Analytical Approaches for the Management of Resistant Cytomegalovirus Infection

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Abstract

Cytomegalovirus is a member of the herpesviridae family which is prevalent in the human population. Following primary viral infection, cytomegalovirus persists in a latent state, from which the virus can reactivate resulting in renewed shedding. While cytomegalovirus rarely causes disease in the immunocompetent population, infection in newborns and immunocompromised patients can result in significant morbidity and mortality. Current strategies for the management of cytomegalovirus infection utilise drugs such as ganciclovir which inhibit viral DNA polymerase. As with other viruses, incomplete suppression of replication during long-term use of antiviral drugs favours the development of resistance resulting in drug failure. In the management of resistant cytomegalovirus infection, the determination of adequate drug delivery warrants attention, and thus, a sensitive and selective capillary electrophoresis assay for ganciclovir concentration in plasma was developed and validated. The assay utilises solid phase extraction and detection by ultraviolet absorbance, and has a lower limit of quantification of 40 ng/ml. Along with pharmacokinetic monitoring, another consideration in the management of drug resistance is the identification of viral susceptibility, and quantitative polymerase chain reaction was used to investigate the effects of ganciclovir on cytomegalovirus DNA. For this investigation, molecular cloning was conducted to produce plasmid-virus DNA, which was used as a quantitative standard in assessing the inhibition of cytomegalovirus DNA replication by ganciclovir in reference and resistant virus strains. In addition to the determination of susceptibility, quantitative polymerase chain reaction can also be applied in the screening of novel antiviral agents against cytomegalovirus, which are required due to continued concerns surrounding antiviral resistance.

Table of Contents

List of Tables

List of Figures

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1. Literature Review

1.1 Human Cytomegalovirus

The cytomegaloviruses (CMVs) are a distinct, widely distributed group of viruses belonging to the Herpesviridae family which share common growth features and cause a cytopathology characterised by nuclear and cytoplasmic inclusions.[1] Cytomegaloviruses are the principal members of the Betaherpesvirinae subgroup, which exhibit distinguishing biological characteristics such as strict species specificity, salivary gland tropism, and slow growth in cultured cells.[2] Although they are more closely related to each other than to other herpesviruses, betaherpesviruses also share common herpesvirus characteristics, including virion structure, and the ability to establish and reactivate from latent infection.[3]

Members of the Herpesviridae family are enveloped double-stranded DNA viruses with a capsid and a tegument.[4] Cytomegalovirus has the largest genome (230 kbp) of the herpesviruses. The genome is divided into long (L) and short (S) components, which are linked by internal repeat (IR) sequences, and flanked by terminal repetitive sequences. The sequences located between the repeat regions are unique (U), designated as the unique long (UL) and unique short (US) regions.[5] Through sequencing of the viral genome, it has been determined that the AD169 strain of CMV contains over 200 predicted open reading frames, encoding more than 100 polypeptides.[6] Sequence analysis has also determined

that clinical CMV isolates may carry genes not found in reference laboratory strains.[7]

1.1.1 Epidemiology of Cytomegalovirus

Infection with CMV in humans is common, with transmission occurring through person-to-person contact. In most developed countries, the seroprevalence of CMV increases after infancy with 10 to 20% of children infected before puberty,[8] and children may subsequently be re-infected with different strains of the virus.[9] Infection is also common during adolescence, which corresponds with the beginning of sexual activity.[10] In the adult population, the prevalence of CMV infection ranges from 40 to 100%, and although the virus is distributed world-wide, CMV infection is more common in lower socio-economic areas, due to the closeness of contact in living under such conditions.[8]

1.1.2 Clinical Aspects of Cytomegalovirus Infection

1.1.2.1 Primary Cytomegalovirus Infection in Children and Adults

In the immunocompetent population, infection with CMV is generally asymptomatic and in most cases rarely causes disease. However, an illness which is clinically indistinguishable from infectious mononucleosis caused by Epstein-Barr virus (EBV) is possible, with symptoms including fever, myalgia, cervical lymphadenopathy, and mild hepatitis. Complications including myocarditis, pneumonitis, and aspetic meningitis have been reported, but are rare.[11] As is the case with other herpesviruses, persistent infection follows

primary CMV infection, with sites of viral latency suggested to include bone marrow progenitor cells and peripheral blood monocytes.[8]

1.1.2.2 Congenital Cytomegalovirus Infection

Cytomegalovirus is a leading cause of congenital viral infection, with an average incidence rate of 1% resulting in an estimated 40,000 infants born with congenital CMV annually in the United States.[12] Clinical disease occurs in approximately 10% of the infected infants, of which 10 to 20% die.[4] The likelihood and severity of foetal infection are greater when a seronegative mother acquires primary infection early in the pregnancy.[11] Characteristics of congenital CMV infection include petechiae, jaundice, hepatosplenomegaly, and neurological manifestations such as microcephaly and lethargy/hypotonia.[13] Subsequent development of late complications is observed in infants born with symptomatic (80 to 90%) and asymptomatic (8 to 15%) CMV infection, including sensorineural hearing loss, mental retardation, seizure disorder, and chorioretinitis.[12]

1.1.2.3 Cytomegalovirus Disease in HIV-infected Persons

Cytomegalovirus is one of the most frequently observed opportunistic pathogens in patients infected with human immunodeficiency virus (HIV), the virus responsible for Acquired Immune Deficiency Syndrome (AIDS), and is the most common viral infection in this population.[14] The cause of disease is usually reactivation of latent virus rather than primary infection, and a correlation is

observed between the development of CMV disease and the severity of immunodeficiency, with patients with CD4+ cell counts lower than 50 lymphocytes per mm^3 carrying a particular risk.[15] In developed countries, the widespread use of highly active antiretroviral treatment (HAART) against AIDS has made it so that there are relatively few patients with such low CD4+ counts. Prior to the introduction of HAART, the most common manifestation of CMV infection was retinitis, characterised by haemorrhagic retinal necrosis spreading along retinal vessels, resulting in sight-threatening disease as necrosis approached the macula.[16]

1.1.2.4 Cytomegalovirus Disease in Bone Marrow Transplant Recipients

Cytomegalovirus disease is a major clinical problem in bone marrow transplant (BMT) recipients, with 30 to 50% incidence of significant infection. Seropositivity for CMV carries risk of disease, and the risk can be eliminated when both donor and recipient are seronegative, and CMV seronegative blood products are used.[11] The most serious manifestation of CMV infection after BMT is pneumonitis with progression to respiratory failure. Graft *versus* host disease (GVHD) may play a role in lung injury, and it is suggested both that CMV infection may predispose to GVHD, and vice versa.[17]

1.1.2.5 Cytomegalovirus Disease in Solid Organ Transplant Recipients

The risk and severity of CMV disease in transplant recipients vary depending on the type of transplant received, the degree of immunosuppression given, and the

serostatus of both the donor and recipient.[18] Seronegative recipients receiving grafts from seropositive donors are at a 3 to 5 times greater risk of developing disease, and disease is likely to be more severe.[11] To reduce the risk, institutions may attempt to match seronegative donors to seronegative recipients, but this approach is limited by organ availability. Disease often presents with specific organ involvement. Cytomegalovirus disease in the gastrointestinal tract includes oesophagitis, gastritis, colitis, and the development of peptic ulcers.[11] Cytomegalovirus has also been associated with particular complications in kidney,[19] heart,[20] and liver[21] transplantation, though a causal relationship has not been definitively determined.

1.1.3 Diagnosis and Monitoring of Cytomegalovirus Infection

Active infection with CMV is diagnosed by viral isolation, or by determining the presence of the virus through immunologic or molecular methods. However, because CMV can establish latent infection, and because excretion of virus can occur during asymptomatic infection, virus identification alone may not prove an etiologic role of CMV in disease, and other factors such as histological evidence and characteristic disease syndrome are required in order for a definitive diagnosis.[4] The viral parameters currently utilised for CMV infection diagnosis and monitoring include viraemia, antigenaemia, DNAaemia, and RNAaemia.[22]

1.1.3.1 Cytomegalovirus Shell Vial Assay

Diagnosis of CMV infection by viraemia employs a rapid virus isolation technique in which peripheral blood leukocytes (PBL) are co-cultured with human fibroblasts. Quantification of infected fibroblasts is done following nuclear staining with a monoclonal antibody against the major CMV immediate early antigen p72, which is representative of the number of infectious foci.[23] This method is rapid and can be used with non-blood samples, but has low sensitivity.[24]

1.1.3.2 Cytomegalovirus pp65 Antigenaemia Assay

The CMV antigenaemia assay employs direct staining of polymorphonuclear leukocytes (PMN) with monoclonal antibodies against the lower matrix protein pp65 (UL83).[25] The procedure includes isolation, fixation, and staining of PMNs, followed by slide reading and quantification. This method is a rapid and sensitive method of estimating CMV load, despite the subjective nature of slide interpretation for quantification.[24]

1.1.3.3 DNA-based Diagnostic Methods

The development of polymerase chain reaction (PCR) technology has enabled the detection of minute quantities of viral nucleic acid material. More recently, quantitative PCR methods have been used to determine CMV DNA copy numbers in PBLs,[26] whole blood,[27] and plasma[28] with high sensitivity. Other DNA-based methods which have been used to quantify CMV DNA include a branched-DNA signal amplification assay[29] and a hybrid capture DNA assay.[4]

1.1.3.4 RNAaemia Assays

Determination of CMV mRNA as direct markers of viral replication has been performed. Reverse transcription-polymerase chain reaction (RT-PCR) has been used to detect transcripts of immediate early CMV genes,[30] and nucleic acid sequence-based amplification (NASBA) technology has been utilised for the qualitative and quantitative determination of immediate early and late genes.[31,32]

1.2 Antiviral Therapy of Cytomegalovirus Infection

There are four established strategies for the management of CMV infection and disease. Aside from antiviral treatment of manifest disease, specific strategies have been developed with the aim of disease prevention, and these include prophylactic, suppressive, and pre-emptive therapy.[33] Prophylactic strategies are started in the absence of both detectable virus and disease. Suppressive and pre-emptive treatment strategies are started in the absence of disease but in the presence of detectable virus, with the difference being the site of viral detection: suppressive therapy refers to treatment given after peripheral detection, whereas pre-emptive therapy is treatment given after systemic detection.[8]

The characteristics of drugs selected for prophylaxis may be different from those of drugs used for the treatment of active disease. A balance between the risk of CMV disease and the acceptable level of drug-induced toxicity must be

considered. Patients being treated for established infection would be at high risk of dying from CMV disease, and thus, selection of a drug regimen associated with high toxicity is acceptable, in that it may still be beneficial to the patient overall. Contrary to this, if the risk of CMV disease is low, drug toxicity should be minimal.[33]

1.2.1 Ganciclovir for the Treatment of Cytomegalovirus Infection and Disease

Ganciclovir (GCV) is a nucleoside analogue of guanosine, and a homologue of the antiviral agent acyclovir (ACV), which is used for the treatment of herpes simplex infections. Ganciclovir inhibits all the herpesviruses and was the first antiviral drug shown to be effective in the treatment of CMV disease in humans.[34]

1.2.1.1 Mechanism of Action

In vivo, ganciclovir is converted to its active form, ganciclovir triphosphate, through the actions of both viral and host cellular kinases. Ganciclovir triphosphate competes with deoxyguanosine triphosphate as a substrate for viral DNA polymerase, encoded by UL54 in CMV. Following the release of pyrophosphate, ganciclovir monophosphate is incorporated into the elongating chain of viral DNA, thereby slowing replication.[35] Unlike acyclovir, ganciclovir is not an obligate chain terminator, and thus, short, subgenomic, non-infectious particles of CMV DNA continue to be synthesised within virus-infected cells in the presence of ganciclovir.[36] *In vitro*, ganciclovir inhibits plaque formation by

laboratory strains and clinical isolates of CMV with EC_{50} values ranging from 0.1 to 1.6 μg/ml. Ganciclovir also exhibits antiviral activity against other herpesviruses, including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus, and human herpesvirus-6.[34]

1.2.1.2 Phosphorylation of Ganciclovir

In cells infected with herpes simplex virus (HSV), ganciclovir is monophosphorylated by the enzyme thymidine kinase, and cellular enzymes convert ganciclovir monophosphate to its active triphosphate form.[35] However, unlike HSV, CMV does not possess a thymidine kinase, but a phosphotransferase enzyme encoded by the UL97 gene.[37,38] The insertion of the CMV UL97 gene into a ganciclovir-insensitive vaccinia virus renders the recombinant virus susceptible to ganciclovir.[39]

On a molar level, acyclovir triphosphate is more effective than ganciclovir triphosphate in the inhibition of CMV DNA polymerase, but in CMV-infected cells, the concentrations of ganciclovir triphosphate achieved are ten times that of acyclovir triphosphate.[35,40] In addition to this, another advantage of ganciclovir for the inhibition of CMV replication is the long intracellular half-time of ganciclovir triphosphate (16.5 hours)[41] in CMV-infected cells.

1.2.1.3 Ganciclovir Toxicity

The common adverse effects associated with ganciclovir are haematological, with anaemia, neutropenia, and thrombocytopenia being the primary toxicities.[42] Due to such haematological effects, caution is required if administering ganciclovir with other drugs that have toxic effects on marrow. Other observed adverse effects include increased levels of serum creatinine, and it is recommended that renal function be monitored in patients receiving ganciclovir, especially if other nephrotoxic drugs are also being administered.[42] Azoospermia and nervous system adverse effects have also been reported.[34,42]

1.2.2 Other Anti-Cytomegalovirus Drugs Available in Clinical Practice

1.2.2.1 Valganciclovir

The low oral bioavailability (6 to 9%)[34] of ganciclovir has led to the development of valganciclovir, a valyl ester prodrug. When administered, valganciclovir is rapidly absorbed and hydrolysed to ganciclovir. Pharmacokinetic studies have shown that the systemic levels of ganciclovir achieved with valganciclovir are similar to those of intravenous ganciclovir,[43] resulting in comparable antiviral efficacy.[44]

1.2.2.2 Foscarnet

Foscarnet is an analogue of pyrophosphate which directly inhibits CMV DNA polymerase by interfering with the release of pyrophosphate during substrate incorporation of viral DNA elongation. Unlike ganciclovir, foscarnet does not require enzymatic conversion to its active form.[45]

1.2.2.3 Cidofovir

Cidofovir is a phosphonate nucleotide analogue which does not require monophosphorylation by UL97 phosphotransferase. The activity of cellular kinases modulates the conversion of cidofovir to its active diphosphate derivative, which inhibits CMV DNA polymerase.[45]

1.2.3 Cytomegalovirus Resistance to Antivirals

1.2.3.1 Emergence of Drug-resistant Cytomegalovirus

The presence of resistant strains of CMV has been determined in immunocompromised patients following therapy with antiviral drugs.[46] Major factors which favour the emergence of resistant strains are high viral titre, and long duration of antiviral therapy.[47]

Anti-CMV drug resistance and its associated problems manifest differently in AIDS patients and transplant recipients. In the past, CMV infection in AIDS patients would require antiviral therapy that consisted of intravenous induction followed by maintenance treatment for the rest of the patient's life. Although HAART has now reduced the need for prolonged antiviral treatment, the potential for development of resistance is still of concern, particularly in patients with suboptimal control of HIV infection. Moreover, it is possible that AIDS patients do not receive antiviral therapy for CMV infection until the virus is

actively replicating and in high titre, which may favour the generation of drug-resistant mutants by chance.[45]

Transplant recipients are often receiving prophylactic regimens or are treated for shorter periods of time, and thus, antiviral resistance is relatively infrequent. However, in cases of long duration of antiviral therapy, or in the use of oral drugs with poor bioavailability or efficacy, there is increased risk of developing mutations associated with clinically significant drug resistance.[48]

1.2.3.2 Mechanisms of Cytomegalovirus Resistance to Ganciclovir

For ganciclovir, the most frequently encountered mechanism of resistance is mutation in the UL97 gene product, rendering the virus incapable of ganciclovir monophosphorylation, and subsequent conversion to its active triphosphate form. The role of UL97 mutation in CMV drug resistance was evidenced by studies in which the cloned UL97 product was used to phosphorylate ganciclovir in bacteria.[38] Further to this, ganciclovir resistance was induced in a previously susceptible laboratory strain following the deletion of four amino acids in the UL97 gene.[37] Clinical investigations have also identified mutations in the UL97 gene as a common cause of ganciclovir-resistant CMV.[49,50] Strains of CMV with mutation(s) in UL97 alone remain susceptible to foscarnet and cidofovir, as these drugs do not require activation by virus-encoded enzymes.

While mutations in UL97 represent the most common mechanism of resistance to ganciclovir in CMV, mutations in UL54, the gene encoding for the viral DNA polymerase, may also confer resistance. Studies of both recombinant CMV containing a mutated DNA polymerase gene[51] and a clinical drug resistant strain[52] indicated that the resistant viruses produced ganciclovir triphosphate at levels comparable to those seen in wild type virus. Mutations in UL54 are rarely observed without a concurrent UL97 mutation, and the specific location of the UL54 mutation determines whether cross-resistance with foscarnet and/or cidofovir is observed.[45]

1.2.3.3 Clinical Antiviral Resistance and Treatment Failure

In addition to the mutations discussed, other factors, such as the failure of drug delivery, may play a role in the failure of antiviral therapy against CMV infection. Failure of drug delivery may be due to issues of compliance, or the inability to achieve sufficient drug concentrations in the necessary tissues. The use of antiviral agents with poor bioavailability or the achievement of subtherapeutic doses is unfortunately common in transplant recipients.[45] It is recognised that resistant mutants are selected when CMV replication continues in the presence of subtherapeutic drug concentrations, and this is particularly problematic with poorly bioavailable drugs such as oral ganciclovir.[53]

1.3 Investigational Objectives

The existing body of evidence suggests that clinical CMV resistance to antiviral agents, and specifically, ganciclovir, continues to be an issue of concern, particularly in the context of long-term therapeutic strategies utilising drugs which may not completely inhibit virus replication.[54] In the management of drug resistant CMV, several factors require consideration, including adequate delivery of the drug, and identification of virus susceptibility.[45] The ability to quantify plasma drug concentrations enables physicians and investigators to determine whether administered drugs are achieving adequate therapeutic levels, and phenotypic determination of drug susceptibility is required for both the detection of resistant viruses and the screening of novel compounds with antiviral therapeutic potential. To this end, the objectives of this thesis are:

1. To optimise and validate a capillary electrophoresis bioanalytical assay for ganciclovir in human plasma that is clinically applicable for pharmacokinetic studies and therapeutic drug monitoring.
2. To study the susceptibility of wild-type and resistant strains of CMV to ganciclovir through the reduction of viral DNA as measured by real-time polymerase chain reaction.

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2. Development and Validation of a Capillary Electrophoresis Assay for the Quantitative Analysis of Ganciclovir in Human Plasma

2.1 Introduction

2.1.1 Capillary Electrophoresis for the Bioanalysis of Drugs

Electrophoresis is a separation method in which compounds are separated based on differential migration rates in a buffer solution upon application of an electric field. The principles of electrophoretic mobility dictate that positively charged cations migrate toward the negative electrode while negatively charged anions migrate toward the positive electrode.

Historically, high performance liquid chromatography (HPLC) has been the most widely used separation method for pharmaceuticals. During the 1980s, there was increased interest in high efficiency compound separation through the application of voltage across capillaries, leading to the advent and development of capillary electrophoresis (CE) as a separation technique.[1] Since its introduction, CE has undergone rapid growth and is gaining in popularity for the analysis of pharmaceuticals. The advantages of CE such as high resolution and low sample volume requirements make it a suitable method for the quantification of drug levels when limited sample is available. Further to this, the minimal solvent consumption of CE makes it an attractive alternative or complementary technique to HPLC.[2] However, the analysis of drugs in body

fluids is complicated due to the presence of other potentially interfering compounds in the sample matrix, and low concentrations of the analyte(s) of interest.[3]

2.1.2 Analysis of Ganciclovir in Plasma

For pharmacokinetic studies and therapeutic monitoring of patient drug levels, the ability to quantify plasma drug concentrations is required. Several methodologies for the analysis of ganciclovir in biological matrices by HPLC which use various procedures for sample cleanup including deproteinisation with acid or organic solvents have been described. Methods utilising detection by direct ultraviolet (UV) absorbance generally require between 0.5 and 1 ml of biological fluid to be analysed, and exhibit a limit of quantification (LOQ) of 50 ng/ml.[4,5] An HPLC method employing detection by fluorescence has also been described, in which lower sample volumes (0.25 ml) are required, and higher sensitivity (LOQ of 40 ng/ml) is achieved.[6] Liquid chromatography using pulsed amperometric detection[7] and coupled with electrospray ionisation and selected reaction monitoring/mass spectrometry[8] have also been used for the quantitative analysis of ganciclovir.

While other nucleoside analogue antiviral drugs including acyclovir[9,10] and penciclovir[11] have been successfully analysed using CE, to date, there have been no reported assays for ganciclovir. Based on the structural similarity between nucleoside analogues, it is reasonable to suggest that a CE assay would be a

suitable method of analysis for the quantification of ganciclovir in biological fluid, which could be utilised in clinical monitoring and studies.

2.1.3 Requirements for Bioanalytical Methods

In order for bioanalytical methods to be useful, there needs to be the assurance that the data obtained are reliable. For this reason, it is important to validate quantitative biomedical applications, and to ensure that such experiments yield satisfactory results.[12] In 1990, a panel of experts discussed the validation procedure in order to come to a consensus as to the guidelines for method establishment, validation, and application to drug analysis in biological matrices.[13] Bioanalytical method validation for pharmaceuticals is also influenced by organisations such as the United States Food and Drug Administration (FDA), the Medicines Control Agency (MCA) of the United Kingdom, and similar regulatory bodies in other countries. For chromatographic methods used in biomedical applications, the key analytical parameters are recovery, linearity and range, precision, accuracy, sensitivity, selectivity, and stability.

2.1.3.1 Recovery

Absolute recovery of a method is determined by the ratio of the response of the processed matrix spiked with the analyte of interest, to the response of the unprocessed pure standard. While recovery as close to 100% is desirable to maximise method sensitivity, it may not be achievable, depending on the nature

of the sample processing procedure. When internal standardisation is used, the recovery of the internal standard should be determined independently, and be within 15% of that of the analyte of interest.[12]

2.1.3.2 Linearity and Range

For chromatographic methods, the calibration model used is determined by the relationship between a response function and the concentration of the analyte. The response functions commonly used are peak height and/or peak area, corrected by the response of the internal standard, if applicable.

2.1.3.3 Precision

Precision describes the degree of variation observed in measurements of samples with the analyte of interest at the same concentration, and is expressed as the percentage coefficient of variation (%CV) or relative standard deviation (RSD). For bioanalytical methods, precision should be measured at five determinations at three concentrations within the expected range, and the RSD should be within 15% at all concentrations, with the exception of the lower limit of quantification (LLOQ), at which the acceptable RSD is within 20%.[14] Precision can also take into account the variation that exists within a sequence of analyses (intra-assay), or between sequences when conditions such as analyst, reagents, or equipment may vary (inter-assay).

2.1.3.4 Accuracy

Accuracy is defined as the agreement between the measured and true concentration of the analyte in the sample, and is best expressed as percentage relative error (RE):

$$\% \text{ RE} = [(\text{measured value} - \text{true value}) / \text{true value}] \times 100$$

Measurement of accuracy should be performed with a minimum of five determinations at three concentrations. The mean measured value should be within 15% of the actual values at all concentrations tested except at the LLOQ, at which the relative error should not exceed 20%.[14]

2.1.3.5 Sensitivity

The limit associated with reliable quantification is the LLOQ. On the calibration curve, the lowest standard is accepted as the LLOQ provided that the analyte response is at least five times greater than blank response, and exhibits less than 20% RSD for precision and less than 20% RE for accuracy.[14]

2.1.3.6 Selectivity

Analytical method selectivity is the ability to differentiate the analyte in the presence of other sample components. Selectivity is particularly important in the analysis of biological matrices, as fluids such as plasma or urine may have many potentially interfering compounds present. Thus, sample processing procedures and analytical conditions must be optimised to achieve adequate separation from interfering signals. Selectivity is investigated by comparing the

analysis of extracted spiked matrix with blank matrix, and by measurement of chromatographic retention time.[12]

2.1.3.7 Stability

Because biological samples are often not analysed immediately following sampling, analyte stability needs to be investigated. The stability of the analyte should be established in stock standard solutions, unprocessed biological matrix, and in processed samples (extracts), and factors to be considered include freeze and thaw stability, short- and long-term stability, and post-preparative stability of the extracts.[12]

2.1.4 Experimental Objective

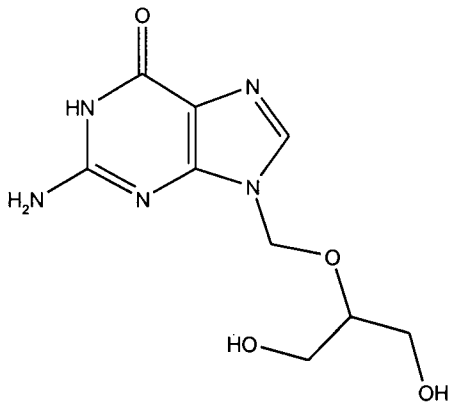
Monitoring plasma ganciclovir concentrations may be beneficial to optimise the management of CMV infection and disease by antiviral therapy. As such, the aim of the following studies was to develop and validate a sensitive CE assay for ganciclovir which meets required criteria for clinically suitable bioanalytical methods.

2.2 Materials and Methods

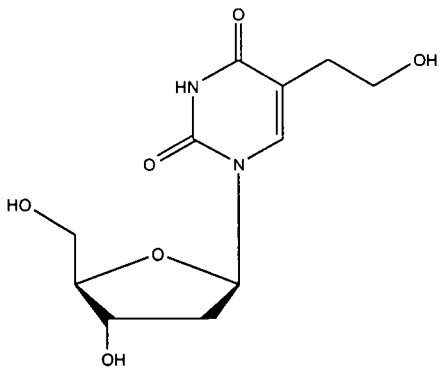
2.2.1 Chemicals and Reagents

Ganciclovir was obtained from Sigma (St. Louis, MO, USA) and the internal standard (I.S.) 5-(2-hydroxyethyl)-2'-deoxyuridine (HEdU) was obtained from Berry & Associates (Dexter, MI, USA). The chemical structures of ganciclovir

and HEdU are shown in Figure 2.1. Analytical grade borax, boric acid, lauryl sulfate (sodium and lithium salts), hydrochloric acid, phosphoric acid, potassium hydroxide, sodium chloride, and sodium phosphate (mono- and dibasic), were also from Sigma. Sodium hydroxide was obtained from Spectrum (New Brunswick, NJ, USA), and pharmaceutical grade hydroxypropyl-beta-cyclodextrin was from Cerestar (Hammond, IN, USA). HPLC grade acetonitrile and methanol were obtained from Aldrich (Milwaukee, WI, USA) and EM Science (Gibbstown, NJ, USA), respectively. Pooled human plasma with disodium EDTA as an anticoagulant was obtained from Valley Biomedical (Winchester, VA, USA). Water used for the preparation of all solutions and buffers was filtered and de-ionised with the ModuLab 2020 system from Continental Water Systems Co. (San Antonio, TX, USA).



Ganciclovir



5-(2-hydroxyethyl)-2'-deoxyuridine (HEdU)

Figure 2.1. Chemical structures of ganciclovir and HEdU.

2.2.2 Experimental Apparatus

The vacuum manifold for the solid phase extraction procedure was obtained from Waters (Milford, MA, USA), and extraction columns were from Waters and Phenomenex (Torrance, CA, USA). Centrifugal filtration units and filtration membranes were obtained from Millipore (Bedford, MA, USA). Electrophoretic analyses were conducted using MDQ capillary electrophoresis systems from Beckman Coulter (Mississauga, ON, Canada). Polyimide-coated bare fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) with internal and external diameters of 75 and 360 μm, respectively, were used. New capillaries were conditioned with the following rinses, each at 20 p.s.i.: methanol (10 min.), 1 M hydrochloric acid (10 min.), water (2 min.), 1 M sodium hydroxide (10 min.), water (2 min.), and background electrolyte (10 min.).

2.2.3 Assay Method Development

The process of method development included the investigation and optimisation of sample preparation, separation, and quantification of ganciclovir in prepared plasma samples. The ability to detect and quantify ganciclovir varied depending on the conditions of the matrix, the buffer, and the mode of CE employed.

2.2.4 Assay Method Validation

2.2.4.1 Standard Solution and Plasma Sample Preparation

Stock solutions of ganciclovir (1.0 mg/ml) and HEdU (0.1 mg/ml) were prepared in water. Serial dilutions were performed to prepare working standard solutions of HEdU (10 μg/ml) and ganciclovir (concentration range from 0.15 to 50 μg/ml). Stock and working standard solutions were stored at 4°C. 200 μl of human plasma with disodium EDTA as an anticoagulant was spiked with 40 μl of HEdU working standard and 40 μl of the appropriate ganciclovir working standard. The final concentration range for ganciclovir in the plasma samples was from 30 to 10000 ng/ml.

2.2.4.2 Solid Phase Extraction Procedure

Oasis® Hydrophilic-Lipophilic Balance (HLB) solid phase extraction columns from Waters were conditioned with 1 ml of methanol and equilibrated with 1 ml of water. Plasma samples were loaded onto the column, and washed with 0.5 ml of 0.5 M phosphate buffer pH 7.2 under vacuum. The samples were eluted from the column with 150 μl of a buffer solution containing 20% acetonitrile, 50 mM phosphate pH 7.2, and 0.2% sodium chloride. Eluted samples were filtered by centrifugation at 14000 RPM for 10 minutes through 0.45 μm filtration units and analysed by CE.

2.2.4.3 Electrophoretic Apparatus and Conditions

The total length of the capillaries used for method validation was 60.2 cm, with an effective length of 50 cm from the inlet end to the detector. Prior to each run during an analytical sequence, the capillary was rinsed with 20 mM sodium

dodecyl sulfate (2 min.), water (1 min.), methanol (2 min.), water (1 min.), 1 M NaOH (2 min.), water (1 min.), and background electrolyte (3 min.), each at 20 p.s.i.

To prepare the background electrolyte buffer, borate (160 mM) was adjusted to pH 8.8 with boric acid (160 mM). This borate buffer was filtered through a 0.45 μm filtration membrane, and was used to dissolve the additives dodecyl lithium sulfate (LDS), hydroxypropyl-beta-cyclodextrin (HPβCD), and sodium chloride (NaCl). The final separation buffer consisted of 160 mM borate pH 8.8 containing 90 mM HPβCD, 200 mM LDS, and 0.3% NaCl.

Plasma samples were maintained at 4°C in the CE instrument, and were injected hydrodynamically (100 seconds at 0.5 p.s.i.) into the capillary. Electrophoretic separation was conducted at 20°C using 17 kV, normal polarity, and detection of the analyte and internal standard was by UV absorbance at a wavelength of 254 nm.

2.2.4.4 Analysis of Validation Data

Samples were processed and analysed, and calibration curves were constructed by plotting the relative corrected peak area ratios (RCPAR) *versus* nominal ganciclovir concentrations. The ratios corrected the peak areas (PA) by migration time (MT) as follows:

$$RCPAR = \frac{(Ganciclovir\ PA\ /\ Ganciclovir\ MT)}{(I.S.\ PA\ /\ I.S.\ MT)}$$

The method was validated with respect to selectivity, sensitivity, recovery, linearity, range, accuracy, precision (intra- and inter-day, inter-analyst), and stability.

2.3 Results

2.3.1 Acidic Separation Buffer Conditions

One mechanism of analyte stacking in CE is field-amplified sample stacking (FASS), in which the sample is prepared in a solution of lower conductivity than the separation buffer. As the analyte migrates from the low conductivity sample matrix into the high conductivity buffer zone, the mobility of the analyte is decreased, resulting in focussing of the analyte.[15] These principles were applied in attempt to quantify ganciclovir in plasma using an acidic separation buffer, with results shown in Figure 2.2. It was observed that ganciclovir was not well-separated from interfering plasma, and that the extent of focussing achieved for ganciclovir was low, suggesting that the desired assay selectivity and sensitivity would not be accomplished with this method.

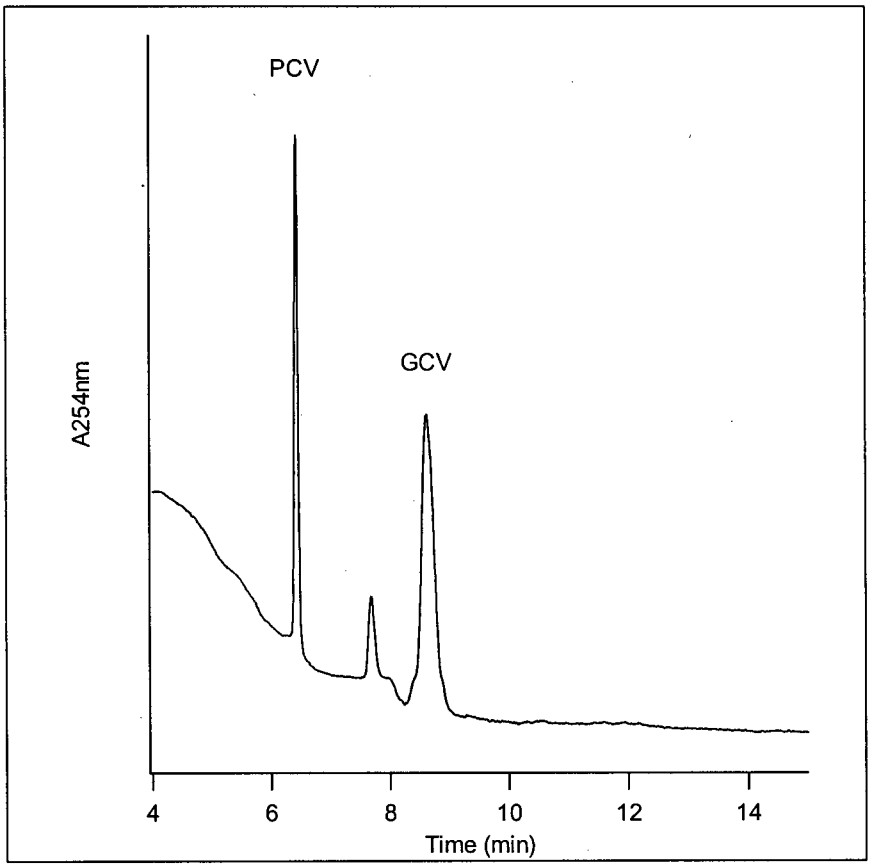


Figure 2.2. Analysis of ganciclovir in plasma following deproteinisation and solid phase extraction under acidic buffer conditions. Ganciclovir (1 μg/ml) and penciclovir (1μg/ml) as internal standard were spiked in 200 μl plasma. The plasma sample was deproteinised with 60% perchloric acid, neutralised with 6 N potassium hydroxide and buffered with 3 M phosphate pH 7.4. Following centrifugation, the neutralised supernatant was loaded onto a C18 solid phase extraction column and eluted with isopropanol. The eluted sample was evaporated under vacuum and re-dissolved in 40 μl water. The re-suspended sample was separated in 100 mM phosphate pH 2.0 under the following electrophoretic conditions:

Sample injection: 10 seconds at 0.5 p.s.i.
Capillary effective length: 31.2 cm
Capillary temperature: 22°C
Separation: 10 kV normal polarity

2.3.2 Basic Separation Buffer with Micellar and Cyclodextrin Additives

A capillary electrophoresis method utilising a basic separation buffer for the quantitative analysis of acyclovir, a nucleoside analogue with similar structure to ganciclovir, has been described.[16] It is hypothesised that basic analytes such as ganciclovir (which possesses a pKa of 9.4) can be introduced into the capillary in large quantities to improve assay sensitivity. The proposed mechanism involves charge-switching of the analyte from neutral in the sample matrix to partially negative as it enters the separation buffer zone, with focussing occurring as analyte migration is slowed.

The inclusion of additives to CE separation buffers is often done to improve sensitivity and separation from potential interference. The addition of SDS to the separation buffer enables the aggregation of SDS micelles which can interact with the analyte in a mode of CE known as micellar electrokinetic chromatography (MEKC or MECC). Separation is achieved due to differences in interaction between ganciclovir and plasma components with the micelles.[17] A cause of concern in the inclusion of micelles is the increase in conductivity of the separation buffer, resulting in high operating currents during the application of voltage. It was observed that the use of lithium dodecyl sulfate (LDS) produced the same separation effects as SDS but produced a lower operating current, and thus, LDS was used during the development of the method. The effects of LDS concentration in the separation are shown in Figure 2.3.

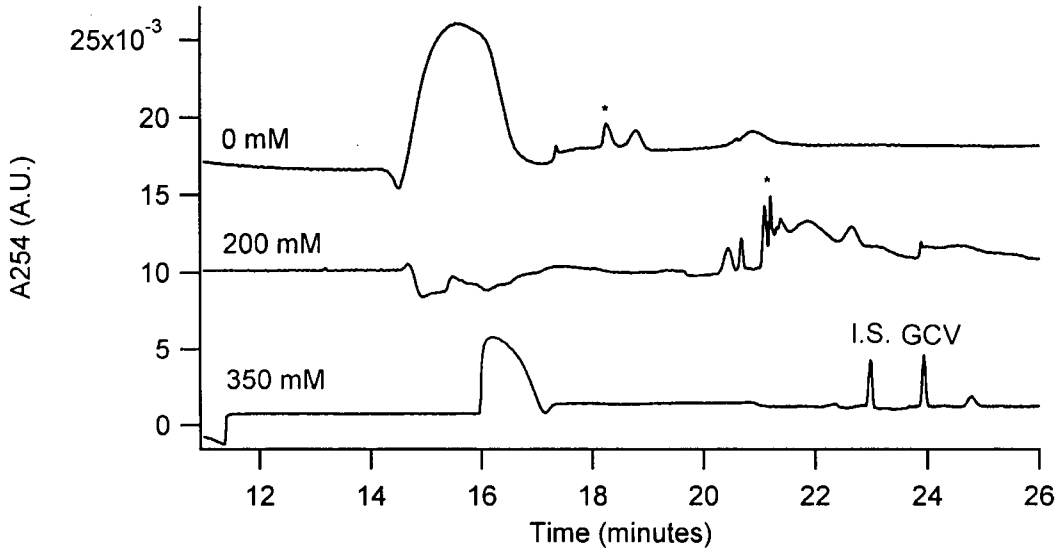


Figure 2.3. Effect of LDS on the separation of ganciclovir from other compounds in plasma. Ganciclovir (1 μg/ml) and HEdU (2 μg/ml) were spiked in 200 μl plasma and processed by solid phase extraction as described in the text in section 2.2.4.2. Samples were separated using 90 mM borate pH 8.8 containing 100 mM HPβCD and the concentrations of LDS indicated, under the following electrophoretic conditions:

Sample injection: 100 seconds at 1.5 p.s.i.
Capillary effective length: 60 cm
Capillary temperature: 22°C
Separation: 20 kV normal polarity

Cyclodextrins are also commonly employed additives in CE separation buffers. Cyclodextrins are linked oligosaccharide rings of glucopyranose whose surfaces are hydrophilic and central cavities are relatively hydrophobic.[18] The formation of cyclodextrin-analyte complexes enables online analyte concentration during separation, thus enhancing the detection sensitivity. Hydroxypropyl-β-cyclodextrin (HPβCD) is a seven glucopyranose unit with a neutral side chain, which was shown to interact with ganciclovir resulting in increases in peak height, as indicated in Figure 2.4. The use of HPβCD in the MEKC system provided a suitable framework upon which the method could be further developed and optimised.

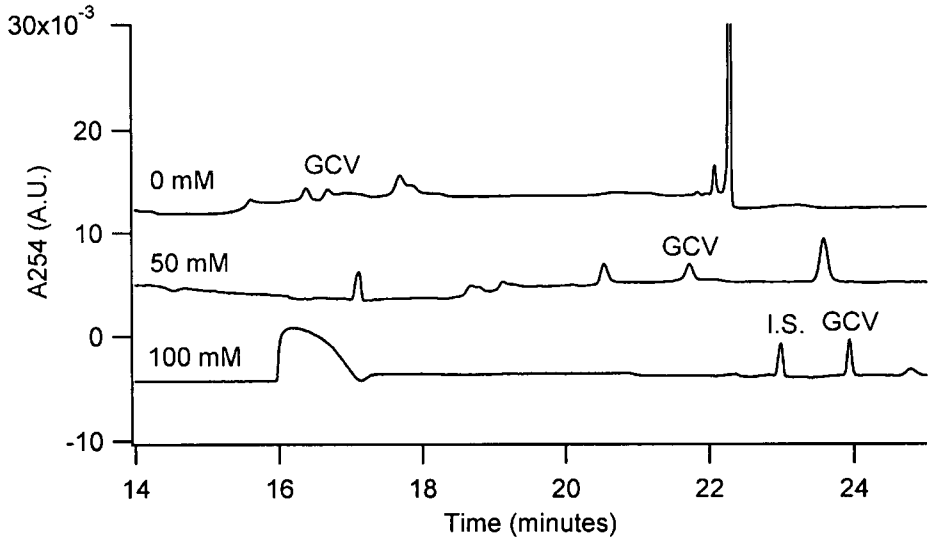


Figure 2.4. Effect of HPβCD on the sensitivity of detection of ganciclovir in plasma. Ganciclovir (1 μg/ml) and HEdU (2 μg/ml) were spiked in 200 μl plasma and processed by solid phase extraction as described in the text in section 2.2.4.2. Samples were separated using 90 mM borate pH 8.8 containing 350 mM LDS and the concentrations of HPβCD indicated, under the following electrophoretic conditions:

Sample injection: 100 seconds at 1.5 p.s.i.
Capillary effective length: 60 cm
Capillary temperature: 22°C
Separation: 20 kV normal polarity

2.3.3 Bioanalytical Assay Validation

2.3.3.1 Recovery of Ganciclovir and Internal Standard

Recovery was determined by comparative analysis of extracted plasma samples containing ganciclovir and HEdU with standard samples prepared in 120 mM phosphate pH 7.2 at concentrations of 100, 1000, and 10000 ng/ml. The average recovery of ganciclovir was 86.8 ± 5.3%, and the average recovery of HEdU was 85.8 ± 1.8%.

2.3.3.2 Linearity and Range

Linearity was determined by plotting the relative corrected peak area ratios (RCPAR) *versus* the corresponding concentrations of ganciclovir in the plasma samples. Five separate calibration curves demonstrated linearity between the response and ganciclovir concentration over the range of 40 to 10000 ng/ml, with analysis by linear regression showing $R^2 > 0.998$.

2.3.3.3 Precision

The precision of the method was determined by assessing variation in the assay both within one sequence of analyses and between multiple sequences. Analysis of low, medium, and high concentration samples on the same day was conducted to calculate intra-day precision, and analysis of samples on different days enabled the determination of inter-day precision. Also considered was the variation in assay performance when conducted by analysts using different

instruments, capillaries, and batches of background electrolyte buffer (inter-analyst precision). Analysis of intra- and inter-day (Table 2.1) and inter-analyst (Table 2.2) precision determined that relative standard deviation was less than 15% at the concentrations tested, indicating the reproducibility of the assay method on same and different days, as well as when performed by different analysts.

Table 2.1. Intra- and inter-day variability of the method for the determination of plasma ganciclovir concentrations, expressed as relative standard deviation (RSD). 200 µl plasma samples were spiked with 2 µg/ml HEdU and the concentrations of ganciclovir indicated, and extracted and separated as described in sections 2.2.3.2 and 2.2.3.3.

	Intra-day Variability			Inter-day Variability		
GCV Conc. Added (ng/ml)	GCV Conc. Found, mean ± SD (ng/ml)	RSD (%)	n	GCV Conc. Found, mean ± SD (ng/ml)	RSD (%)	n
100	90.6 ± 5.9	6.5	5	112.2 ± 12.6	11.2	9
1000	1009 ± 43	4.3	5	1024 ± 36	3.5	10
10000	$(1.063 \pm 0.080) \times 10^4$	7.5	5	$(1.061 \pm 0.058) \times 10^4$	5.5	10

Table 2.2. Inter-analyst variability of the method for the determination of plasma ganciclovir concentrations, expressed as relative standard deviation (RSD). 200 µl plasma samples were spiked with 2 µg/ml HEdU and the concentrations of ganciclovir indicated, and extracted and separated as described in sections 2.2.3.2 and 2.2.3.3.

GCV Concentration Added (ng/ml)	GCV Concentration Found, mean ± SD (ng/ml)	RSD (%)	n
100	100.6 ± 13.7	13.6	9
1000	1038 ± 24	2.3	10
10000	$(1.018 \pm 0.063) \times 10^4$	6.2	9

2.3.3.4 Accuracy

The accuracy of the method was determined by calculating the relative error of mean ganciclovir concentrations as measured by the assay compared with nominal concentrations. Accuracy was found to be within acceptable limits at low, medium, and high concentrations of ganciclovir, with relative error less than 15% (Table 2.3).

Table 2.3. Accuracy of the method for the determination of plasma ganciclovir concentrations, expressed as relative error.

GCV Concentration Added (ng/ml)	GCV Concentration Found, mean ± SD (ng/ml)	Relative Error (%)	n
100	90.6 ± 5.9	9.4	5
1000	1009 ± 43	0.9	5
10000	$(1.063 \pm 0.080) \times 10^4$	6.3	5

2.3.3.5 Sensitivity

The lower limit of quantification (LLOQ) of the assay was defined as the lowest ganciclovir concentration which could be measured with relative standard deviation (RSD) and relative error (RE) both less than 20%. While ganciclovir at 30 ng/ml was detectable, the RSD and RE values at this concentration were determined to be 50.1% and 21.7%, respectively, and therefore not within the acceptable range for the LLOQ. It was determined that 40 ng/ml was the lowest concentration to meet the acceptance criteria (Figure 2.5), with absolute RE of 5.3% and RSD of 15.8%.

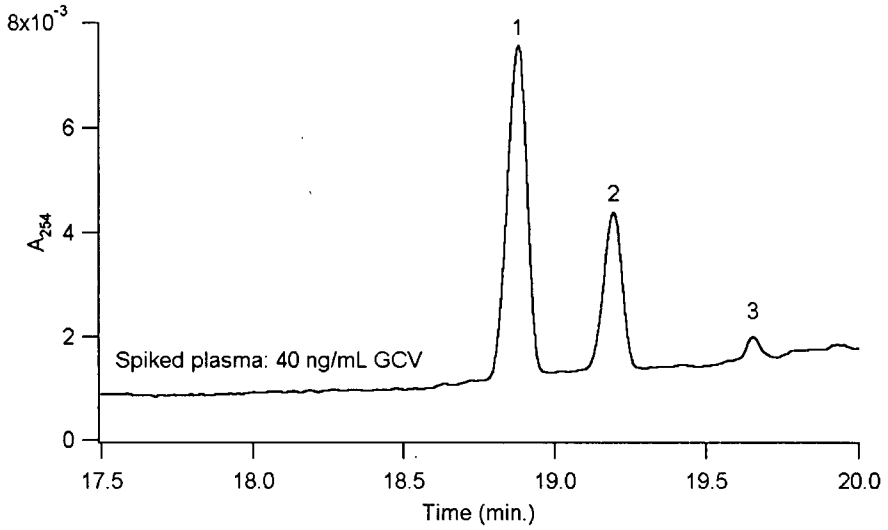


Figure 2.5. Lower limit of quantification of the assay for the determination of plasma ganciclovir concentration. 200 µl plasma samples were spiked with 2 µg/ml HEdU and 40 ng/ml of ganciclovir, and extracted and separated as described in sections 2.2.3.2 and 2.2.3.3. Peak identification: 1 = HEdU, 2 = plasma peak, 3 = ganciclovir.

2.3.3.6 Selectivity

Assay selectivity was determined by analysis of blank samples to ensure that there was no co-migrating interference with either the internal standard or ganciclovir, as shown in Figure 2.6. Both peaks were well resolved from the nearest eluting plasma peak, with average resolution values greater than 1.6 for HEdU, and greater than 2.5 for ganciclovir at the lower limit of quanitification.

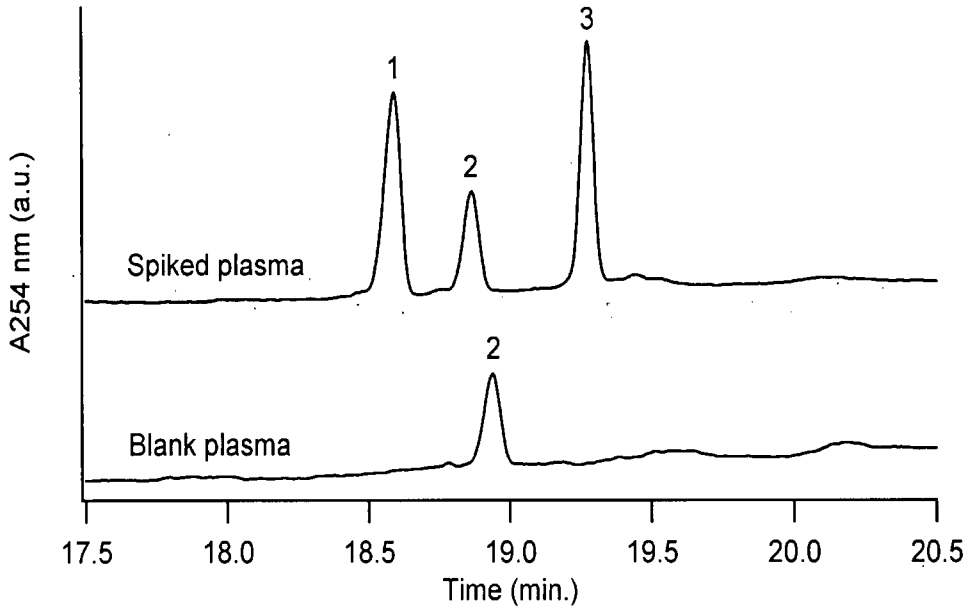


Figure 2.6. Selectivity of ganciclovir analysis in plasma samples. 200 µl plasma samples were spiked with 2 µg/ml HEdU and 1 µg/ml of ganciclovir, and extracted and separated as described in sections 2.2.3.2 and 2.2.3.3. Peak identification: 1 = HEdU, 2 = plasma peak, 3 = ganciclovir.

2.3.3.7 Stability

No significant changes in ganciclovir and internal standard concentration were observed in the working solutions after one month of storage at 4°C. The stability of prepared plasma samples was determined following three freeze-thaw cycles over a period of three days, with no significant changes in ganciclovir concentration. Short-term and long-term stability of the prepared plasma samples were assessed, and it was observed that samples were stable for at least 4 hours when maintained at room temperature, and for at least 3 months when kept frozen at -80°C. The stability of the processed extracts was also assessed, and it was determined that the samples were stable when stored at 4°C in the instrument overnight. Assessment of plasma sample stability was by comparison with freshly prepared samples, with both relative standard deviation and absolute relative error of less than 15%.

2.3.4 Analysis of Clinical Samples

The validated assay method was used to analyse clinical plasma samples from allogeneic stem cell transplant recipients receiving pre-emptive ganciclovir therapy.[19] In this study, several patients on pre-emptive therapy exhibited sustained and/or recurrent antigenaemia, although none of the common UL97 mutations associated with ganciclovir resistance were detected. Measurement of plasma ganciclovir concentrations in plasma samples was performed by both the described CE method and a separate HPLC method, and analysis was conducted to determine the degree of correlation between the results obtained from the two

methods (Table 2.4, Figure 2.7). A positive correlation was observed ($r = 0.977$), which was shown to be statistically significant by the two-tailed Student's t-test ($p < 0.001$).

Table 2.4. Ganciclovir (GCV) concentrations in plasma samples determined by HPLC and CE.

Sample	[GCV] (ng/ml) as Determined by HPLC	[GCV] (ng/ml) as Determined by CE
1	1836.0	3671
2	4931.7	7746
3	242.6	482.2
4	331.0	536.6
5	0	109.6
6	2651.8	5297
7	1352.7	2373
8	221.5	435.0
9	1246	817.9
10	0	156.8
11	0	0

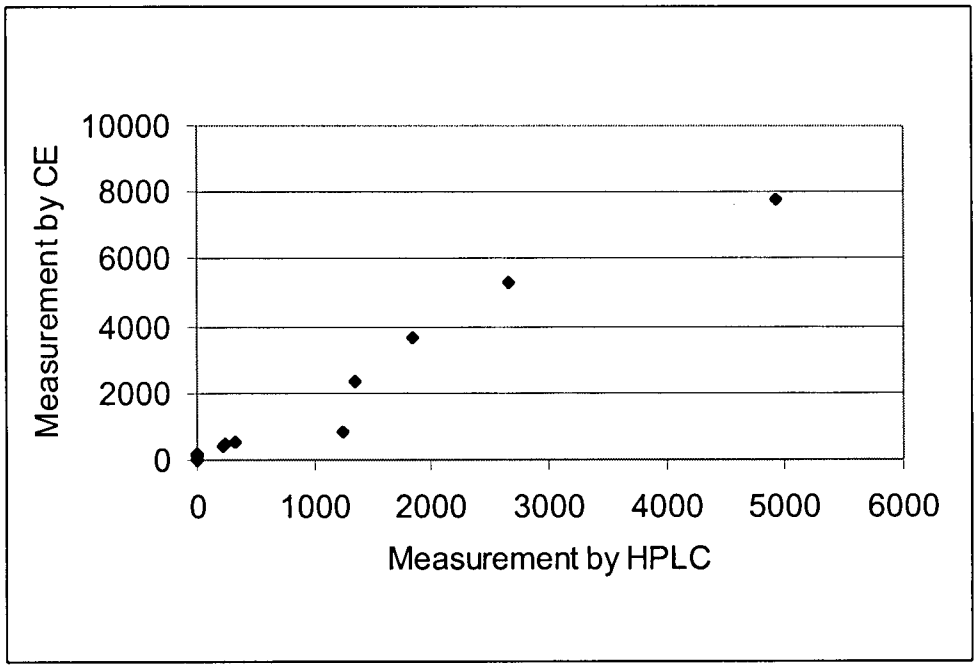


Figure 2.7. Correlation of plasma ganciclovir concentrations as determined by HPLC and CE analysis. $r = 0.977$, $p < 0.001$

2.4 Discussion

2.4.1 Development of Optimal Assay Conditions

The analysis of antiviral compounds in biological matrices can be difficult due to structural similarity with endogenous substances, and thus, methods for the bioanalysis of such compounds need to be sensitive and selective. The described capillary electrophoresis method employs strategies to improve both the sensitivity of the assay and separation from endogenous compounds, and has been validated and applied for the quantitative analysis of ganciclovir in clinical samples.

The validated procedure uses a small plasma volume of 200 µl, and thus, the method is suitable for the measurement of clinical samples in which plasma volume is limited, such as in paediatric studies. The pH and ionic strength of the solid phase extraction wash buffer is optimised for best sample cleanup and analyte recovery, and it was determined that washing the plasma samples with 0.5 M phosphate buffer pH 7.2 provides the best baseline and sample recovery.

The elution buffer (50 mM phosphate pH 7.2 / 20% acetonitrile / 0.2% NaCl) was developed to obtain the best recovery of ganciclovir without plasma interference. It also provides a pH differential between the sample matrix and the separation buffer zone, thus facilitating charge-switching for ganciclovir to enable greater analyte focussing. Acetonitrile is necessary for the elution of ganciclovir and HEdU from the solid phase extraction column. In addition to

this, the inclusion of both acetonitrile and sodium chloride in the sample matrix enables online sample concentration through injection of a large sample volume (100 second injection), which has been previously described for acetonitrile-salt sample mixtures.[20] Furthermore, the presence of phosphate, sodium, and chloride ions in the sample matrix produces a conductivity in the sample zone that is homogenous with that of the separation buffer. The condition of homogenous conductivity has been observed to increase peak height and sharpness in CE separations employing neutral cyclodextrins in the buffer and its mechanism is currently under investigation.

The components of the separation buffer were also optimised for sensitivity and separation from plasma components. It was determined that the sensitivity of the assay was improved by increasing the concentration of borate, but concentrations greater than 160 mM resulted in interference with the ganciclovir peak. The inclusion of HPβCD in the separation buffer also improved the height and sharpness of the ganciclovir peak, which is suggested to be due to the hydrophobic interactions between ganciclovir and neutral HPβCD. However, HPβCD alone did not enable separation of ganciclovir from plasma interference, and thus, LDS was also included in the separation buffer. In this system, the formation of LDS micelles allowed for the separation of ganciclovir from plasma interference, but this came at the expense of some detection sensitivity, as previously shown.[16] It was determined that a balance of sensitivity and

selectivity could be best achieved with 90 mM HPβCD and 200 mM LDS in 160 mM borate pH 8.8.

Determining drug concentrations in biological matrices can be done through a host of methods. Commonly monitored drugs are often measured by commercially available immunoassay kits, and monitoring of compounds for which kits are unavailable is often accomplished by HPLC. As a bioanalytical tool, CE is proving to be an increasingly popular technique for use in pharmaceutical analysis and therapeutic drug monitoring.[21] Separation can be achieved on the basis of charge, hydrophobicity, and stereospecificity, with separation efficiency superior to that achievable by HPLC. Additional advantages of CE include smaller sample volume requirements, shorter times required for the completion of assays, and the use of lower quantities of buffers and solvents, resulting in less consumable expenses.

The most common disadvantage associated with CE is its low detection sensitivity, due to the short optical path length of the capillaries used for separation. For this reason, strategies to enhance sensitivity are of interest in CE research and application. When ultraviolet detection is used, detection sensitivities are often in the μM range.[2] The LLOQ of 40 ng/ml described here for ganciclovir exceeds that of 50 ng/ml reported for HPLC methods which employ UV detection,[4,5] and equals that of an HPLC method which utilises fluorescent detection.[6] Furthermore, sensitive quantification is achieved by the

CE method using a low sample volume of 200 µl, which may be of clinical benefit in circumstances where sample availability is limited, such as in neonatal drug level monitoring.

2.4.2 Rationale for the Clinical Monitoring of Ganciclovir Levels

Drug monitoring is the measurement of therapeutic agents in a clinical setting to address potential questions of therapeutic efficacy or drug toxicity. In the context of CMV infection and disease management, it may be of clinical benefit to monitor patient ganciclovir levels to ensure that adequate circulating drug concentrations are achieved with a given drug regimen. In a study conducted in AIDS patients infected with acute CMV retinitis, treatment failure was observed in some patients despite conventional schedules of intravenous ganciclovir.[22] Another study investigated oral ganciclovir for the maintenance therapy of CMV retinitis in AIDS patients, and it was observed that earlier recurrence of retinitis was associated with low trough plasma concentrations of ganciclovir, with patients exhibiting trough levels below 0.6 µg/ml at higher risk of disease progression.[23] These studies suggest that treatment failure with ganciclovir may occur when plasma concentrations do not reach therapeutically efficacious levels, and as such, monitoring ganciclovir levels in conjunction with virological markers may be beneficial in optimising antiviral treatment regimens against CMV.

In addition to ensuring that administered drug levels are within range to avoid treatment failure, monitoring of ganciclovir concentrations may be a useful strategy in the management and prevention of drug resistance in CMV. Resistant mutant strains of CMV are selected when the virus maintains replication in the presence of subtherapeutic drug concentrations,[24] and given the low bioavailability of ganciclovir (6 to 9%),[25] this could be of significant concern. Because ganciclovir exhibits low protein binding (1 to 2%)[26] and does not undergo metabolic conversion other than enzymatic phosphorylation, plasma ganciclovir concentration is a suitable representation of the amount of delivered drug circulating in the body. Monitoring of ganciclovir levels would be one approach to ensure that the drug is being delivered in adequate quantities to maintain antiviral activity, without causing dose-related adverse effects.

2.4.3 Summary

A CE assay for ganciclovir has been developed and validated. The assay exhibits a linear range of 40 to 10000 ng/ml, with a lower limit of quantification of 40 ng/ml. This assay, the first reported CE method for ganciclovir, is suitable for clinical bioanalytical applications such as pharmacokinetic studies and therapeutic drug monitoring, which may be of clinical benefit in the management of CMV disease and drug resistant virus.

2.5 *References*

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3. Investigation of Cytomegalovirus Susceptibility by Real-Time LightCycler Polymerase Chain Reaction

3.1 Introduction

3.1.1 Monitoring Cytomegalovirus Response to Antiviral Treatment

Physicians and clinical virologists require timely identification of drug resistant viruses when faced with failure of antiviral treatment. Correct monitoring of the response to antiviral treatment is vital for detecting the emergence of drug resistant CMV strains. When antiviral therapy is initiated in patients harbouring susceptible strains of CMV, a decrease in the indices of viral growth is observed. Contrary to this, a lack of decrease, or possibly an increase in such indices, is associated with the emergence of drug resistant virus strains.[1] Management of emerging drug resistant CMV first requires identification of such strains, and both genotypic and phenotypic approaches are used for this purpose.

3.1.2 Genotypic Determination of Antiviral Drug Resistance

As discussed in Chapter 1, the most common mutations that confer drug resistance in CMV occur in the sequences encoding for viral phosphotransferase (UL97) and viral DNA polymerase (UL54). Genotypic methods of resistance determination analyse differences in the viral genome that may be associated with drug resistance.

3.1.2.1 Restriction Fragment Length Polymorphism (RFLP)

Analysis of RFLP is commonly used in the identification of UL97 mutations conferring drug resistance. Mutations are detected by analysing endonuclease-digested products of the polymerase chain reaction (PCR). In RFLP analyses, modifications in the restriction site[2] and deletions[3] resulting in changes in the size of digested fragments can be detected as markers for viral mutations.

3.1.2.2 Specific Amplification and Hybridisation in Polymerase Chain Reaction

The use of primers designed to anneal to a specific sequence (wild type or mutant) of CMV DNA during PCR or ligase chain reaction (LCR) has been used to identify mutations in UL97.[4] The use of specific hybridisation probes to determine nucleotide substitutions has also been reported.[5]

3.1.2.3 Gene Sequencing

Sequencing of UL97 and UL54 genes is the reference technique for identifying viral mutations associated with drug resistance. However, its use is limited by equipment cost and personnel requirements.[1]

3.1.3 Phenotypic Determination of Antiviral Drug Resistance

Phenotypic assays for susceptibility measure the inhibition of viral growth in the presence of antiviral agents. Generally, these methods are culture-based, with a

known amount of infectious virus grown in different concentrations of the drug in question.[6]

3.1.3.1 Plaque Reduction Assay

The plaque reduction assay has been considered the gold standard of antiviral susceptibility testing for many viruses, including CMV.[7] A known titre of stock virus is inoculated into cultures and incubated in the presence of the antiviral drug, and the cultures are monitored for the development of plaques. Quantification of the plaques enables the determination of EC_{50}, the concentration at which plaque formation is reduced by 50%.

One of the limits of the plaque reduction assay is its labour-intensive nature, resulting in excessive time required to perform antiviral susceptibility testing. Completion of the plaque reduction assay requires a process that includes:

1. Virus isolation in culture
2. Virus stock preparation by sequential passage in culture
3. Titration of prepared virus stocks
4. Incubating virus in the presence of drug
5. Termination of viral growth and plaque staining and quantification

Despite being the reference method for antiviral susceptibility determination, true standardisation of the plaque reduction assay is lacking, and interpretation

and comparison of results is difficult. Factors which make standardisation difficult for CMV include the use of different cell lines to propagate the virus, the difference between the use of cell-free *versus* cell-associated CMV, the technique used for plaque staining, and the selection of appropriate reference strains for use as controls.[1] Further to this, sequential passage of virus to prepare stocks may select CMV strains which may not be representative of the original virus population,[6] and variability in plaque formation exists between different strains and isolates of CMV, which can lead to subjectivity in reading and interpreting test results.[8] In an attempt to advance the standardisation process, the CMV Resistance Work Group of the AIDS Clinical Trials Group (ACTG) has put forth effort to develop a consensus assay to determine drug susceptibility for clinical CMV isolates.[9]

3.1.3.2 Virus Yield Reduction Assay

The inhibition of viral replication by antiviral agents can be determined by measuring the reduction in virus titres. The extremely time-consuming nature of yield reduction assays makes their use undesirable, especially for viruses such as CMV which exhibit slow growth in culture.

3.1.3.3 Susceptibility by Reduction of Viral Antigen Production

The reduction of CMV antigen production in virus-infected cells can be utilised to determine antiviral susceptibility. Enzyme-linked immunosorbent assays (ELISA) have been used to measure the reduction of CMV late antigen

production, which is indicative of antiviral inhibition of virus replication. The advantages of ELISA include the ability to titrate the virus and determine antiviral susceptibility simultaneously.[10,11] Detection of immediate-early and late antigen in CMV-infected cells has also been achieved by flow cytometry.[12,13]

3.1.3.4 Susceptibility by Reduction of Viral DNA

DNA hybridisation can be used to measure the effects of antiviral agents on the synthesis of CMV DNA in culture. Following the incubation of virus in the presence of drug, cells are lysed, and DNA is extracted and hybridised to probes labelled with iodine-125, and quantified by counting gamma emission. DNA hybridisation eliminates the variability attributed to subjective interpretation, but requires the use of radiolabelled probes and dedicated facilities and equipment. Good correlation has been observed between results obtained from DNA reduction and plaque reduction assays.[14]

3.1.4 LightCycler Polymerase Chain Reaction

As discussed in Chapter 1, quantitative polymerase chain reaction is a method capable of determining CMV DNA copy numbers with high sensitivity. The LightCycler (LC) technology enables rapid real-time PCR with product analysis within a closed capillary system. LightCycler employs the principles of PCR to amplify a specific region of target DNA. Quantification of the PCR product utilises dye-labelled donor and acceptor probes which hybridise with the

accumulating product in close proximity. Upon activation of the instrument light source, fluorescence is emitted as energy is transferred from the donor to acceptor probe through the process of fluorescence resonance energy transfer (FRET). The increase in fluorescence is measured, and is representative of the increase in PCR product formation, thus enabling quantification.[15]

3.1.5 Experimental Objective

Quantification of CMV DNA by real-time PCR is a phenotypic method through which antiviral susceptibility can be determined. The aim of the studies described here was to develop CMV DNA standards to be used for quantification, and to investigate the susceptibility of wild-type and resistant strains of CMV to ganciclovir through real-time PCR.

3.2 Materials and Methods

3.2.1 Molecular Cloning of CMV DNA

The development of CMV DNA standards for use in quantification was a process of isolation of the CMV gene product, transformation of susceptible bacteria, and preparation of the plasmid-CMV DNA.

3.2.1.1 Isolation and Amplification of the CMV Gene Product

The AD169 strain of human CMV was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human embryonic lung (HEL 299) fibroblasts were infected with the virus, and cultured in minimum

essential medium (MEM) from Gibco (Burlington, ON, Canada) which was supplemented with 4% foetal bovine serum, 0.1 mM non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture supernatant containing cell-free CMV was concentrated by filtration through centrifugal devices from Millipore (Medford, MA, USA). Viral DNA was isolated by extraction with DNAzol Reagent (Invitrogen, Burlington, ON) according to manufacturer's protocols.

Selected regions of the CMV genome were amplified by PCR. The PCR reaction mixture (50 μl) contained 5 μl of the extracted DNA, 0.2 mM dNTP mix, 1 unit of Taq DNA polymerase, 1.5 or 3 mM $MgCl_2$, and 10 pmol of primers. Two pairs of primers obtained from TIB Molbiol LLC (Adelphia, NJ, USA) were used. The first pair amplified a fragment of the CMV genome corresponding to the gB glycoprotein, with the following sequences:

gB for: 5'-ATA GGA GGC GCC ACG TAT TC-3'

gB rev: 5'-TAC CCC TAT CGC GTG TGT TC-3'

The second pair of primers amplified a 152 base pair fragment corresponding to nucleotides 135176 to 135326 of the CMV AD169 genome:[16]

CMV-01: 5'-GGC AGC TAT CGT GAC TGG-3'

CMV-02: 5'-GAT CCG ACC CAT TGT CTA AG-3'

The amplification procedure consisted of one cycle of 94°C (2 minute), 55°C (1 minute), 72°C (1 minute), followed by 39 cycles of 94°C (30 seconds;

denaturation), 55°C (45 seconds; annealing), and 72°C (45 seconds; extension). The PCR products were visualised by gel electrophoresis.

3.2.1.2 Bacterial Transformation with Cytomegalovirus DNA

The PCR products obtained from Section 3.2.1.1 were excised and purified from the gel with a QIAquick extraction kit from QIAGEN (Mississauga, ON, Canada) according to the manufacturer's protocol. The purified CMV DNA fragment was ligated to a 3000 base pair pGEM-T vector from Promega (Madison, WI, USA), and the recombinant plasmids were used to transform susceptible *E. coli* DH5α cells. Identification of bacterial colonies which contained the recombinant plasmids was performed by testing for α-complementation.[17]

3.2.1.3 Preparation of Plasmid DNA

Bacterial colonies transformed with the recombinant CMV DNA-plasmid were selected and plasmid DNA was prepared as described by Sambrook et al.[18] Preparation was performed by the alkaline lysis method, followed by purification by precipitation with polyethylene glycol. The purified plasmid DNA was subjected to digestion by the restriction enzyme Pst I obtained from Promega. The linearised DNA (pCMV-PstI) was analysed by gel electrophoresis, and the amount of pCMV-PstI produced was determined by measurement of UV absorbance on an Ultrospec 2000 spectrophotometer from Pharmacia Biotech (Cambridge, England).

3.2.2 LightCycler PCR Protocol Development

The primers CMV-01 and CMV-02 described in Section 3.2.1.1 were also used for LightCycler PCR (LC-PCR) to amplify the 152 base pair fragment of the CMV AD169 genome. Dye-labelled probes with the following sequences were obtained from TIB Molbiol LLC:

CMV-03: 5'-CGA CGG TGA TTC GTG GTC GT-3'

CMV-04: 5'-CCA ACT GGT GCT GCC GGT CG-3'

The 3' end of CMV-03 is coupled to the donor fluoroscein dye, and the 5' end of CMV-04 is coupled to the acceptor LC Red640 dye. Upon hybridisation, fluoroscein and LC Red640 are separated by only two base pairs, enabling the energy transfer process and measurement of the fluorescent signal.

The total reaction volume for PCR was 20 µl per capillary, with master mixes based on FastStart DNA Master Hybridization Probes from Roche Diagnostics Corporation (Indianapolis, IN, USA). 5 µl of extracted DNA were added to each reaction mix containing 2 pmol each of the primers CMV-01 and CMV-02, 2 pmol of the CMV-03 probe and 4 pmol of the CMV-04 probe. $MgCl_2$ between 1.5 and 4.5 mM were tested to determine the condition which produced optimal results. The reaction capillaries were sealed, centrifuged and transferred to the temperature cycling chamber of the LightCycler instrument. Following an initial denaturation step at 95°C for 7.5 minutes, 45 cycles of denaturation (95°C for 5 seconds), annealing (55°C for 10 seconds), and extension (72°C for 10

seconds) were performed. Temperature transition ramp rates were 20°C/s from denaturation to annealing, 5°C/s from annealing to extension, and 20°C/s from extension to denaturation.

3.2.3 Determination of Cytomegalovirus Susceptibility to Ganciclovir

3.2.3.1 Cell Culture

Human embryonic lung (HEL 299) fibroblasts were cultured in minimum essential medium (MEM) supplemented with 10% foetal bovine serum, 0.1 mM non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin.

3.2.3.2 Virus Infection and Drug Incubation

Monolayers of HEL 299 cells were seeded in 24-well culture plates. The cells were infected with cell-associated CMV (AD169, C73593, and C75098 strains). Unabsorbed virus was removed following 2 hours of incubation at 37°C and cell monolayers were washed with phosphate-buffered saline (PBS). Cultures were then incubated at 37°C in MEM supplemented with 4% foetal bovine serum, 0.1 mM non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin, containing ganciclovir at concentrations of 0, 0.005, 0.05, 0.5, 5, and 50 μg/ml. Following 3 days of incubation, the virus was released from cells by 3 freeze-thaw cycles.

3.2.3.3 Isolation and Quantification of Viral DNA

400 µl of media containing released virus were digested with 0.5 mg/ml Proteinase K. Viral DNA was isolated from the digestion mix by phenol-chloroform extraction. Extracted DNA samples were re-suspended in 20 µl water and kept at -20°C until analysis by LC-PCR. Quantification by LC-PCR was performed as described in Section 3.2.2, using a final $MgCl_2$ concentration of 3 mM.

3.3 Results

3.3.1 Molecular Cloning of CMV DNA

3.3.1.1 Isolation of CMV Gene Product for Standard Preparation

The PCR products obtained from extracted CMV DNA were separated by gel electrophoresis. From Figure 3.1, it can be seen that samples 2 and 4, which utilised the CMV-01 and CMV-02 primer pair, produced bands that were between 100 and 200 base pairs in size.

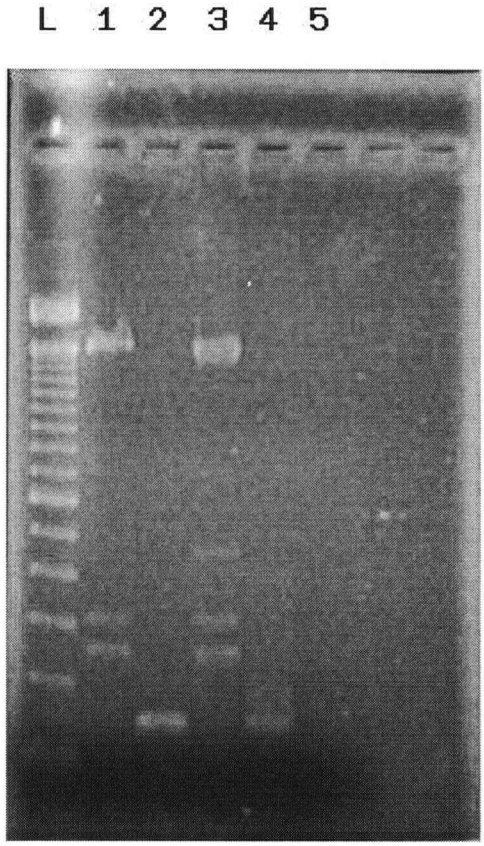


Figure 3.1. Amplification of DNA from CMV AD169. Gel electrophoresis of the PCR products from CMV AD169 was conducted using 2% agarose. Samples were loaded at the cathode end and electrophoresis was conducted at 100 V. The bands were stained with ethidium bromide. Two sets of primers and two $MgCl_2$ concentrations were used. The conditions used for each well are:

L: 100 base pair ladder
1: gB for / gB rev primer pair; 1.5 mM $MgCl_2$
2: CMV-01 / CMV-02 primer pair; 1.5 mM $MgCl_2$
3: gB for / gB rev primer pair; 3 mM $MgCl_2$
4: CMV-01 / CMV-02 primer pair; 3 mM $MgCl_2$

3.3.1.2 Preparation of CMV-plasmid DNA

Eight bacterial colonies transformed with the recombinant plasmid containing CMV DNA were used for the small-scale preparation of plasmid DNA. Following the isolation of plasmid DNA by phenol-chloroform extraction and restriction enzyme digestion by Pst I, the eight samples were analysed by gel electrophoresis, with results shown in Figure 3.2. Based on the expected size of the recombinant plasmid, the observed bands suggested that the harvested bacterial colonies included the CMV DNA insert.

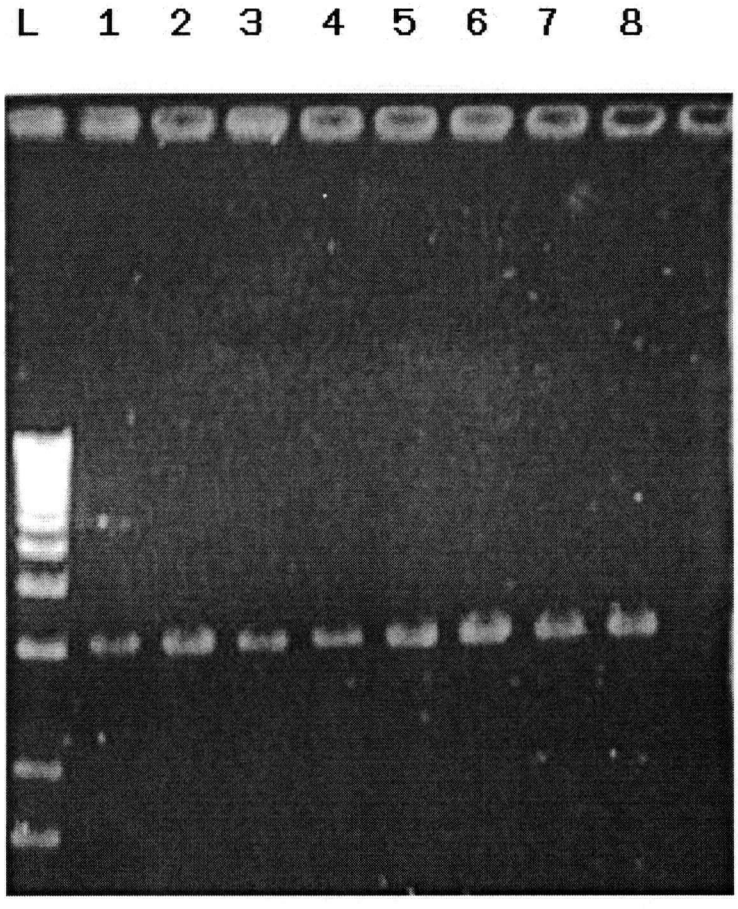


Figure 3.2. Preparation of CMV-plasmid DNA. Gel electrophoresis of plasmid-DNA obtained from *E. coli* DH5α cells transformed with purified DNA from CMV AD169. Electrophoretic separation was conducted at 80 V in 1% agarose gel. Bands were stained with ethidium bromide. Identification of each well:

L: 1 kb ladder

1 through 8: DNA obtained from each selected bacterial colony

3.3.1.3 Spectrophotometric Determination of Plasmid DNA Concentration

A bacterial colony transformed with the recombinant plasmid containing CMV DNA was used for the large-scale preparation of plasmid DNA. Following the isolation of plasmid DNA by phenol-chloroform extraction, the DNA pellet was re-suspended in TE buffer and the concentration was determined spectrophotometrically by UV absorbance (Table 3.1). The concentration of DNA was calculated as follows:

$$[\text{DNA}] = A_{260} \times \text{Dilution factor} \times 50\ \mu\text{g/ml}$$

The average concentration of plasmid DNA was determined to be 348.3 μg/ml.

20 μg of DNA were digested by Pst I restriction enzyme and purified by phenol-chloroform extraction. Absorbance measurements were taken, and the concentration was determined as above (Table 3.2). The average concentration of pCMV-PstI was determined to be 224 μg/ml. Based on the size of the plasmid DNA and the conversion factor of 660 g/mol per base pair, the converted concentration of the pCMV-PstI stock was determined to be 6.48 x 10^{10} copies/μl.

Table 3.1. Spectrophotometric determination of CMV-plasmid DNA concentration.

Dilution Factor	A_{260}	[DNA] (μg/ml)
50	0.137	342
20	0.340	340
10	0.725	362

Table 3.2. Spectrophotometric determination of CMV-plasmid DNA concentration following Pst I digestion.

Dilution Factor	A_{260}	[DNA] (μg/ml)
20	0.238	238
40	0.112	224
40	0.109	218
40	0.108	216

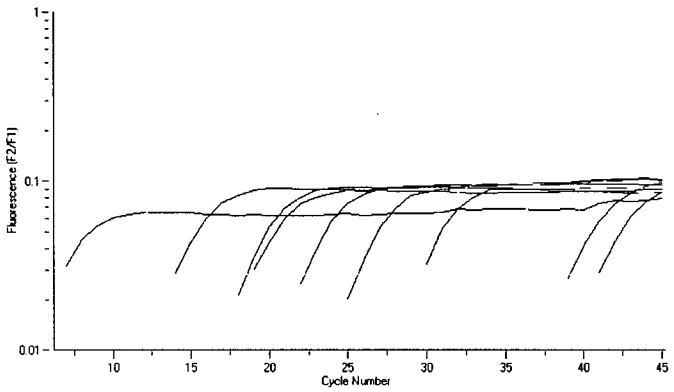
3.3.2 Optimisation of LightCycler PCR Conditions

3.3.2.1 Quantification Sensitivity and Specificity

The pCMV-PstI DNA was serially diluted and quantified with LightCycler PCR. 5 µl samples containing between 10 and 1 x 10^{10} copies of DNA were prepared and analysed. The analytical sensitivity and linearity of the quantification is indicated in the standard curve presented in Figure 3.3.

To determine specificity, DNA extracted from uninfected cells and other viruses was tested for amplification. No cross-amplification was observed for DNA extracted from HSV-1 strain F, HSV-2 strain G, Varicella zoster virus (VZV) strain Ellen, and Epstein-Barr virus (EBV). Cross-amplification of DNA extracted from uninfected HEL 299 cells was also tested, and no product was formed.

1	pCMV 1e10	1.000E+10	8.899E+09	6.682
2	pCMV 1e9	1.000E+09	1.545E+09	9.609
3	pCMV 1e8	1.000E+08	1.132E+08	13.98
4	pCMV 1e7	1.000E+07	7.436E+06	18.53
5	pCMV 1e6	1.000E+06	7.991E+05	22.26
6	pCMV 1e5	1.000E+05	1.067E+05	25.62
7	pCMV 1e4	1.000E+04	9.256E+03	29.71
8	pCMV 1e3	1.000E+03	7.996E+02	33.81
9	pCMV 1e2	1.000E+02	9.126E+01	37.43
10	pCMV 25	2.500E+01	3.300E+01	39.14
11	pCMV 10	1.000E+01	1.136E+01	40.92
12	Hel 299			
13	CMV AD169		6.356E+06	18.79
14	Commercial CMV 5e4		1.972E+04	28.45
15	Water			



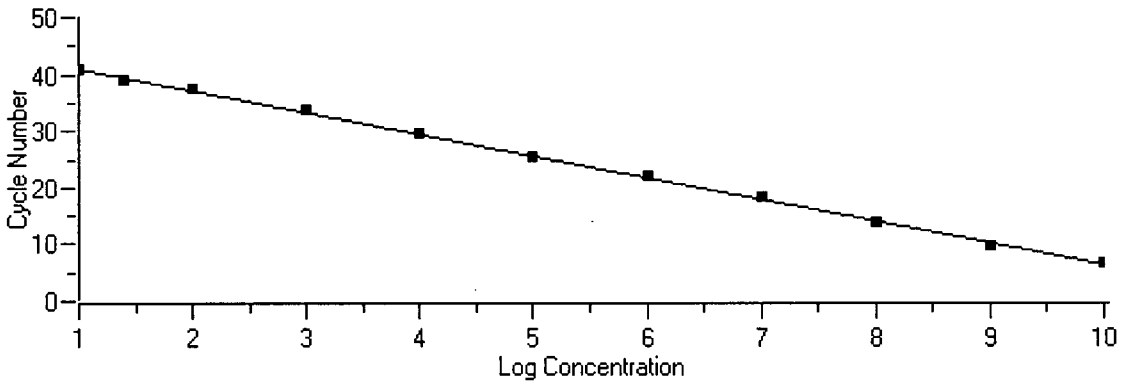


Figure 3.3. Quantification of standard CMV DNA. LightCycler amplification of CMV DNA was performed as described in Section 3.2.2. Quantification of CMV DNA was achieved in samples containing between 10 and 1 x 10^{10} copies. The equation of the curve was determined to be y = -3.85x + 45.0.

3.3.2.2 Magnesium Chloride Concentration

The determination of the optimum $MgCl_2$ concentration for the LightCycler reaction is indicated in Table 3.3. A final $MgCl_2$ concentration of 3 mM enabled both the sensitive detection and accurate determination of CMV DNA copy numbers, and this condition was used for subsequent experiments.

Table 3.3. The effects of $MgCl_2$ concentration on CMV DNA quantification. (ND: Not detected)

Standard CMV DNA (copies)	Calculated CMV DNA Copy Numbers (copies)		
	Final $[MgCl_2]$ (mM)		
	4.5	3	1.5
1×10^8	6.35×10^7	8.90×10^7	1.12×10^8
1×10^6	2.11×10^6	1.01×10^6	9.50×10^5
1×10^4	8.38×10^3	1.13×10^4	7.91×10^3
1×10^2	9.78×10^1	1.47×10^2	1.19×10^2
1×10^1	9.11×10^0	6.67×10^0	ND

3.3.2.3 Reproducibility

The reproducibility of the method was assessed by analysis of multiple standard curves. The cycle number at which the log-line intersects with the noise band during data analysis was compared with CMV DNA copy numbers and is shown in Table 3.4. During LightCycler amplification, a ten-fold increase in DNA copies generally corresponds with a decrease of four cycle number crossing points.

Table 3.4. CMV DNA quantification reproducibility. (n = 6)

Standard CMV DNA (copies)	Average Cycle Number Crossing Point	Standard Deviation
1×10^8	13.2	1.5
1×10^7	17.2	1.1
1×10^6	21.3	1.1
1×10^5	25.2	1.2
1×10^4	28.9	1.0
1×10^3	33.2	1.6
1×10^2	37.4	1.1
1×10^1	39.9	1.6

3.3.3 Cytomegalovirus Susceptibility to Ganciclovir

The susceptibility of cytomegalovirus strains to ganciclovir was evaluated by determining the reduction of CMV DNA by LightCycler PCR. The inhibition of CMV AD169 DNA replication is indicated in Table 3.5 and Figure 3.4. Being one of the reference laboratory strains of CMV, strain AD169 was effectively inhibited by ganciclovir as expected. In an attempt to quantify the relationship between ganciclovir concentration and reduction of CMV DNA, the data were analysed by linear regression using Statview (SAS; Cary, NC, USA) to estimate the concentration of ganciclovir required to reduce CMV DNA replication by 50% (EC_{50}). By interpolation of the linear region of the curve produced when CMV DNA (copies) is plotted *versus* the logarithm of ganciclovir concentration (μg/ml), the EC_{50} was estimated to be 0.14 μg/ml.

The effects of ganciclovir on CMV C73593 and C75098 DNA are shown in Table 3.6 and Table 3.7, respectively. Qualitatively, both strains exhibited reduced susceptibility to ganciclovir compared with AD169, and it was observed that the production of CMV DNA could not be reduced to near-zero levels for either C73593 or C75098, even at the highest concentration of ganciclovir tested. However, the relationship between ganciclovir concentration and CMV DNA was not quantifiable, and EC_{50} values were not determined for these strains.

Table 3.5. The antiviral effect of ganciclovir on CMV AD169.

[GCV] (μg/ml)	**0**	**0.005**	**0.05**	**0.5**	**5**	**50**
Average DNA (copies; n = 3)	4.40×10^5	1.25×10^5	2.61×10^5	1.83×10^5	2.46×10^4	6.33×10^3
Standard Deviation (copies)	3.90×10^5	1.80×10^5	1.69×10^5	1.59×10^5	2.01×10^4	9.46×10^3

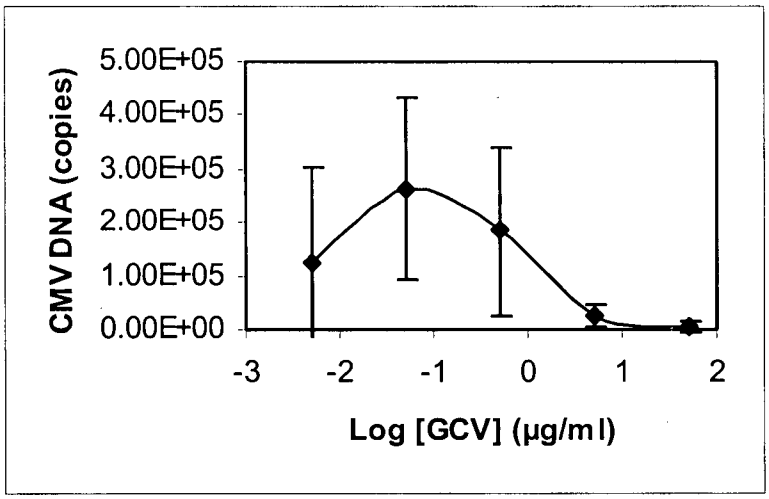


Figure 3.4. The effect of ganciclovir on CMV AD169 DNA. CMV AD169 was grown and incubated in the presence of ganciclovir as described in Section 3.2.3. The results are expressed as copy numbers of CMV DNA *versus* the logarithm of ganciclovir concentration (in µg/ml), n = 3.

Table 3.6. The antiviral effect of ganciclovir on CMV C73593.

[GCV] (μg/ml)	0	0.005	0.05	0.5	5	50
Average DNA (copies; n = 3)	3.11×10^4	4.13×10^4	1.87×10^4	2.16×10^4	2.35×10^4	1.65×10^4
Standard Deviation (copies)	1.73×10^4	2.42×10^4	1.64×10^4	1.25×10^4	1.99×10^4	9.97×10^3

Table 3.7. The antiviral effect of ganciclovir on CMV C75098.

[GCV] (μg/ml)	**0**	**0.005**	**0.05**	**0.5**	**5**	**50**
Average DNA (copies; n = 3)	5.33×10^4	6.79×10^4	4.93×10^4	4.08×10^4	2.08×10^4	3.01×10^4
Standard Deviation (copies)	2.05×10^4	8.41×10^4	2.56×10^4	3.02×10^4	1.27×10^4	2.71×10^4

3.4 Discussion

3.4.1 Identification of Virus Susceptibility by LightCycler PCR

The susceptibility of cytomegalovirus strains to ganciclovir was investigated using copies of CMV DNA as a phenotypic endpoint. Compared with the reference strain AD169, reduction of CMV DNA in strains C73593 and C75098 was limited, even upon incubation of the virus at the highest ganciclovir concentration.

The standard deviations observed in the average copy numbers of CMV DNA suggest a high degree of variation between replicate experiments. One factor which may contribute to this is the difficulty in attaining accurate virus titres due to the highly cell-associated nature of CMV. As such, virus inoculums may vary between experiments even when equivalent dilutions of the same virus stock are used. However, even with such variation, the trend observed indicates that ganciclovir treatment resulted in ten-fold reduction of CMV DNA in strain AD169, although not in strains C73593 and C75098. Lessons learned from viral genome quantification of HIV infection suggest that reductions of such magnitude may be associated with a lowered risk of disease progression following antiviral therapy.[19]

The use of quantitative PCR for the determination of antiviral susceptibility may be advantageous in that subjective variation is reduced. Culture-based methods such as the plaque reduction assay are prone to errors that result from subjective

plaque interpretation and counting by different individuals. Further to this, some strains of CMV show poor ability or inability to form plaques,[20] and this difficulty may be overcome by quantifying drug effects on the viral genome.

3.4.2 Practical Considerations of Susceptibility Testing

Methods used to investigate the degree to which drugs inhibit viral replication *in vitro* should provide evidence of susceptibility or resistance to antiviral agents. However, the interpretation of antiviral resistance studies can be difficult even when common methods and virus strains are used. An example of this can be seen in the determination of EC_{50} for ganciclovir against CMV AD169 by plaque reduction assay, as EC_{50} values ranging from 1 to 10 μM have been reported.[21,22,23,24] It is evident that variability exists among methods and across laboratories, and can be attributed to factors such as differences in cell lines and viral inoculums employed.[6]

A lack of consensus over the selection of controls has complicated the standardisation of phenotypic assays for antiviral susceptibility. While reference laboratory strains of CMV including AD169 and Towne are commonly used, they have been shown to possess differences in genetics and viral characteristics when compared to isolates of CMV found in clinical settings, such as in viral kinetics (fast *versus* slow growth rates) and viral transmission in culture (cell-free *versus* cell-to-cell).[1,25] Despite such differences, susceptible reference strains are necessary in determining antiviral resistance because they provide a

point of comparison for the degree of inhibition of viral replication achieved. Indeed, susceptibility and resistance are relative measures, and although efforts have been made to establish cut-off concentrations to define CMV drug resistance,[26,27,28] such cut-off values are not yet available for all drugs against all viruses.

3.4.3 Summary

As the problem of drug-resistance in CMV persists, the ability to detect emerging resistant strains is undoubtedly required. Further to this, increasing drug resistance may lead to progressive inefficacy of the antiviral agents currently available for the treatment of CMV, and thus, new drugs against CMV continue to be in development[29,30] As with DNA hybridisation methods for susceptibility testing, quantitative PCR methods reduce the variation associated with subjective plaque interpretation, but can be performed without the inconveniences of using radiolabelled probes. The use of quantitative PCR adds to the arsenal of methods available for the monitoring of infection and response to therapy, and can be beneficial in the screening of novel agents against CMV.

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4. Conclusions

The resistance of cytomegalovirus to antiviral agents is well-documented, and the issue continues to be of concern especially among the immunocompromised population and with prolonged administration of antiviral drugs. Significant advances in the understanding of the mechanisms underlying drug resistance in CMV have been made, enabling the assessment of resistance in the clinical setting.

One approach to the management of resistant infection is to prevent the development of resistance. Mutations rendering resistance are favoured when there is incomplete suppression of CMV replication, which may occur due to suboptimal dosing of antiviral drugs. Assessment of pharmacokinetic factors such as plasma ganciclovir levels by a sensitive and selective capillary electrophoresis method enables the monitoring of drug delivery to ensure the achievement of therapeutic concentrations.

The ability to determine the susceptibility of CMV to antiviral drugs is also necessary for the management of resistant infection. Resistant virus strains can be identified through the quantification of the viral genome by real-time polymerase chain reaction and comparison with susceptible reference strains following drug administration. Furthermore, as all of the currently available antiviral agents against CMV are inhibitors of viral polymerase, the ability to

quantify DNA reduction may prove to be beneficial in the development and evaluation of novel antiviral agents against CMV.

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In addition to the preceding chapters of this thesis, the manuscripts presented in Appendix I and Appendix II were also completed during the course of the degree programme, and both manuscripts have since been published.

Appendix I: Au E, Sacks SL. Antivirals in the prevention of genital herpes. Herpes 2002; 9:74-7.

Appendix II: Au E, Sacks SL. Therapeutic options for herpes simplex infections. Current Infectious Disease Reports 2003; 5:22-7.

Appendix I

ANTIVIRALS IN THE PREVENTION OF GENITAL HERPES

KEY WORDS

Genital herpes, herpes simplex virus, prevention, antivirals, transmission, asymptomatic shedding

SUMMARY

Genital herpes remains one of the most common sexually transmitted infections (STIs). Despite best efforts at prevention of STIs in the era of AIDS, the incidence of genital herpes continues to rise. Evidence that HSV-2 is a significant cofactor in the transmission of HIV infection is mounting. Because of the common psychosocial consequences of genital herpes transmission, prevention modalities against transmission of genital herpes are urgently required—from both personal and public health perspectives. Most discordant couples are either unaware of genital herpes in one partner or unaware of clinical activity of the virus during sexual exposure that results in

transmission. Yet, the body of evidence available suggests that the antiviral drugs currently used in the treatment of genital herpes are effective in suppressing asymptomatic virus shedding, and thus, could play a future role in reducing transmission. The first study to examine that possibility will be made public this year.

INTRODUCTION

Genital herpes is a very common STI for which no cure is known. It has been estimated that 48 million people in the United States, alone, have genital herpes, with most cases due to infection with herpes simplex virus type 2 (HSV-2), and an increasing incidence of genital herpes caused by HSV type 1 (HSV-1).[1] Despite the efforts directed toward preventing the spread of AIDS and other STDs, the incidence of HSV-2 infection has increased, as evidenced by serum sample collection during the National Health and Nutrition Examination Surveys (NHANES) II and III, which showed a 30% increase in HSV-2 seroprevalence.[2]

Because of the concern associated with increased HSV prevalence and disease, strategies to prevent further transmission of HSV infection are required. However, the specifics of such strategies are unknown, and it is likely that multiple methods will be required to effectively reduce transmission of genital herpes.[3] Behavioural aspects are of great importance for individuals with recognised HSV infection, as lesion-to-skin contact should be avoided during active infection, and safer sex procedures such as the use of condoms should be practiced with sexual contact.[4] Along with behavioural considerations, future strategies for the prevention of genital herpes could include

approaches to the protection of the susceptible partner, through the use of vaccines, topical microbicides, and/or pre-emptive antivirals. The major focus of this review will be the role of antiviral drugs in preventing genital herpes transmission. Although no clinical trials have yet proven the efficacy of this approach — i.e., reduction of transmission through the quantitative suppression of viral reactivations in the source partner – each therapeutic antiviral against genital herpes has been shown to exert significant suppressive capabilities against both symptomatic and asymptomatic viral reactivations in people with genital herpes.

VACCINES AND TOPICAL MICROBICIDES FOR THE PREVENTION OF GENITAL HERPES

The aim of vaccine development is to confer immunity in susceptible individuals such that infection of the genital tract does not occur, and to protect sensory ganglia from latent infection. Vaccines against HSV that are currently under development can be classified as either live or inactive vaccines. Live vaccines include non-pathogenic attenuated forms of the virus, mutant viruses incapable of replication, and replication vector vaccines. Inactive vaccines include killed/inactivated virus, protein subunit, and nucleic acid vaccines. The challenge of developing these vaccines is determining the balance between the benefits and potential detriments involved with each candidate, as factors such as the degree of immunogenicity, safety, and stability need to be considered. Both live and inactive developmental vaccines have been evaluated in clinical investigations.[5] A gDgB-based vaccine developed by the Chiron Group was not effective in preventing herpes transmission.[6] However, a much larger

study of a recombinant gD2-based vaccine was shown to be efficacious in reducing acquisition of HSV-2 by HSV seronegative women.[7]

Another potential approach to preventing genital herpes is through the use of topical microbicides, an area of recent research interest. The first generation, non-specific microbicides include sulphated polymers, surfactants, and acid buffers. Each class of compounds confer protection through a unique mechanism, as sulphated polymers prevent pathogen-target attachment through pathogen and/or target binding, whereas surfactants function through disruption of membranes and viral envelopes, and acid buffers maintain the acidic vaginal environment, thus protecting against acid-sensitive pathogens. The first generation microbicides of each class are currently under investigation, and we await the results of trials designed to study their clinical efficacy.[8]

ANTIVIRALS USED IN THE TREATMENT OF HSV INFECTIONS

The most commonly used antiviral drugs in the treatment of HSV diseases are the nucleoside analogues, of which acyclovir was the first effective agent in human trials.[9] In cells infected with HSV, acyclovir is monophosphorylated by viral thymidine kinase (TK), and subsequently converted to di- and triphosphate forms by host cellular enzymes. Acyclovir triphosphate, the active form of the drug, competes with deoxyguanosine triphosphate as a substrate for HSV-encoded DNA polymerase, resulting in obligate termination of viral DNA replication, and thus inhibiting viral growth.[10] Acyclovir has been shown to be effective and safe,[11] but poor bioavailability

and inconvenient dosing schedules have led to the introduction of nucleoside prodrugs with enhanced absorption characteristics, including valaciclovir and famciclovir.

Valaciclovir is the 1-valyl ester prodrug of acyclovir. This agent is much better absorbed than acyclovir when administered orally with an oral bioavailability (for acyclovir) of about 55% compared with about 15% for the parent drug. Valaciclovir is metabolised to L-valine and acyclovir and raising the C_{max} approximately 4 times compared with similar doses of acyclovir.[12] The pharmacokinetics and safety profiles of acyclovir from valaciclovir or the parent nucleoside are essentially identical.[13] Clinical trials of valaciclovir have shown it to be equally effective with acyclovir in suppressing clinical recurrences of genital herpes.[14] Apparently the result of enhanced bioavailability, valaciclovir 1000 mg once daily has been shown to be equally effective to acyclovir 400 mg twice daily in suppressing recurrences of clinical episodes. For those patients experiencing fewer than 10 episodes of recurrent herpes annually in the absence of suppressive therapy, valaciclovir 500 mg once daily was considered an appropriate dosage format for clinical suppression. Famciclovir is the prodrug of penciclovir, another nucleoside analogue with antiviral activity against HSV. Although penciclovir is not an obligate chain terminator, inhibition of HSV DNA elongation is attained. It has been shown that the intracellular half-life of penciclovir triphosphate, the active drug form, is significantly longer than that of acyclovir triphosphate in cells infected with HSV-2.[15] For suppressive therapy, however, these agents may spend the majority of their active circulating time *in vivo* in their unphosphorylated nucleoside extracellular state between episodes of reactivation. In this case, trace quantities of

nucleoside may be all that is available during the early hours of herpes reactivation, suggesting that intracellular phosphorylation may take on less importance than potency of the nucleoside triphosphate against viral DNA polymerase. Overall, acyclovir and valaciclovir are favored because of their triphosphate potencies, while famciclovir is favored because of its intracellular half-life and excess production. Overall, these advantages are balanced among the different antivirals. However, in selected patients, these may be used to advantage in identifying the most effective approach. Both episodic[16] and suppressive[17] therapy with famciclovir have been shown to be effective and safe in the treatment of symptomatic genital herpes.

ASYMPTOMATIC VIRAL SHEDDING AND TRANSMISSION

In discussing the use of antivirals in the prevention of genital herpes, it is necessary to consider the factors involved in transmission of HSV. It is well established that condoms do not provide sufficient protection against transmission when HSV is actively growing on skin or mucous membranes, and thus, lesion-to-skin contact should be avoided during times of active infection. A recent Seattle study lent scientific support to the standing recommendations favouring safer sexual practices for contact between active episodes by demonstrating significant efficacy in preventing transmission of HSV-2 to susceptible females in discordant heterosexual couples.[18] In this same study, however, men were not afforded protection. This study has led to some controversy because it was not possible to power it sufficiently to analyze male protection and because condoms were assessed as a factor if used for more than 25% of sexual encounters—an objective far short of what public health authorities would recommend

for proper protection. Furthermore, it is clear that male condom latex protection will frequently fail to fully physically obstruct contact between the most common sites of herpes reactivation in women. This anatomical difference, alone, could account for a lack of observed efficacy in protecting men. Finally, the psychosocial barriers to proper condom use are clear by the consistently small proportion of proper condom use even in studies of discordant couples concerned with transmission who are regularly counselled about proper methods. Of course, the definitive study for condom protection is probably too large to contemplate, however, it is clear that condom use is not a complete story and that other prevention measures are going to be required to get a handle on this continuing pandemic.

People who are aware of their diagnosis of genital herpes form only a small fraction of the total of affected seropositive individuals capable of transmitting infection.[19] People with herpes can shed virus and thus transmit infection even when lesions or other signs of active infection are not present. For HSV-seropositive individuals who do not recognise that they have herpes, the diagnosis can be made with accurate blood tests such as the Western blot.[20] Through counselling and education concerning the various presentations of lesions, they can learn to recognise active infection, and be encouraged to both control the disease and decrease the risk of HSV transmission. However, it is well understood that genital herpes can be transmitted from a seropositive individual in the absence of lesions through asymptomatic shedding, when virus is on the skin and a mild episode of herpes occurs but goes unnoticed. The extent to which asymptomatic shedding occurs varies across studies, depending on

factors such as individuals' recognition of symptomatic herpes, and the sensitivity of the laboratory test for viral detection.[4]

To assess the risk of sexual transmission of genital herpes, prospective studies of factors associated with HSV-2 transmission have been conducted. In one study of 57 discordant couples, it was determined that women were at a greater risk of acquiring genital herpes than men, and that the risk was greater in women who did not already have antibodies against HSV-1. In addition to this, it was found that 11 of the 57 individuals who believed they did not have genital herpes were actually seropositive for HSV-2 at the beginning of the study, underscoring the fact that people with genital herpes often do not recognise that fact.[21] In another prospective study, discordant couples participating in a clinical trial were studied, and it was determined that there was significant risk of transmission despite identification of genital herpes in the source partner. The study concluded that women were more likely to acquire genital herpes than men, with individuals possessing antibodies to HSV-1 less likely to be infected with HSV-2, and that asymptomatic shedding appeared to play a role in transmission in 70% of patients.[22]

EFFECT OF ANTIVIRALS ON ASYMPTOMATIC SHEDDING

As the potential for asymptomatic transmission has been demonstrated, studies have been conducted to evaluate the ability of antiviral drugs to reduce asymptomatic HSV shedding. In a double-blind, placebo-controlled, crossover clinical trial of women with genital herpes for less than 2 years, acyclovir 400 mg twice daily for 70 days was

shown to significantly reduce subclinical HSV shedding, which was defined as isolation of virus in the absence of lesions. Subclinical shedding occurred on 0.3% of days among patients on acyclovir, compared to 6.9% of days among participants given placebo, and the frequency of subclinical shedding was diminished in all patients and at all measured anatomic sites.[23] These findings were confirmed by re-analysis of data obtained from an earlier clinical study on the effect of acyclovir on virus shedding and recurrent genital herpes, which had previously reported that acyclovir therapy had minimal effect on asymptomatic shedding.[24] However, upon re-analysis using positive viral culture in the absence of lesions as the definition of asymptomatic shedding, an 82% reduction in asymptomatic shedding was observed.[25] The investigators in both of these studies concluded that acyclovir can decrease but not completely eliminate asymptomatic shedding.

Valaciclovir has also been evaluated in a clinical trial for its efficacy in the reduction of subclinical shedding. A double-blind, crossover study of men and women comparing valaciclovir 500 mg twice daily, acyclovir 400 mg twice daily and placebo was conducted, and measurement of virus shedding was performed with both viral culture techniques and HSV DNA polymerase chain reaction (PCR), a method which provides higher sensitivity.[20] Subclinical shedding was reduced from 15.3% of days in the placebo group to 0.7% of days in patients given valaciclovir, as measured by viral culture. Measurement by PCR showed a decrease in shedding from 40.0% of days on placebo to 7.5% of days on valaciclovir.[26]

Studies have also shown the ability of famciclovir to decrease HSV shedding, as measured by both viral culture techniques and PCR.[27] Clinical trials have been conducted to evaluate famciclovir for the suppression of asymptomatic shedding. In a study of women with recurrent genital herpes, rates of asymptomatic shedding from any anatomic site, defined as positive viral culture with no related symptoms or signs, were 0.52% and 0.41% of days for participants taking famciclovir three times daily at 125 mg and 250 mg, respectively. This was a significant reduction when compared with individuals in the placebo group, in which an asymptomatic shedding rate of 3.1% of days was observed.[28] In a subsequent placebo-controlled study in men with a history of recurrent genital herpes, it was determined that famciclovir 250 mg twice daily significantly reduced asymptomatic shedding from any site, with rates of 0.08% of days for the famciclovir group compared to 1.09% for placebo.[29] A double-blind, placebo-controlled crossover study of famciclovir on HSV reactivation in HIV-infected persons also showed that asymptomatic shedding was reduced from 5.1% of days in patients receiving placebo, to 1.2% of days in patients taking famciclovir 500 mg twice daily.[30]

The data available indicate that the antiviral drugs currently used in the treatment of genital herpes are effective in reducing asymptomatic shedding, and provide a compelling rationale to suggest that transmission due to asymptomatic viral shedding could be decreased as well. This possibility is currently being under investigation with once daily valaciclovir 500 mg in patients with fewer than 10 annual recurrences, compared with placebo, given to the source partner. Unfortunately, transmission trials are difficult to conduct due to cost and complexity, as well as and the

requirement of long-term participation by monogamous couples.[4] Currently, a full clinical episode transmission study is required by the US FDA for an antiviral agent to find an indication for this purpose. Although many clinicians have elected to provide chronic suppressive therapy for this reason, its efficacy in this setting awaits proof. It is not clear whether it will be appropriate to extend any future findings to other drugs or other dosage formats of valaciclovir. Much future work in this area is recommended.

FURTHER CONSIDERATIONS

Further issues and questions will be raised as studies continue to evaluate the use of antiviral drugs for the prevention of genital herpes. If an effective drug is identified, its use can complement safer sex practices as a strategy to prevent HSV transmission. Data obtained from a clinical trial indicated that condom use offers protection against HSV-2 infection in susceptible women, but more data are required to determine the efficacy of condom use for the prevention of HSV-2 in men.[31]

If antivirals are shown to be effective in preventing transmission, another point of consideration would be the potential *de novo* acquisition of drug resistance, as has been previously described,[32] with increased use of antiviral agents. Strains of acyclovir-resistant HSV have been identified, with resistance conferred by viral mutations resulting in thymidine kinase (TK)-deficient strains[33] or altered enzyme activity without loss of virulence.[34] As the active, triphosphate forms of acyclovir and penciclovir depend upon monophosphorylation by viral TK, HSV strains lacking TK would not be subjected to inhibition of viral DNA replication by these nucleoside analogues.

Mathematical modelling has been utilised to predict the potential changes in resistance patterns of HSV, and it has been suggested that difficulties associated with increased usage of antivirals would be minimal.[35] Nonetheless, antivirals such as cidofovir and foscarnet, whose activities do not depend on viral thymidine kinase activity, have been developed,[36] and recent publications have described the in vitro and in vivo anti-herpesvirus activities of new agents which target HSV helicase-primase, and such enzyme inhibitors may have future therapeutic potential.[37,38]

CONCLUSIONS

As the prevalence of genital herpes continues to rise, the approaches to reducing its transmission must continue to be developed. The antiviral drugs currently used for the treatment of HSV infection – acyclovir, valaciclovir, and famciclovir – have all been shown to significantly reduce asymptomatic viral shedding, and it is reasonable to suggest an associated decrease in genital herpes transmission through the use of antivirals, based on the established connection between asymptomatic shedding and transmission. The use of antivirals, in conjunction with safer sex practices, provides a viable strategy for preventing the spread of genital herpes.

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Appendix II

THERAPEUTIC OPTIONS FOR HERPES SIMPLEX INFECTIONS

ABSTRACT

Herpes simplex viruses are responsible for a number of disease states in infected individuals. Capable of establishing latent infection, herpes simplex can reactivate, causing pain, discomfort, and psychosocial consequences. As no cure is available, treatment modalities for herpes simplex infection are required, from both personal and public health standpoints. To date, therapy has centered around the use of antiviral drugs to control infection and suppress recurrences. To expand the scope of available treatments, efforts have focused on the development of vaccines against herpes simplex virus and new agents such as immune response modifiers. Recent data suggests that these new agents are promising in their therapeutic potential.

INTRODUCTION

The herpesviridae family, officially designated as human herpes viruses 1 through 8 (HHV-1 through HHV-8), consists of eight viruses capable of producing pathogenic infection in humans [1]. HHV-1 and HHV-2 are commonly referred to as herpes simplex viruses type 1 and type 2 (HSV-1 and HSV-2), respectively. HSV-1 and HSV-2 are enveloped double-stranded DNA viruses, distinguished by differences in glycoproteins expressed on the viral envelope. It is estimated that 85% of the population is seropositive for HSV-1 [2], and though this virus is most often associated with herpes labialis (cold sores), the incidence of herpes genitalis (genital herpes) caused by HSV-1 is on the rise [3]. HSV-2 is the major cause genital herpes, one of the most common sexually transmitted infections (STI). Despite the efforts aimed at preventing the spread of AIDS and other STIs, the incidence of HSV-2 infection has increased, as evidenced by the National Health and Nutrition Examination Surveys (NHANES) II and III, which showed a 30% increase in HSV-2 seroprevalence [4]. Along with being the major causative agent of genital herpes, HSV-2 is also associated with neonatal herpes.

HSV initiates infection through direct contact with mucosal surfaces or abraded skin. Following infection, the virus is transported to the dorsal root ganglia, where latent infection is established. Stimuli such as stress, fever, and exposure to ultraviolet light can cause reactivation from latency, upon which active infection occurs near the point of entry into the host [5]. Signs of recurrent infection include a prodrome of pain, burning, tingling or discomfort, which precede the development of classic herpes

lesions characterized by vesicle formation on a red patch of skin. The vesicular lesions evolve into erosions, with ensuing crusting over and scabbing [2].

There is no known cure for herpes simplex infections. As the incidence of infection continues to rise, and with mounting evidence that HSV-2 is a significant cofactor in the transmission of HIV, it is imperative to develop therapeutic options for HSV disease. This review discusses the use of antiviral drugs currently used in episodic and suppressive therapy. The increased use of antivirals may play a role in the selection of drug-resistant HSV, and therapies for resistant virus strains are discussed. In addition, vaccines against HSV are considered, as well as topical treatment modalities and other potential targets for HSV chemotherapy.

ANTIVIRALS IN THE TREATMENT OF HERPES SIMPLEX INFECTIONS

The major treatment modality for HSV infections is the use of antiviral nucleoside analogues. Common across this class of medication is the requirement of activation by viral-encoded thymidine kinase (TK). The first nucleoside analogue shown to be effective agent in clinical trials was acyclovir [6], which is available in oral, topical, and intravenous preparations. In cells infected with HSV, acyclovir is phosphorylated to acyclovir monophosphate by TK, and subsequently converted to di- and triphosphate forms through the actions of host cellular enzymes. The active form of the drug is acyclovir triphosphate, which inhibits HSV-encoded DNA polymerase through competition with deoxyguanosine triphosphate as a substrate for the enzyme. This results in obligate termination of HSV DNA replication, and thus inhibition of

viral growth [7]. Oral acyclovir is approved by the US Food and Drug Administration (FDA) for the treatment of genital herpes infection. Primary infection is effectively treated with 200 mg five times daily for five to ten days, and episodic drug therapy with 200 mg five times daily for five days is used for the treatment of recurrent infection [6]. An alternative regimen recommended by the US Centers for Disease Control is 400 mg three times daily for the treatment of both primary and recurrent infections [8]. A two-day regimen of acyclovir has also been successfully used for episodic treatment of recurrent infection [9]. For patients with frequent recurrences, 400 mg twice daily is effective in continuous suppressive therapy of genital herpes [6], although dosages of 200 mg two to five times daily have also been effectively used [10, 11]. Acyclovir may also be of some benefit in the treatment of herpes labialis, though there are no FDA-approved treatment regimens for this indication [12-14]. Though acyclovir has been demonstrated to be both effective and safe, its inconvenient dosing schedules leading to issues in compliance, and low bioavailability (approximately 15%) have led to the development of the nucleoside prodrugs valaciclovir and famciclovir, which exhibit improved absorption characteristics.

Valaciclovir, the 1-valyl ester prodrug of acyclovir, is metabolized to L-valine and acyclovir. Its safety and pharmacokinetics are essentially identical to those of acyclovir as the parent nucleoside, but valaciclovir is much better absorbed than acyclovir, with an oral bioavailability of about 55%. The peak plasma concentrations of acyclovir achieved are approximately 4 times greater when valaciclovir is administered in similar doses to acyclovir [15]. Oral valaciclovir is efficacious in the treatment of

genital herpes, and is indicated for primary (1000 mg twice daily for ten days) and recurrent (500 mg twice daily for three to five days) infections [6]. It is also approved for continuous suppressive therapy of genital herpes at a dosage of 500 mg once daily for patients with ten or fewer recurrences per year, and 1000 mg once daily for patients with greater than ten recurrences annually [6]. Doses of 250 to 500 mg twice daily have also been shown to be effective in suppressive therapy [16, 17].

Recent studies have demonstrated the therapeutic potential of valaciclovir for the treatment of herpes labialis. High dose valaciclovir at the first prodromal symptom for 1 day (2000 mg twice) or 2 days (2000 mg twice on the first day, followed by 1000 mg twice on the second day) were effective in reducing episode duration and lesion healing time [18]. It has also been recently reported that suppressive valaciclovir therapy is effective in reducing transmission of genital herpes among heterosexual HSV-2 discordant couples – a significant finding in that it is the first controlled trial demonstrating the ability of antiviral therapy to reduce sexual transmission of infection [19].

Famciclovir is the oral prodrug of penciclovir, another nucleoside analogue possessing antiviral activity against HSV. Though penciclovir and acyclovir are both dependent on viral TK, penciclovir differs from acyclovir in that HSV DNA chain termination is attained with penciclovir despite the presence of a 3' hydroxyl group, whereas acyclovir lacks the 3' hydroxyl group making its chain termination "obligate". It has been shown that the active drug form, penciclovir triphosphate, is made in excess

and has a much longer intracellular half-life (20 hours) than acyclovir triphosphate (1 hour) in cells infected with HSV-2. However, penciclovir triphosphate is proportionally less potent against viral DNA polymerase [20]. A topical 1% cream preparation of penciclovir, applied once every two hours for four days, is approved by the FDA for the treatment of recurrent herpes labialis.

Famciclovir, administered at 125 mg twice daily for five days, is approved for the episodic treatment of recurrent genital herpes. It is also effective in the suppression of recurrent infection, approved at a dose of 250 mg twice daily [6]. The dosing of famciclovir is often confusing to clinicians but results from the intracellular pharmacokinetic advantage in making the triphosphate. This provides a dosing advantage during active treatment, but that advantage disappears between active episodes. The use of famciclovir in the treatment of primary genital herpes infection has been investigated in clinical studies. It was determined that when administered at 250 mg, 500 mg, and 750 mg three times daily for five days, famciclovir was as efficacious as acyclovir at doses approved for the treatment of primary infection [21], and has the practical advantage of reduced dosing frequency.

In assessing the advantages of the antivirals currently available for the treatment of HSV infection, acyclovir and valaciclovir are favored for the potency of acyclovir triphosphate, whereas famciclovir has a dosing advantage during episodic therapy only due to its long intracellular half-life. The advantages of each drug provide multiple

therapeutic options, and the most effective treatment can be selected for individuals infected with HSV.

RESISTANCE TO ANTIVIRALS

A concern regarding the use of antiviral medications for the treatment of HSV infections has been the development of drug resistance. Strains of HSV that are resistant to acyclovir have been identified, with a higher rate of occurrence in immunocompromised individuals (4 to 11%) when compared to the general population (0.4%) [2]. Resistance is conferred by viral mutations resulting in strains of HSV which are deficient in thymidine kinase [22], or strains which exhibit altered enzyme activity without loss of virulence [23]. As the active, triphosphate forms of the nucleoside analogue antivirals require phosphorylation by viral thymidine kinase, strains lacking the enzyme are not susceptible to inhibition of DNA replication by these agents.

Drugs which have been developed for the treatment of acyclovir-resistant HSV include foscarnet and cidofovir. Foscarnet exhibits antiviral activity against a broad range of viruses, without requiring conversion to an active form by HSV thymidine kinase. It acts by inhibiting the cleavage of pyrophosphate groups from deoxynucleoside triphosphates, thereby interfering with viral DNA chain elongation [24]. Foscarnet is approved by the FDA for the treatment of acyclovir-resistant HSV infections in immunocompromised patients, however its use is limited to intravenous administration due to poor oral bioavailability. Drug toxicity is a dose-dependent

concern with foscarnet, as potential adverse effects include renal dysfunction, gastrointestinal disturbances, hypocalcaemia, and genital ulcers [25].

Another broad-spectrum antiviral agent under investigation for the treatment of acyclovir-resistant HSV is cidofovir, an analogue of deoxycytosine monophosphate. Like foscarnet, the antiviral activity of cidofovir does not depend on viral-encoded thymidine kinase. Cidofovir is metabolized to its active diphosphate form through the actions of cellular enzymes, and exerts its antiviral activity through interaction with viral DNA polymerase. Incorporation of one cidofovir molecule causes a decrease in chain elongation efficacy, and incorporation of two cidofovir molecules separated by one nucleotide results in complete termination of DNA synthesis [24]. Though limited by intravenous adverse effects and a poor oral bioavailability of less than 5% [6], the use of topical cidofovir gel for the treatment of HSV infections has been shown to have a significant antiviral effect. A trial of 1, 3, and 5% cidofovir gel for the treatment of genital herpes lesions in immunocompetent patients showed a dose-dependent decrease in median time to negative virus culture compared to placebo, however adverse effects included dose-dependent genital ulcers in men [26]. Cidofovir gel has also been tested in a small study in immunocompromised patients, and was shown to possess both antiviral and clinical efficacy [27]. Unfortunately, this gel is not approved and is not available from a commercial source. Some pharmacies have used the intravenous preparations to prepare the gel formulation in critical clinical situations.

DEVELOPMENT OF HSV VACCINES

Another approach to control HSV infection is the prevention of viral transmission, which can be difficult for two reasons: individuals infected with HSV are often unaware of the fact, and transmission can occur during asymptomatic shedding, during which infection is active but goes unnoticed [28]. An ideal vaccine against HSV would be capable of inducing immune responses such that infection could be prevented. It follows, then, that if primary infection can be prevented, there would be no virus available to cause recurrent HSV infection, or to be transmitted to susceptible individuals. However, it is difficult to be certain as to whether such goals are attainable, and thus, developmental vaccines may be considered effective according to different clinical endpoints, such as the ability to alleviate primary episodes, prevent ganglia colonisation, reduce severity or frequency of recurrences, and reduce viral shedding [29]. Much of the data currently available for HSV vaccines comes from clinical trials designed to study the prevention of genital herpes. If an effective vaccine for genital herpes were to become available, it may also have implications in the management of other HSV diseases [30].

Vaccines against HSV currently under development can be classified as either live or inactive vaccines [30]. The challenge of the developmental process is to balance the advantages and disadvantages of each potential candidate, taking into account factors such as immunogenicity and safety profile. Live vaccines include non-pathogenic attenuated virus forms, replication-limited mutants, and also replication vectors for the delivery of HSV antigens. Advantages of live vaccines that make them

preferable over inactive vaccines include the generation of a broader immunity which would be longer-lasting because of vaccine replication in the recipient. However, there are concerns regarding their safety and stability, especially with non-pathogenic strains of HSV derived from cell culture which have been shown to revert to pathogenic forms [31].

Inactive vaccines include killed or inactivated virus forms, nucleic acid vaccines, and protein subunit vaccines. Subunit vaccines, usually based on HSV envelope glycoproteins gB and gD, have been developed in attempt to increase antigenic concentration thereby inducing a stronger immune response, while maintaining a good safety profile by not requiring whole live virus and eliminating viral DNA [29]. While the degree of immunogenicity achieved with subunit vaccines may be less than that of live vaccines, immunogenicity can be improved through the addition of adjuvants in the formulation.

Various subunit vaccine candidates have been evaluated in clinical trials. An early study of an HSV-2 purified envelope glycoprotein vaccine developed by Merck, Sharp & Dohme showed that it was poorly immunogenic and failed to provide protection from HSV infection, with acquisition rates of infection being similar in groups receiving vaccine (10.7%) versus placebo (8%) [31]. Subsequently, clinical trials have been conducted to investigate the efficacy of a vaccine developed by the Chiron group – a gD_2 and gB_2-based vaccine containing MF59, a squalene oil-in-water emulsion adjuvant. In these studies, a benefit was observed for the initial five months

of the study, as HSV-2 acquisition rates were 50% lower among vaccine recipients compared to placebo during this period. Although high levels of HSV-2 neutralizing antibodies were induced in patients receiving the vaccine, the overall efficacy was determined to be 9%. Therefore, the authors concluded that high titres of specific neutralising antibodies alone are insufficient to provide sustained protection from HSV-2 infection [33]. A recombinant gD_2 subunit vaccine developed by GlaxoSmithKline has also been evaluated in clinical trials. This vaccine, which is combined with the adjuvant alum plus monophosphoryl lipid A, was shown to be efficacious in protecting HSV double-seronegative women from both infection (43%) and disease (72%). However, double-seronegative men and individuals seropositive for HSV-1 were not afforded protection [34].

OTHER TREATMENT MODALITIES FOR HSV INFECTION

Resiquimod

Resiquimod belongs to class of therapeutic agents known as immune response modifiers (IRM). Unlike the nucleoside antivirals which directly target HSV, IRMs do not possess direct antiviral effects. Rather, they exert their actions through modulation of certain immune cells, resulting in production of cytokines that regulate the immune response against viral infection. Resiquimod is more soluble and potent in stimulating Th1 cell-mediated immune responses than imiquimod, a first-generation synthetic IRM [35]. Resiquimod has been shown to stimulate the production of interferon-alpha, interferon-gamma, and interleukin-12, as well as enhance dendritic cell antigen presentation [36, 37]. *In vivo* studies on the mechanism of resiquimod action suggest

that it exerts its effects through activation of immune cells via the Toll-like receptor 7 (TLR7)-MyD88-dependent signaling pathway [38].

A recent clinical study was conducted to evaluate the effects of resiquimod applied during a recurrence of genital herpes. In this study, patients with frequently recurring genital herpes applied topical resiquimod gel (0.01%, two or three times weekly; or 0.05%, once or twice weekly) or vehicle gel to lesions for three weeks. During the six month observation period, resiquimod was shown to decrease recurrences, with median days to first recurrence of 169 days for the pooled resiquimod group, versus 57 days for the vehicle group. It was also determined that 32% of patients receiving resiquimod completed the observation period without any recurrences, compared to 6% of patients receiving vehicle. With respect to tolerability, resiquimod 0.05% twice weekly caused inflammation at lesion sites, whereas other regimens were well tolerated [39]. Resiquimod is currently being evaluated in phase III clinical trials.

n-Docosanol

n-Docosanol, a 22-carbon primary alcohol, has been shown to inhibit a broad range of enveloped viruses including HSV-1 and HSV-2, and is approved by the FDA as an over-the-counter topical 10% cream for the treatment of cold sores. n-Docosanol exerts its antiviral effects through inhibition of viral fusion with host cell membranes, thus preventing nuclear localisation and limiting viral replication [40]. Evidence suggests that the anti-HSV actions of n-docosanol require cellular uptake and metabolism of the drug [41]. The clinical efficacy of topical n-docosanol for the

treatment of herpes labialis has been studied in a multicenter trial. In this study, patients with histories of herpes labialis were randomized to receive either n-docosanol 10% cream or placebo five times daily, and began therapy in the prodrome or erythema stage of an episode, with treatment continuing until healing occurred. It was determined that the mean time to healing in individuals treated with n-docosanol was 18 hours shorter than in those given placebo. n-Docosanol was also shown to be efficacious with respect to other endpoints of clinical significance, as both the time to relief from pain and other symptoms and the duration of the most severe stage of the lesion were reduced [42].

Microbicides

An area of recent research interest has been the development of topical microbicides with broad-spectrum activity against various sexually transmitted pathogens, including HSV. First-generation microbicides, each of which confer protection through a unique mechanism of action, have been evaluated in animal models of genital herpes [43]. Buffergel, an acidic buffer microbicide which acts by inactivating acid-sensitive pathogens, showed protective effects against HSV-2 transmission in a mouse infection model [44]. Protection against genital herpes was also observed with the naphthalene sulphonate polymer PRO 2000, which prevents infection by blocking pathogen-target attachment [45]. These results suggest that the use of microbicides in the prevention of herpes is promising, and further studies designed to study their efficacy are warranted. With the increasing incidence of HSV-2

infection, and because it increases the risk of HIV acquisition, all attempts to reduce transmission need to be examined.

Potential Approaches for Antiviral Therapy

Drugs currently available for the treatment of HSV infections inhibit viral DNA replication by targeting DNA polymerase. In the development of new therapeutic agents, it would be beneficial to consider drugs capable of blocking DNA replication through inhibition of other molecular targets, and to consider other stages of the viral cycle, such as attachment, entry, uncoating, and protein synthesis, and assembly [36]. Enzymes of interest include HSV protease, which is essential in protein cleavage during virus capsid maturation. Other potential targets include ribonucleotide reductase, the enzyme which catalyses deoxyribonucleotide formation, and uracil-DNA glycosylase, which functions in post-replicative DNA repair [37]. Recently, inhibitors of HSV helicase-primase were shown to possess both *in vitro* and *in vivo* anti-HSV properties [38, 39].

CONCLUSION

Herpes simplex viruses cause diseases for which there are no cures. The major treatment modality remains the oral administration of nucleoside analogue antivirals – acyclovir, valaciclovir, and famciclovir – for genital herpes infection, and a topical penciclovir preparation for orolabial herpes. Advances in immunological therapy include a subunit vaccine showing a significant protective effect against HSV infection and disease in double-seronegative women, and a reduction of recurrent infection in

patients receiving topical resiquimod, an immune response modifier. Docosanol has recently been approved for the treatment of herpes labialis. Other potential therapeutic options have been identified, and we await the results of further trials to determine the clinical benefit of these newer agents.

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