ELSEVIER

Antiviral Research 32 (1996) 35–42

Antiviral Research

A rapid assay for determination of antiviral activity against human cytomegalovirus

Paul J. Hippenmeyer[a,*], Vickie M. Dilworth[b]

[a]*Department of Molecular and Cellular Biology, G.D. Searle, c/o Monsanto Company, 700 Chesterfield Village Parkway-North, St. Louis, MO 63198, USA*

[b]*Department of Infectious Disease Research, G.D. Searle, c/o Monsanto Company, 700 Chesterfield Village Parkway-North, St. Louis, MO 63198, USA*

Received 27 February 1996; accepted 3 May 1996

Abstract

RC256, the recombinant human cytomegalovirus (HCMV) which expresses β-galactosidase, was used as a tool for rapid screening of compounds for antiviral activity. The effective concentration of antiviral compound needed to inhibit RC256 was identical to the concentrations needed to inhibit other strains of HCMV as measured by plaque reduction assay or virus yield reduction assay. Measurement of β-galactosidase activity in infected cell lysates allowed determination of effective concentrations 48 h postinfection with results comparable to the longer, more laborious assays.

Keywords: Antiviral; Cytomegalovirus; RC256; β-galactosidase

1. Introduction

Human cytomegalovirus (HCMV) causes morbidity and mortality in part of the population, particularly those with compromised immune function (Alford and Britt, 1993). Ganciclovir and foscarnet are currently the drugs of choice for treatment of HCMV infections; however both have dose-limiting side effects (Markham and Faulds, 1994; Wagstaff and Bryson, 1994). Therefore, more effective treatments for HCMV are needed. Antiviral drug discovery relies on cell culture assays of HCMV replication. The traditional assays are the plaque reduction assay and the virus yield reduction assay. Because of the slow growth of HCMV in cell culture, these two assays each take 1–2 weeks to complete. The quantitation of antiviral effect is labor intensive and the number of samples that can be analyzed is limited. Newer assays such as the immunological detection of HCMV antigens in cell culture by

* Corresponding author. Tel.: +314 5376866; fax: +314 5376480; e-mail: pjhipp@ccmail.monsanto.com

PII S0166-3542(95)00976-X

enzyme-linked immunosorbent assays (ELISA) have increased the number of samples that can be analyzed, the ease of quantitation of antiviral effect and the sensitivity of the assay. (Tatarowicz et al., 1991).

While ELISA systems are relatively simple, they still require multiple reagents that need to be optimized. To further simplify the detection and, perhaps, to increase the sensitivity in antiviral screens we have investigated the use of a recombinant HCMV. Recombinant virus RC256 has the *Escherichia coli* β-galactosidase gene under control of the major early HCMV promoter integrated into one copy of the major early gene (Spaete and Mocarski, 1987). Since this early gene is one of the most abundantly expressed genes, the infected cells synthesize large amounts of β-galactosidase. Cells were infected in the presence or absence of test compounds and β-galactosidase was measured 48 h postinfection in a 96-well format. This assay was a fast, reliable and very simple indicator of antiviral activity.

2. Materials and methods

2.1. Cells and viruses

RC256 was obtained from Dr. E. Mocarski (Stanford University). Virus strains AD169, Towne and Davis were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Primary human fibroblasts were obtained from human foreskin samples from Washington University, St. Louis, MO. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) containing 5% heat-inactivated (1 h, 55°C) fetal bovine serum (FBS) (Life Technologies) and 2 mM extra glutamine (Life Technologies) at 7.5% CO_2. The cells were maintained at low passage by splitting the cells 1 : 5 to 1 : 10 once or twice a week. Media were changed twice a week.

2.2. Reagents

Reporter Lysis Buffer (catalog number E153A) was from Promega (Madison, WI). 2 × β-galactosidase buffer consisted of 120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 4.4 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG, catalog number N-1127, Sigma, St. Louis, MO). X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside), acyclovir and phosphonoformate (PFA) were purchased from Sigma. Ganciclovir (sodium salt) was a gift from Syntex. Human immune globulin (GAMMAR, Armour Pharmaceutical Co., Kankakee, IL; NDC 0053-7595-02) was obtained via a local retail pharmacy.

2.3. Infection

Human fibroblasts were trypsinized, counted and resuspended in DMEM/5% FBS at the desired concentration, typically 3.5×10^5 cells/ml. Virus was added to give a specific multiplicity of infection (MOI). The MOI was based on the titer of infectious units derived from a standard plaque assay of the virus on monolayers. Therefore, the true MOI in suspension may vary. The cell/virus mixture (100 μl) was then plated into wells of a 96-well tissue culture plate (Falcon) that had previously been loaded with 100 μl of specific concentrations of test compound in DMEM/5% FBS. Test compounds were typically made as a 20 mM stock in dimethyl sulfoxide (DMSO) and diluted such that the final concentration of DMSO, once cells were added, was 0.5% at the highest concentration. Two columns of wells contained media without any test compound. One received infected cells and served as the positive control and one column received uninfected cells and served as the negative control. The plates were incubated for various lengths of time at 37°C, 7.5% CO_2.

2.4. β-galactosidase assay

Medium was aspirated from the wells and 50 μl of Reporter Lysis Buffer (1 ×, diluted in water) was added to each well. Plates were maintained for 20–30 min at room temperature. 50 μl of 2 × β-galactosidase assay buffer was added and the reaction proceeded at room temperature for 30 min. The reaction was stopped with 100 μl of 1 M CAPS, pH 11. The absorbance was determined at 410 nm. The EC_{50} was determined on

semilog plots by determining the dose of compound that decreased the absorbance 50% between the maximum absorbance (cells that received virus only) and the minimum absorbance (cells that were not infected with virus). In situ staining of cells was according to Lim and Chae (1989).

2.5. Plaque reduction assay

Human foreskin cells were plated in 12-well plates (Costar, Cambridge, MA) at 2×10^5 per well in DMEM/10% heat-inactivated FBS. The cells were plated 24 h before use and incubated at 37°C in 5% CO_2. Virus was diluted to 250 plaque forming units (PFU) per ml in DMEM/10% FBS with 0.2 ml added per well and adsorbed for 1 h at 37°C with rocking of the plates every 15 min.

At the end of adsorption, the inoculum was removed and 3 ml medium with 1% GAMMAR and compound was added to triplicate wells. Freshly diluted compound was added twice a week and stocks were prepared once a week. Water-soluble compounds were dissolved in 100% DMSO to make a 20 mM stock. Plates were incubated for 10–13 days. The supernatant was aspirated and 1 ml methanol was added per well. Giemsa stain (Fisher Scientific, Pittsburgh, PA, catalog number SG-28500D) was diluted 1 : 8 in phosphate buffered saline (PBS) and added to the wells for at least 2 h. The wells were rinsed with water, dried and plaques counted using 40 × magnification. The concentration of chemical which reduces the number of plaques to 50% of the control was calculated according to Langford et al. (1981).

2.6. Virus yield reduction assay

Human fibroblasts were infected at a MOI of 3 PFU/cell and then plated into wells containing various concentrations of ganciclovir. These primary plates were incubated at 37°C for 6 days with one addition of fresh ganciclovir and then frozen at −80°C to disrupt cells and release intracellular virions.

For the second phase of the assay, supernatants from each concentration of ganciclovir from the primary plates were diluted in DMEM/5% FBS and adsorbed for 1–2 h onto fresh cell monolayers along with infected and uninfected controls. The inoculum was aspirated, DMEM/5% FBS/1% GAMMAR was added and plates were incubated for 8 days. Monolayers were fixed and stained with 0.1% crystal violet in 10% formalin (Fisher, catalogue number SF93-4) and plaques counted. The EC_{50} for virus yield reduction for ganciclovir was calculated as described for the plaque reduction assay.

3. Results

3.1. Growth of RC256

Human foreskin fibroblasts were mixed with various amounts of RC256 and plated in wells of 96-well plates. The cells were lysed and assayed for β-galactosidase activity at various times after plating. Culture conditions for detection of β-galactosidase activity were 3.5×10^4 cells per well with a MOI of 0.05 PFU per cell. β-galactosidase activity increased in the wells with increasing time (Fig. 1). No activity was detected at 7 h postinfection. However, from 24 to 72 h postinfection, there was a linear increase in β-galactosidase activity. To show that the increase in β-galactosidase activity was due to an increase in the number of infected cells and not just more β-galactosidase activity from the originally infected cells, the cells were fixed and stained with X-gal at the same time points. The number of blue cells increased with time in clusters, consistent with the spread of virus (data not shown).

3.2. Inhibition of RC256 by antiviral compounds

The EC_{50} value for ganciclovir against RC256 was 1.6 μM as determined by plaque reduction assay. EC_{50} values for other virus strains (Towne, AD169 and Davis) ranged from 1.6 μM to 5.2 μM with the mean being 3.0 μM (S.D. = 1.3). Therefore, the plaque reduction EC_{50} value for ganciclovir against RC256 was within the range of other strains of HCMV on the human fibroblasts.

If the increase in β-galactosidase activity shown in Fig. 1 were due to spread of virus, the increase

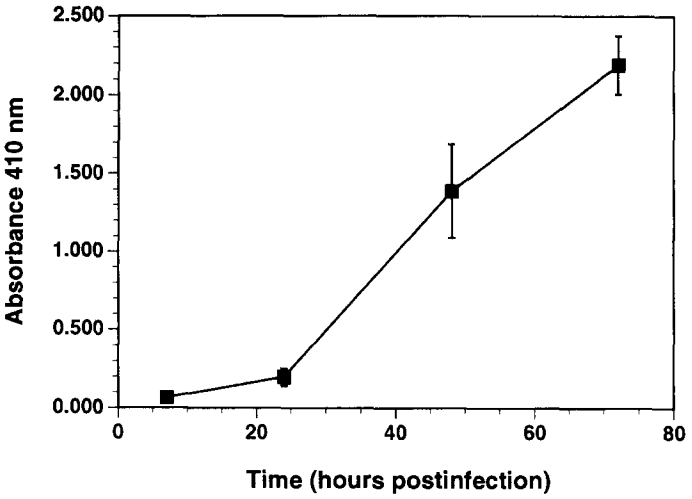

Fig. 1. β-galactosidase activity in cells infected with RC256. Human foreskin fibroblasts 3.5×10^5 per ml were mixed with RC256 at a MOI of 0.05 PFU/cell. 100 μl were plated in wells of a 96-well plate. At various times the cells were lysed and β-galactosidase activity was determined on 12 wells for each time point. Error bars represent the S.D. of the mean. The absorbance at 7 h postinfection (0.065) is background.

in activity should be inhibited by compounds that inhibit replication of HCMV. However, the β-galactosidase activity in the initially infected cells would not be inhibited by compounds that block virus replication after expression of early genes. Ninety-six-well plates were preloaded with serial dilutions of various inhibitors of HCMV replication. RC256 and cells were mixed at the appropriate ratio and added to the wells. At 48 h postinfection, the media were aspirated and the wells were assayed for β-galactosidase activity. There was a dose-dependent inhibition of β-galactosidase activity using ganciclovir (Fig. 2), acyclovir and foscarnet (not shown). The effective concentration necessary to inhibit 50% of the β-galactosidase activity (EC_{50}) for various inhibitors is shown in Table 1. The EC_{50} values for ganciclovir as determined by β-galactosidase activity were slightly higher than the values determined by plaque reduction. EC_{50} values determined by β-galactosidase activity for acyclovir and foscarnet were within the ranges seen on other virus strains (Markham and Faulds, 1994). These results with three different inhibitors suggest that the inhibition of β-galactosidase activity was not due to inhibition of β-galactosidase enzyme activity per se or due to inhibition of β-galactosidase gene expression without concomitant inhibition of virus replication. To confirm these results, a virus yield reduction experiment was done using RC256 in the presence of ganciclovir. The virus harvested after infection in the presence of ganciclovir was plated on two sets of fresh fibroblasts. One set was used to determine plaque number and the other was used to determine β-galactosidase activity. The virus yield at each ganciclovir concentration is shown in Fig. 3. The decrease in virus yield as measured by plaque assay and the decrease in β-galactosidase activity parallel each other over the concentrations of ganciclovir tested. The amount of β-galactosidase activity in the 33.3- and 100 μM ganciclovir samples was below the limit of detection using ONPG. No virus was detected by the plaque assay at 100 μM ganciclovir. Therefore, the decrease in β-galactosidase activity corresponds to the decrease in infectious virus yield from the treated cultures, indicating that β-galactosidase activity can be used as an accurate measure of virus replication.

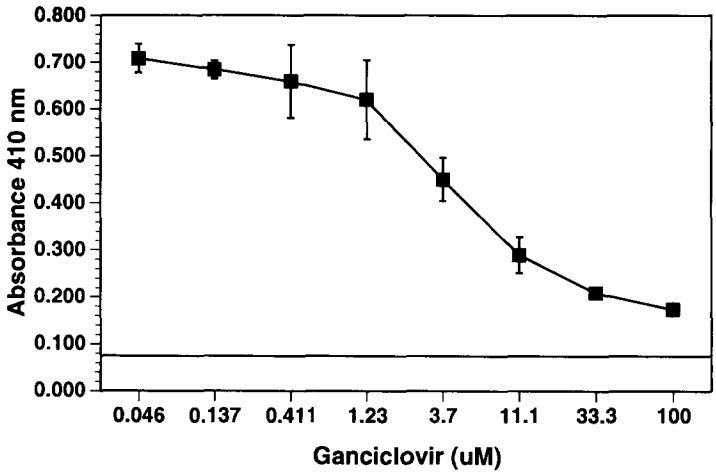

Fig. 2. Ganciclovir inhibition of β-galactosidase activity in RC256-infected cells. Human fibroblasts were infected with a MOI of 0.05 PFU/cell with RC256 in the presence of serial dilutions of ganciclovir. β-galactosidase activity was determined at 48 h postinfection. The error bars represent the S.D. of the mean of values from triplicate wells. The horizonal line at the bottom is the background optical density (OD) on uninfected cells.

3.3. Assay performance

To determine the performance of the assay over time, the log of the EC_{50} values was determined as a function of assay replicates with one assay being done per week. For each assay, three wells were used for each concentration of inhibitor. Six wells were used for determination of maximum β-galactosidase activity (cells with virus) and six wells were used to determine the background (cells without virus). The control chart is shown in Fig. 4. In general, the performance of the assay was very good, with only two of the assays falling outside the control limits (± 3 S.D. from the mean).

Table 1
EC_{50} for inhibition of β-galactosidase activity in RC256-infected cultures [a]

Inhibitor	EC_{50} (μM)	S.D.	n
Ganciclovir	8.9	1.7	24
Acyclovir	45	15	3
PFA	80	8.7	3

[a] The EC_{50} is the concentration of inhibitor that reduces the optical density (OD) 50% of the range between the maximum OD (cells and virus without inhibitor) and the minimum (cells alone).

4. Discussion

The standard assays for testing in vitro efficacy of compounds for activity against HCMV are the plaque reduction assay and the virus yield reduction assay. Both assays are labor intensive and time consuming and therefore not amenable to screening a large number of samples in a short period of time. Recent developments such as ELISA have decreased the time and increased the number of samples that can be analyzed by antiviral assays (Tatarowicz et al., 1991). To further simplify antiviral screening, we tested the use of a recombinant HCMV in a rapid 96-well assay. RC256 has a deletion/insertion such that the *E. coli* β-galactosidase gene is expressed from an early HCMV promoter. Overexpression of β-galactosidase does not inhibit the growth of the virus compared to the wild-type parental strain Towne (Spaete and Mocarski, 1987). Since β-galactosidase is under control of a strong, early promoter, enzyme activity can be detected soon after infection. Expression of β-galactosidase in the presence of ganciclovir is not inhibited in the initially infected cells since ganciclovir inhibits viral DNA replication, an event that occurs after expression of early genes. The initially infected

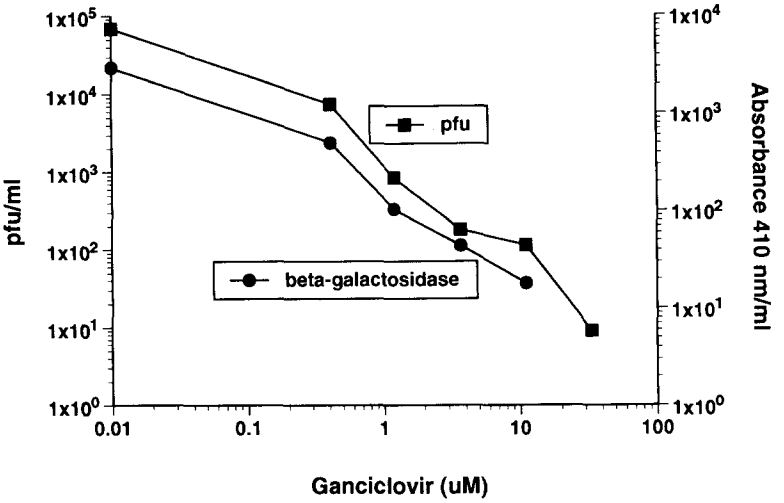

Fig. 3. Virus yield reduction assay measured by plaque assay and β-galactosidase assay. Human fibroblasts were infected with RC256 at a MOI of 3 PFU/cell in the presence of various concentrations of ganciclovir. Six days later, the cells were freeze–thawed three times and the lysates were serially diluted onto fresh fibroblasts. The total PFU of HCMV at each ganciclovir concentration was determined by plaque assay scored on day 8. The same serially diluted lysates were cultured on a matched set of fibroblasts and β-galactosidase activity was determined. ■, PFU/ml; ●, β-galactosidase activity. The limit of detection for the plaque assay was 3 PFU. The limit of detection of β-galactosidase activity was 0.3 OD units/ml. Vehicle-treated cultures yielded 6.9×10^4 PFU/ml and 3×10^3 OD units/ml.

cells in the culture will contribute to a background, but that level of activity is low compared to the level of activity due to amplification of expression due to virus spread. This background results in a maximum inhibition of 80–90% by optical density (OD) using ganciclovir. Use of a lower MOI may decrease the background even further; however the length of time to get an acceptable signal may need to be increased. Alternatively, engineering of HCMV to put the marker enzyme under control of a strong late gene promoter may also decrease background. Testing of chemical libraries or natural product inventories for HCMV antiviral activity is readily accomplished using RC256. Compounds that are positive in the assay are verified by plaque reduction to confirm that the decrease in signal was due to a decrease in viral replication and not inhibition of β-galactosidase enzyme by residual compound on the plate.

Our results suggest that ganciclovir inhibits replication of RC256 at concentrations similar to those that inhibit other strains of HCMV as measured by plaque reduction assay. Therefore, engineering of the virus did not alter its susceptibility to inhibitors of viral DNA synthesis. Virus yield reduction assays showed that the inhibition of β-galactosidase activity in the rapid screening assay is not due to inhibition of the enzyme itself or inhibition of expression of the β-galactosidase gene (without inhibition of virus replication). In general, the EC_{50} values determined by β-galactosidase activity in the microplate assay were slightly higher than the values obtained by plaque reduction, although within the norm for different literature values for the inhibitors tested on different cell lines (Freitas et al., 1985; Kern, 1991; Markham and Faulds, 1994; Tatarowicz et al., 1991). Importantly, the results were consistent over time. The response of ganciclovir over time appears to be cyclical. There is no obvious explanation for this. There is no correlation between the EC_{50} pattern and cell passage, virus stock, or ganciclovir stock. Another compound assayed in the same set of assays did not show this cyclical pattern (data not shown).

The amount of time needed to determine antiviral efficacy is much decreased over the plaque

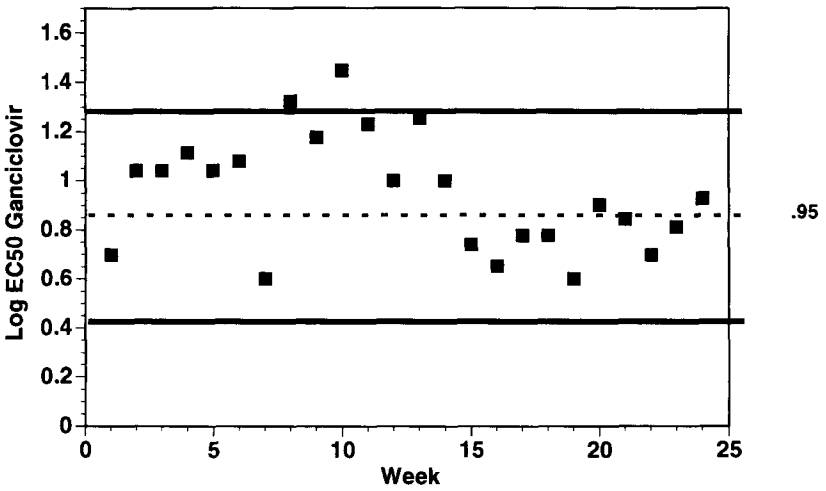

Fig. 4. Control chart for ganciclovir inhibition of RC256. The $\log_{10}$ of the EC_{50} as determined by β-galactosidase activity is plotted as a function of weekly assays. The control limits shown as the dark horizontal bars represent $\pm$ three times the S.D.. The mean is shown as the dotted line.

reduction assay and even the ELISA. In our experience, the ELISA format required 4 days from infection to antigen determination (M. Highkin, V. Dilworth, unpublished). Formatting of the assay required examination of several parameters such as choice of primary and secondary antibodies, concentrations of antibodies, times of incubation, types of blocking reagents, number of washings, types of enzyme substrate and development times. Development of the plates required many hours of antibody incubations, washing and reading of plates. Using the RC256 assay, the total time required is 3 days, while the reagents needed for β-galactosidase determination are both inexpensive and simple to use. More recently, a radioimmunoassay was developed for screening compounds for activity against HCMV (Gangopadhyay et al., 1994). Although more rapid than the standard plaque reduction or cytopathic effect assay (Adler et al., 1994), the use of radioactivity makes it less desirable than the assay we describe. In summary, the use of recombinant HCMV in screening for inhibitors of replication is accurate, reproducible and saves time, labor and reagent expense.

Acknowledgements

We wish to thank Dr. E. Mocarski for RC256, M. Bryant, R. Wiegand and D. Lansky for helpful discussions, M. Highkin, B. Holwerda and A. Rankin for helpful discussions, cell lines and plaque reduction data, L. Pennelly for ganciclovir and T. Warren and R. Wiegand for critical reading of the manuscript.

References

Adler, J., Mitten, M., Norbeck, D., Marsh, K., Kern, E.R. and Clement, J. (1994) Efficacy of A-73209, a potent orally active agent against VZV and HSV infections. Antiviral Res. 23, 93–105.

Alford, C.A. and Britt, W.J. (1993) Cytomegalovirus. In: B. Roizman, R.J. Whitley and C. Lopez (Eds), The Human Herpesviruses, pp. 227–255. Raven Press, New York.

Freitas, V.R., Smee, D.F., Chernow, M., Boehme, R. and Matthews, T.R. (1985) Activity of 9-(1,3-dihydroxy-2-propoxymethyl)guanine compared with that of acyclovir against human, monkey and rodent cytomegaloviruses. Antimicrob. Agents Chemother. 28 (2), 240–245.

Gangopadhyay, N.N., Whitley, R.J. and Chatterjee, S. (1994) A rapid protein A binding radioimmunoassay for the evaluation of antiviral agents. J. Virol. Methods 48, 273–279.

Kern, E.R. (1991) Value of animal models to evaluate agents with potential activity against human cytomegalovirus. Transplant. Proc. 23 (Suppl. 3(3)), 152–155.

Langford, M.P., Weigent, D.A., Stanton, G.J. and Baron, S. (1981) Virus plaque-reduction assay for interferon: microplaque and regular macroplaque reduction assays. Methods Enzymol. 78, 339–346.

Lim, K. and Chae, C.-B. (1989) A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for β-galactosidase. Biotechniques 7 (6), 576–579.

Markham, A. and Faulds, D. (1994) Ganciclovir. An update of its therapeutic use in cytomegalovirus infection. Drugs 48 (3), 455–484.

Spaete, R.R. and Mocarski, E.S. (1987) Insertion and deletion mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad. Sci. USA 84, 7213–7217.

Tatarowicz, W.A., Lurain, N.S. and Thomson, K.D. (1991) In situ ELISA for the evaluation of antiviral compounds effective against human cytomegalovirus. J. Virol. Methods 35, 207–215.

Wagstaff, A.J. and Bryson, H.M. (1994) Foscarnet. A reappraisal of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with viral infections. Drugs 48 (2), 199–226.