection. Mock-infected cells (control) have an initial growth advantage when compared to the infected cells incubated in the presence of compounds (Fig. 2a,b, day 4). This advantage is amplified after 7 days of incubation since several cell division cycles are allowed to proceed. The positive effect of HPMPC and DHPG on cell viability was observed 6–7 days post-infection (Fig. 2a,b). This is most likely related to the antiviral mechanism of action of the two nucleotide analogs, which is exhibited during the major peak of replication cycle of HCMV DNA, which does not occur until 72–92 h post-infection (Stinski, 1990). This would result in a reduced amount of infectious virions available for the second round of infection. In addition, this could be related to the toxicity of the compound on the log phase growing infected cells which subsides as the cells reach confluence.

The EC_{50} values obtained with both DHPG and HPMPC were in good agreement with the plaque reduction assay results. A higher level of inhibitory effect was achieved with HPMPC in both antiviral assays. Several 1,6-naphthyridine compounds with various degrees of potency against HCMV (Jin et al., 1997) have been tested at least twice in both CPE and plaque reduction assays and a similar trend of inhibitory potency was observed in the two test systems (data not shown). The results obtained using this chemically distinct class of compound further support the reliability of the presented methodology. It will be interesting to perform antiviral susceptibility testing of some representative drug resistant HCMV strains as an additional means to validate the assay as well as to compare the present assay with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) or neutral red uptake assays.

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