TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES ON AMIDE DERIVATIVES OF 5-METHOXYMETHYL-2-DEOXYCYTIDINE

SHAJAN MANNAI

1999
TOXICOLOGICAL AND PHARMACOLOGICAL STUDIES ON AMIDE DERIVATIVES OF 5-METHOXYMETHYL-2'-DEOXYCYTIDINE

A Thesis Submitted to the College of Graduate Studies and Research
In Partial Fulfillment of the Requirements For the Degree of Master’s of Science In the Toxicology Graduate Program
University of Saskatchewan
Saskatoon

By
Shajan Mannala
Fall 1999

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DEDICATION

I dedicate this thesis to my late grandfather Abraham Mannala, Esq. who passed away in India, when I was involved in pursuit of knowledge and skills presented in this study.

His inspirational guidance and loving care during my formative years and encouragement to excel in academics and life has left a lasting shadow of his person for years to come.

May the Almighty rest his soul in peace.
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Toxicity has been a major problem in the development of drugs for the treatment of viral infections. Our hypothesis was that deoxycytidine analogs should have lower toxicity profile, because these pharmacophores would act selectively on virus infected cells.

The antiviral activity of amide derivatives of 5-Methoxymethyl-2'-deoxycytidine (MMdCyd), namely, acetyl-MMdCyd, propanoyl-MMdCyd, butanoyl-MMdCyd and hexanoyl-MMdCyd against Herpes simplex virus type 1 (HSV-1), in A549 cells and VERO cells was determined using the plaque reduction assay. The potency of MMdCyd and amide derivatives was higher in A549 cells as compared to VERO cells. Butanoyl-MMdCyd (But-MMdCyd) was the most potent compound in both cell lines. Concentrations of MMdCyd and But-MMdCyd required to inhibit 50% virus plaque formation (ED$_{50}$) were 6.69 ± 0.70 and 0.87 ± 0.54 µM, respectively, in A549 cells. Butanoyl-MMdCyd was very effective in preventing cytopathogenic effects of HSV-1 and decreasing the production of infectious virus particles.

The acute and delayed cytotoxicity of MMdCyd and But-MMdCyd was determined using CEM cells. Dideoxycytidine (ddC) was used as a positive control drug. At a concentration of 0.5 µM, ddC increased the doubling time of the cells, from the fourth day onward. MMdCyd and But-MMdCyd did not alter the doubling time of the cells up to 2000 µM (highest concentration tested).
The acute and subacute toxicity of But-MMdCyd was studied in CD-1 male mice. No morbidity or mortality was observed in But-MMdCyd treated mice up to a concentration of 1000 mg/kg (acute trial) or in a repeat dose study using a dose of 100 mg/kg for 15 days (subacute). No gross or histopathological lesions attributed to drug treatment were observed in the tissues examined from the mice from the acute or subacute toxicity studies. The hematological (red blood cell count, white blood cell count, hemoglobin, hematocrit value, platelet count and total protein) and clinical chemistry parameters (alanine amino transferase, aspartate amino transferase, creatinine, sodium and potassium ion concentrations) examined showed no statistically significant difference (P values 0.25 to 0.83) between treated animals and control group, in either the acute or subacute toxicological studies. The low order of animal toxicity of But-MMdCyd and potent antiviral activity warrants additional toxicological studies on this compound for further development.
ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. V.S. Gupta for his inspirational and patient guidance, financial support, and constant encouragement throughout the entire duration of my studies. Without his cooperation this study would not have been possible.

I also extend my sincere appreciation to Dr. A. Saxena (co-supervisor), for his guidance and support. His tireless assistance and constructive criticism contributed much to the completion of this thesis. I thank members of my advisory committee, Drs B.R. Blakley, C.S. Sisodia, and K. Liber for valuable advice and constructive criticism.

My sincere appreciation and gratitude is extended to Dr. Sashi Kumar for synthesizing compounds, Mrs. Jeanette Heis, Department of Microbiology, College of Medicine, for her guidance on tissue culture methodology, Dr. J. Kalra and the staff of the Department of Pathology for assistance with the histopathology and clinical chemistry studies and Drs. A. Stuart, Roy Skariah Udyamputhhor and Ruili Shi, for their cooperation and encouragement.

My deepest thanks to the Toxicology Centre and the University of Saskatchewan for awarding me a graduate scholarship to undertake this study.

I would sound ungrateful, if deep appreciation and thanks were not extended to my wife Sally, and our daughters, Samantha and Sonya. Their unending support and undeviating encouragement has been my strength. Finally I wish to say thank you to my dear family in India, for teaching me the value of learning. My parents constantly prayed for me and encouraged me to keep on going even during the toughest times.
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<th>Full Form</th>
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<td>ACV</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Amino Transferase</td>
</tr>
<tr>
<td>ANA</td>
<td>Antiviral Nucleoside Analogs</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis Of Variance</td>
</tr>
<tr>
<td>ANP</td>
<td>Acyclic Nucleoside Phosphonates</td>
</tr>
<tr>
<td>Ara-A</td>
<td>Arabinofuranosyladenine</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Amino Transferase</td>
</tr>
<tr>
<td>AZT</td>
<td>3'-Azidothymidine</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood Urea Nitrogen</td>
</tr>
<tr>
<td>BV-araU</td>
<td>1-β-D-arabinofuranosyl-E-5-(2-bromovinyl) uracil</td>
</tr>
<tr>
<td>BrVdUrd</td>
<td>(E)-5-(2-bromovinyl)-2'-deoxyuridine</td>
</tr>
<tr>
<td>CC50</td>
<td>Cytotoxic concentration required to reduce cell growth by 50%</td>
</tr>
<tr>
<td>CEM cells</td>
<td>A human T lymphoid cell line</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>ddC</td>
<td>Dideoxycytidine</td>
</tr>
<tr>
<td>dCyd</td>
<td>2'-Deoxycytidine</td>
</tr>
<tr>
<td>ddI</td>
<td>Dideoxyinosine</td>
</tr>
<tr>
<td>DP</td>
<td>Diphosphate</td>
</tr>
<tr>
<td>dThd</td>
<td>Deoxythymidine</td>
</tr>
</tbody>
</table>
dUrd 2'-deoxyuridine
EBV Epstein Barr Virus
EtdUrd 5-ethyl-2'-deoxyuridine
EtdCyd 5-ethyl-2'-deoxycytidine
ED\textsubscript{50} Concentration required to inhibit virus replication by 50%
ED\textsubscript{90} Concentration required to inhibit virus replication by 90%.
FCV Famciclovir
FDA Food and Drug Administration
FIA Focal immunoassay
GCV Gancyclovir
GGT Gamma Glutamyl Transferase
GLP Good Laboratory Practice
H\textsubscript{4}dUrd Tetra hydrodeoxy uridine
HCT Hematocrit
HHV Human Hepatitis Virus
HIV Human Immunodeficiency Virus
HSV Herpes Simplex Virus
IFN Interferon
IdUrd Idoxuridine
i.p. Intraperitoneal
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean Corpuscular Volume</td>
</tr>
<tr>
<td>MMdCyd</td>
<td>5-methoxymethyl-2'-deoxycytidine</td>
</tr>
<tr>
<td>MMdUrd</td>
<td>5-Methoxymethyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>PCV</td>
<td>Penciclovir</td>
</tr>
<tr>
<td>PFA</td>
<td>Phosphonoformic acid</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque Forming Unit</td>
</tr>
<tr>
<td>PRA</td>
<td>Plaque Reduction Assay</td>
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<tr>
<td>RBC</td>
<td>Red Blood Corpuscles</td>
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<tr>
<td>SI</td>
<td>Ratio of CC&lt;sub&gt;50&lt;/sub&gt;/ED&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Tissue culture infective dose</td>
</tr>
<tr>
<td>TFT</td>
<td>Trifluridine</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine Kinase</td>
</tr>
<tr>
<td>TP</td>
<td>Total Protein</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella Zoster Virus</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Corpuscles</td>
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<td>WHO</td>
<td>World Health Organization</td>
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</table>
1. **INTRODUCTION**

Viruses are obligatory intracellular parasites that cause diseases in humans and animals. Viral infections are either localized to mucosal or cutaneous tissues or generalized by viremia or neurogenic spread to distant organs. Because of their intracellular location and total dependence on the host's synthetic machinery, viral infections may induce profound changes in the body's physiological processes (Babe and Craik, 1997). Many viral infections can be controlled by immunization. In some cases the potentiation of the immune system results in life-long immunity. The common viral infections of man and animals amenable to immunization are listed in Table 1.1. Antiviral chemotherapy has been successfully used for the control of some viral infections. The most important viral infections for which therapeutic measures are the only means of control are listed in Table 1.2.

The cardinal principle of chemotherapy is selectivity. Therefore, selective antiviral agents must inhibit virus-specific replicate events to minimize toxicity to the host. Preferential inhibition of the virus-induced enzymes or their utilization resulting in the production of altered metabolites, has proven to be a useful approach in the discovery of chemotherapeutic agents for the treatment of viral infection (Jones, 1998). Serependity and more recently in-depth knowledge of the molecular mechanisms of viral replication have led to the discovery of novel antiviral drugs. Virus genome is comprised of nucleic
acid (DNA or RNA) surrounded by a protein shell or capsid. Some viruses (Herpetoviridae and Togaviridae) also possess a lipoprotein envelope with antigenic properties. The pathogenic DNA viruses of humans and animals include poxviruses, herpes viruses, adenoviruses, and papillomaviruses. Replicative cycle of DNA viruses is similar. Briefly, DNA viruses, enter into the host cell nucleus, where the viral DNA is transcribed into mRNA by host cell mRNA polymerase; mRNA is translated in the usual host cell fashion into virus-specific proteins (Hayden, 1995). The poxvirus has its own RNA polymerase and consequently replicates in host cell cytoplasm. Replicative cycle of herpes viruses is shown in Fig 1.1.

Theoretically, replication of DNA viruses can be inhibited by interfering at any of the steps involved in the process viral replication, namely, adsorption, penetration, transcription, virion assembly, maturation and/or release. The different classes of inhibitors and the site at which they interfere are summarized in Table 1.3. (Hayden, 1995). Most compounds endowed with antiviral activity reported until early seventies were toxic to the host and thus the medical community held the view that all antivirals will have a high order of toxicity. This myth however has been laid to rest with the discovery of many new chemotherapeutic agents with selective action against Herpes viruses (HSV, VZV and CMV) and Human Immunodeficiency Virus (HIV). These drugs also have a low order of host toxicity.
Table 1.1 Common Virus Infections amenable to control through active Immunization

<table>
<thead>
<tr>
<th>Man</th>
<th>Animals</th>
</tr>
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<tbody>
<tr>
<td>Small Pox</td>
<td>Marek’s Disease</td>
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<td>Poliomyelitis</td>
<td>Foot and Mouth Disease</td>
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<td>Measles</td>
<td>Rhinderpest</td>
</tr>
<tr>
<td>Rubella</td>
<td>Canine Distemper</td>
</tr>
<tr>
<td>Mumps</td>
<td>Feline Leukemia Virus Infection</td>
</tr>
<tr>
<td>Rabies</td>
<td>Rabies</td>
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<tr>
<td>Yellow fever</td>
<td>Parvo virus Infection</td>
</tr>
<tr>
<td>Hepatitis (A and B)</td>
<td>Hepatitis</td>
</tr>
<tr>
<td>Chicken Pox</td>
<td>Avian leukosis</td>
</tr>
<tr>
<td></td>
<td>Ranikhet disease</td>
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</tbody>
</table>

Table 1.2 Viral infections amenable to chemotherapy

Herpes Virus infection

Herpes Simplex Virus (HSV-1, HSV-2)
Varicella Zoster Virus (VZV)
Cytomegalovirus (CMV)

Human Immuno Deficiency Virus (HIV-1, HIV-2)

Hepatitis B Virus (HBV)

Influenza Virus
Table 1.3 Stages of Virus Replication and Targets of Antiviral Agents

<table>
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<th>Stages of replication</th>
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<td>Soluble receptor decoys</td>
</tr>
<tr>
<td>2. Virus penetration</td>
<td>Antireceptor antibodies</td>
</tr>
<tr>
<td>3. Virus uncoating</td>
<td>Ion channel blockers</td>
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<td></td>
<td>Capsid stabilizers</td>
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<td>4. Transcription of viral genome</td>
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<td>Reverse transcriptase inhibitors</td>
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<td></td>
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<td>5. Translation of viral proteins</td>
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<td>Antisense oligonucleotides</td>
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<td>Ribozymes</td>
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<td></td>
<td>Inhibitors of regulatory proteins</td>
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<td>6. Viral assembly</td>
<td>Interferons</td>
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<td></td>
<td>Assembly-protein inhibitors</td>
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<td>7. Post-translational modifications</td>
<td>Protease inhibitors</td>
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<td>8. Release, budding and cell lysis</td>
<td>Antiviral-antibodies</td>
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<td></td>
<td>Cytotoxic-lymphocytes</td>
</tr>
</tbody>
</table>

1. Adapted from Hayden, 1995.
Fig. 1.1 Replicative cycle of herpes viruses, showing the site of action of antiviral agents.
1. Attachment
2. Uncoating and transfer of viral DNA to host nucleus
3. Synthesis of viral DNA
4. Transcription into viral mRNA
5. Protein synthesis by host cell ribosome
6. Assembly of virions
This figure was adapted from Hayden, 1995.
1.1 Antiviral Chemotherapy.

1.1.1 Historical perspective

Viral diseases were largely untreatable 40 years ago and the true origins of antiviral drugs can be traced to the early 1950s. The development of antiviral agents has been a very slow process compared to antibacterial or antiparasitic compounds. The often-elusive aim was to develop antiviral agents that are safe, selective, and able to cross blood-brain barrier. The progress of antiviral chemotherapy can be divided into three chronological periods: (1) the past era of early antivirals (idoxuridine, vidarabine and trifluorothymidine): (2) the present era dominated by the discovery of acyclovir and other drugs (dideoxynucleoside analogs) with selectivity against Herpes simplex virus (HSV) and Human Immunodeficiency virus (HIV), respectively and (3) hopefully the future era of very selective virucidal therapeutic agents. The lessons learned from the past and present research on the antivirals paves the way for the development of better antivirals in the future.

Viruses are obligatory intracellular parasites and utilize many of the host cell biochemical pathways for their replication and survival. The development of antiviral compounds has been hindered because of the lack of knowledge of steps involved in viral replication, absence of systems for testing compounds for antiviral activity and the apathy of the scientific community. The general belief in the scientific and medical community was that it would be impossible to develop selective chemotherapeutic agents because of the intimate virus-host relationship. Antiviral agents available produced severe impairment
of host metabolism resulting in host toxicity on systemic administration (De Clercq, 1987; Aduma, 1989; Parker and Cheng, 1994; Hayden, 1995; Martin et al., 1998). A brief review of the toxicity problems observed with the use of first generation of antivirals illustrates this point. Idoxuridine (IdUrd) was the first antiherpes compound licensed for use in herpes infection (only for topical application). Although this compound proved very effective in the treatment of HSV keratoconjunctivitis, its lack of selectivity resulted in host tissue toxicity (Kaufman and Heidelberger, 1964). The toxicity of IdUrd in systemic therapy was well demonstrated when it was used for treatment of HSV encephalitis in the early 1970s (Boston Interhospital Virus and the NIAID Cooperative Antiviral study Group, 1975). This toxicity or precisely the lack of selective toxicity precluded its use for systemic antiviral therapy. During this period of antiviral drug development, other medications came to the forefront that provided valuable insight in the development of antiviral chemotherapy. Parallel with the development of IdUrd for the treatment of herpes infection, amantidine was developed for the prophylaxis of influenza infections (Davies et al., 1964; Becker 1976). This drug had an over 70% success in preventing influenza out breaks. Introduction of arabinofuranosyl adenine (Vidarabine, Ara-A) for the treatment of herpes encephalitis was the beginning of the era of successful systemic antiviral therapy. Vidarabine was shown to be effective for the treatment of HSV encephalitis, neonatal HSV disease, and VZV infections in patients. The reduction in mortality from debilitating and often fatal herpes encephalitis from 70% in untreated cases to 40% in Ara-A treated cases was a significant step in the
advancement of antiviral chemotherapy (Buchanan and Heiss, 1985). The major drawbacks for the use of Ara-A were: (i) it was deaminated by adenosine deaminase to arabinosyl hypoxanthine, a less potent antiviral compound and (ii) Ara-A is not orally bio-available and is poorly soluble, thus requiring continuous intravenous administration of large volumes of solution. The requirement for continuous and frequent intravenous administration as well as evidence of limited but well documented toxicity (pain, pruritus, inflammation and edema involving the eye or lids) severely impaired this drug’s potential as a much-sought ideal antiviral (Kaufman, 1980). Thus clinical studies of Ara-A in the late 1970s and early 1980s marked an end to the first generation of antiviral drugs because of the lack of selective action.

The lessons learned from the use of idoxuridine, trifluorothymidine and later vidarabine stressed the need for the development of antiviral agents, which would affect viral functions to a greater extent than the host cell functions. Several other 5-substituted deoxyuridine analogs were also synthesized and shown to have similar characteristics (Bubbar and Gupta, 1970; De Clercq and Torrence, 1978; De Clercq et al., 1981). Although, at this time, mechanisms responsible for the selective action of these compounds were not known, nonetheless, these compounds did indicate that it might be possible to develop “selective” antiviral agents. The development of assay systems using cell lines in tissue cultures enabled researchers to assess the antiviral activity of potential drug candidates. The era of present antiviral therapy began in the 1980s with the introduction of acyclovir, for the treatment of systemic HSV infections (Collum et al.,
1980; Saral et al., 1981; Prober et al., 1982; Douglas et al., 1988; Prentice et al., 1994).

The discovery of 5-substituted deoxyuridine analogs in early eighties, namely, 5-methoxymethyldeoxyuridine (Meldrum et al., 1980; Gupta et al., 1987) and 5'-amino-5-iododeoxyuridine (Prusoff, 1980) was a significant event in the path of selective antiviral drug therapy. These compounds were shown to have potent activity against HSV and low cytotoxicity. The use of acyclovir for the treatment of genital herpes in patients demonstrated safety, efficacy and oral bioavailability (Whitley et al., 1992). The success of acyclovir in the clinics demonstrated that it was possible to develop safe and effective medicines to combat viral diseases, without generating serious toxicity to the host. Subsequently, molecular pharmacology studies demonstrated that the selective action of acyclovir was due to its anabolism by a virus-specific enzyme (Sullivan et al., 1992; Littler, 1994).

Since the recognition of Acquired Immunodeficiency Syndrome (AIDS) in the early 1980s and the successful use of antiviral drugs for combating HIV infection, the field of antiviral chemotherapy has acquired a new status and research for the development of selective chemotherapeutic agents for combating viral infections is being pursued around the world. Prior to AIDS epidemic, the major emphasis of antiviral research programs centered on the development of drugs primarily for the treatment of infection due to herpes viruses because of their considerable importance as human pathogens (Johnson et al., 1989). During the past decade safe and effective antiviral therapy for the treatment of a number of viral infections has been accomplished. Many
viral infections now can be managed with the use of specific antiviral drugs. Antiviral agents are now classified based on their structure, stage of virus cycle affected by these compounds, spectrum of viral activity and their mechanism of action. The advances in molecular biology have enabled a better understanding of the replicative cycle of viruses (Hayden, 1995). Three-dimensional structures of many viruses and antiviral compounds have been elucidated by X-ray crystallography (Bossart-Whittakar et al., 1993; Lavie et al., 1997; Jones, 1998). Thus advances in the fields of molecular biology of viruses and topology of active molecules has been very helpful in understanding the molecular mechanisms of action of many antiviral compounds.

In this review, I will primarily focus on compounds with activity against herpes viruses and their toxicity. Nucleoside analogs with selective antitherpes activity will be discussed in greater detail because the subject matter of this thesis is in this area.

1.1.2 Nucleoside analogs

The development of nucleoside analogs with selectivity was a major breakthrough for the treatment of infection caused by HSV. Acyclovir, the first compound of a new generation of HSV inhibitor was found to be approximately 10 times more active than IdUrd and 600 times more active than Ara-A with very low cytotoxicity (Hayden, 1995). Since the introduction of acyclovir, large number of nucleoside analogs with selectivity against herpes viruses have been described (Kinchington et al., 1997). These compounds are selectively and actively phosphorylated by a viral induced thymidine kinase and are not converted to their active form in un-infected cells. This unique property confers selective
mechanism of action against virus. The nucleoside analogs approved for clinical use are listed in Table 1.4.

1.1.2.1. Acyclovir and Valacyclovir (pro-drug):

Acyclovir [9-(2-hydroxy-ethoxy) methyl]-9H-guanine] is an acyclic guanine nucleoside analog that lacks 3'-hydroxyl on the side chain (Hayden, 1995). Acyclovir is available as capsules, as an ointment and as powder to be reconstituted for intravenous use. Acyclovir has potent activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro (ED₅₀ 0.02 to 0.9 µg/ml) and low cytotoxicity (>50 µg/ml). It is also a good inhibitor of varicella-zoster virus (VZV) replication in cell culture (ED₅₀ 0.8 to 4.0 µg/ml) but has little activity against vaccinia virus, adenovirus and cytomegalovirus (Wagstaff, et al., 1994). Acyclovir preferentially inhibits viral DNA synthesis. It is taken up selectively by the virus-infected cell and its uptake is facilitated by HSV-induced thymidine kinase. This viral enzyme phosphorylates ACV to its monophosphate (ACVMP). The affinity of ACV for viral thymidine kinase is about 200-fold greater than that for the mammalian enzyme. Acyclovir can also be activated by other phosphorylating enzymes as evidenced by its activity and phosphorylation in thymidine kinase negative (TK⁻) viruses such as cytomegalovirus (Wingard et al., 1981). The cellular kinases convert ACVMP to acyclovir triphosphate (ACVTP), the active form of the drug. Higher concentrations of ACVTP are found in HSV-infected cells where it competes with endogenous deoxyguanosine triphosphate (dGTP) for incorporation into viral DNA. After incorporation into the DNA, chain termination occurs because of the lack of 3'-hydroxyl
Table 1.4 Nucleoside analogs approved for clinical use

<table>
<thead>
<tr>
<th>Antiviral agent</th>
<th>Generic name</th>
<th>Target virus$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pyrimidine analogs</strong></td>
<td>3Brivudine (BrVdUrd)</td>
<td>HSV-1, VZV</td>
</tr>
<tr>
<td></td>
<td>4Sorivudin (BrV-ara-U)</td>
<td>HSV-1, VZV</td>
</tr>
<tr>
<td><strong>Acyclic nucleoside analogs</strong></td>
<td>Acyclovir (ACV)</td>
<td>HSV-1, HSV-2 and VZV</td>
</tr>
<tr>
<td></td>
<td>Penciclovir (PCV)</td>
<td>HSV-1, HSV-2 and VZV</td>
</tr>
<tr>
<td></td>
<td>Ganciclovir (GCV)</td>
<td>CMV</td>
</tr>
<tr>
<td><strong>Acyclic nucleoside analogs</strong></td>
<td>Cidofovir (HPMPC)</td>
<td>HSV-1, HSV-2</td>
</tr>
<tr>
<td></td>
<td>Adefovir (PMEA)</td>
<td>HSV-1, HSV-2, VZV, CMV, HIV-1 and HIV-2</td>
</tr>
<tr>
<td><strong>Pyrimidine dideoxynucleoside analogs</strong></td>
<td>Zidovudine (AZT)</td>
<td>HIV-1, HIV-2</td>
</tr>
<tr>
<td></td>
<td>Stavudine (d4T)</td>
<td>HIV-1, HIV-2</td>
</tr>
<tr>
<td></td>
<td>Zalcitabine (ddC)</td>
<td>HIV-1, HIV-2</td>
</tr>
<tr>
<td></td>
<td>Lamivudine (3TC)</td>
<td>HIV-1, HIV-2 and HBV</td>
</tr>
<tr>
<td><strong>Purine nucleoside analogs</strong></td>
<td>Didanosine (ddl)</td>
<td>HIV-1, HIV-2</td>
</tr>
<tr>
<td></td>
<td>Ribavirin</td>
<td>RSV</td>
</tr>
</tbody>
</table>

1. Adapted from Kinchington, et al., 1997; Krawczyk and Bischofberger, 1997.
2. Cytomegalovirus (CMV), Hepatitis B virus (HBV), Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2), Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), Respiratory syncytial virus (RSV), and Varicella zoster virus (VZV).
3. Not currently approved for use in the United States and Canada but approved in Europe for the treatment of herpes keratitis.
group in acyclovir. Acyclovir triphosphate is a more potent inhibitor of HSV-1 DNA polymerase compared to cellular DNA polymerases (Allaudeen et al., 1982). The widespread use of ACV in recent years has resulted in the emergence of ACV resistant strains of HSV. The resistance has been linked to mutations in genes coding for viral thymidine kinase and DNA polymerase. These mutations cause reduced or lack of the production of HSV-induced thymidine kinase, or result in the synthesis of thymidine kinase and viral DNA polymerase with altered substrate specificities (Crumpacker, 1980; Field et al., 1995).

**Untoward Effects.** Pre-clinical studies in different species of animals indicated that acyclovir was a fairly safe drug. The major toxic manifestation of ACV is nephrotoxicity (Tucker, 1983). Obstructive nephropathy occurred in rats and mice given 20, 40 and 80 mg/kg/day of acyclovir for 21 days (Tucker et al., 1983). Acyclovir was not mutagenic in standard microbial assays (Griffiths, 1995). At high dosage (greater than or equal to 250 µg/ml), some chromosomal breaks were seen *in vitro* (Clive et al., 1983). Acyclovir was clastogenic in Chinese hamsters at a dose of 2000 mg/kg (400 x human exposure). Cell transformation was seen in one of two assays at high dosage (31-63 x human exposure). Mutagenicity was seen at high dosage (250 x human exposure) in mouse lymphoma assay (Clive et al., 1983; Griffiths, 1995). Acyclovir had no carcinogenic effects in rats or mice given lifetime exposure of 3-6 x (mouse) and 1-2 x (rat) estimated human exposure (5 mg/kg). Acyclovir causes testicular atrophy in rats at very high doses (24-48 x) and aspermatogenesis in dogs [47-317 x human doses
A study in humans did not show evidence of impaired sperm production in patients receiving acyclovir (Douglas et al., 1988). Acyclovir had no effect on fertility or reproduction in mice. Acyclovir is not teratogenic in rats, mice or rabbits in standard tests (Griffiths, 1995). In a non-standard test in rats, head and tail abnormalities and maternal toxicity was seen at high doses (63-125 x human exposure).

In human patients, ACV has shown to produce few side effects. When taken orally acyclovir can cause nausea, diarrhea, rash, and headache in a small number of patients (Goldberg et al., 1993). High doses of acyclovir can cause nephrotoxicity and to a lesser extent, thrombocytopenia. The principal dose-limiting toxicities of intravenous acyclovir are renal insufficiency and central nervous system side effects. Reversible renal dysfunction occurs in approximately 5% of patients, related to high urine levels causing crystalline nephropathy (Sawyer et al., 1988) similar to those seen in animals. Manifestations include nausea, emesis, flank pain, and increasing azotemia. Neurotoxicity occurs in 1 to 4% cases and is manifested by altered sensorium, tremor, myoclonus, delerium, seizures, and/or extrapyrimidal signs (Haefeli et al., 1993). Other side effects seen include phlebitis, extravasation, rash, hypotension and interstitial nephritis.

Valacyclovir (pro-drug) is the L-valyl ester of acyclovir. The toxicity profile of valacyclovir parallels that of acyclovir. In toxicology studies in multiple species, valacyclovir has been shown to have the same favorable toxicological profile as that of acyclovir, with no evidence of gonadal toxicity in standard animal models (Goldberg et al., 1993). In bioassays where valacyclovir was administrated at maximum tolerated doses
for 24 months, there was no evidence of carcinogenicity in either rats or mice. High doses of valcyclovir have been associated with nephrotoxicity and, uncommonly, with severe thrombocytopenic syndromes (Beutner et al., 1995). Valcyclovir triphosphate has a longer half-life intracellularly compared to acyclovir and thus this compound can be given less frequently and this alleviates the dose limiting toxicities due to ACV.

1.1.2.2 Penciclovir (PCV), Famciclovir (FCV) and Ganciclovir (GCV, DHPG).

Penciclovir [(9-(4-hydroxy-3-hydroxymethylbutyl) guanine] is an acyclic guanine nucleoside analog (Boyd et al., 1993). The ED$_{50}$ of penciclovir is cell dependent (0.04 to 2.4 µg/ml). Famciclovir is the diacetyl ester pro-drug of penciclovir. Ganciclovir [9-(1-3-dihydroxy-2-propoxymethyl) guanine] is also an acyclic nucleoside analog that has inhibitory activity against herpes simplex viruses (ED$_{50}$ 0.04 to 1.1 µg/ml) and also CMV [ED$_{50}$ 0.2 to 2.8 µg/ml (Plotkin et al., 1985)]. Penciclovir is phosphorylated intracellularly initially by viral kinase, which after conversion to penciclovir triphosphate (PCVTP) inhibits viral DNA synthesis in a manner similar to that of ACV. Ganciclovir is 'activated' by the virus-induced phosphotransferase to its corresponding monophosphate (GCVMP). The triphosphate form of ganciclovir (GCVTP) inhibits viral DNA synthesis. Resistance to famciclovir and penciclovir occurs due to mutations in genes coding for viral thymidine kinase or DNA polymerase. Resistance to ganciclovir occurs due to mutation in UL97-encoded phosphotransferase, resulting in decreased levels of GCVTP and altered viral DNA polymerase (Sullivan et al., 1992).

Untoward Effects. The toxicity profile of these compounds parallels that of acyclovir
and valacyclovir (Saltzman et al., 1994). Chronic administration of famciclovir caused testicular toxicity and tumors in laboratory animals. In 2-year dosing studies, FCV produced a significant increase in mammary adenocarcinomas and marginal increases in fibrosarcomas or squamous cell carcinomas of the skin in female rats receiving the largest tested dose, but not in those receiving smaller doses (Griffiths, 1995). No such effects were seen in male rats, although male mice showed increases in fibrosarcomas/squamous cell carcinomas similar to those seen in female rats. The largest dose in female rats was equivalent to 1.5 x the human systemic recommended dose of 500 mg three times daily. The equivalent exposure for male mice was 0.4 x human systemic exposure. Famiclovir and penciclovir were not mutagenic in microbial assays. Famiclovir and penciclovir caused chromosomal aberrations in human lymphocytes, Penciclovir, but not famiclovir, produced gene mutations/chromosome aberrations in mouse lymphoma assay at higher concentrations (Saltzman et al., 1994). Famiclovir had no effect on fertility in female rats. In male rats, mice and dogs, testicular toxicity included atrophy of seminiferous tubules, decreased sperm count and/or sperm with abnormal morphology or reduced motility. Toxicity increased with chronic administration and high doses (Griffiths, 1995). The no-observable-effect dose in rats after 26 weeks was equivalent to 0.2 x the human exposure at 500 mg three time daily. Testicular toxicity was also seen following chronic administration to mice (0.4 x human exposure) and dogs (1.7 x human exposure). Famiclovir produced no adverse effects on embryo-fetal development in mice and rats (Hayden, 1995). In humans, oral FCV is
generally well tolerated but may be associated with headache, diarrhea, and nausea.

Ganciclovir is cytotoxic to human bone marrow progenitor cells at a concentration of 0.4 to 1 µg/ml (Sommadossi et al., 1987). Inhibition of human lymphocytic blastogenic response also occurs at concentration in the range of 1 to 10 µg/ml. Neutropenia occurs in about 15% to 40% of patients and thrombocytopenia has been reported in 5% to 20% patients (Faulds et al., 1990). Teratogenicity, embryotoxicity, irreversible reproductive toxicity, and myelotoxicity have been observed in animals at ganciclovir dosages comparable to those used in human beings (Hayden, 1995). The dose limiting toxicity of ganciclovir in humans include myelosuppression, neutropenia, CNS effects and liver function abnormalities (Schwarz and Perez, 1998). Cytotoxic drugs and nephrotoxic agents increases the risk of myelosuppression (Hochester, 1990).

1.1.2.3 Sorivudine (BrV-Ara-U, Brovavir)

Sorivudine [1-β-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil, BrV-Ara-U] is a pyrimidine nucleoside analog. It is a very potent and selective inhibitor of HSV-1 and VZV in cell culture (Gnann, 1993).

Sorivudine is preferentially taken up by virus infected cells and phosphorylated by viral thymidine kinase to the monophosphate, BrV-Ara-UMP (Yokota et al., 1989). Viral thymidylate kinase further phosphorylates the BrV-Ara-UMP to the diphosphate and cellular enzymes metabolize the diphosphate to the triphosphate (BrV-Ara-UTP), which is a competitive inhibitor of viral DNA synthesis with respect to deoxythymidine triphosphate. However, unlike acyclovir BrV-Ara-UTP is not incorporated into viral
Untoward Effects. Side effects associated with the administration of the drug include GI toxicity and hepatotoxicity manifested by elevation of liver enzymes. Long term administration of sorivudine has been associated with hepatic and testicular neoplasms in rodents (Dehertogh et al., 1994). The metabolite bromovinyluracil (BrVU) may enhance the toxicity of 5-fluorouracil (5-FU), an anticancer agent by inhibiting the enzyme dihydropyrimidine dehydrogenase required for 5-FU metabolism. This can lead to fatalities in patients treated with both 5-FU and sorivudine (Littler, 1994). Sorivudine has not been approved for use in the United States and Canada, because of the toxicity problems associated with the systemic administration of the drug.

1.1.2.4 5-Iodo-2'-deoxyuridine (Idoxuridine; IdUrd)

This was the first nucleoside analog licensed for clinical use for the treatment of herpes keratitis (Kaufman, 1962) and provided impetus to pursue research in this area. This drug was synthesized as a part of an anticancer program. Idoxuridine has a broad spectrum of antiviral activity (Babiuk et al., 1975; Prusoff and Ward, 1976; Prusoff, 1980). Inhibitory concentrations for HSV-1 in different cell lines vary from 0.28 to 0.66 µg/ml. The iodine moiety of IdUrd has a Van der Waals atomic radius of 2.15 Å, which is similar to that of the methyl group of thymidine (2.0 Å). Therefore IdUrd acts as a thymidine analog and can substitute readily for thymidine in DNA synthesis. Substitution of iodine at C-5 position of the pyrimidine moiety results in a more acidic dissociation constants for hydrogen atom at the N-3 position (Prusoff and Goz, 1975; Prusoff and Ward, 1976). At
pH 7.4, the proportion of the enolic form of IdUrd is greater than that of thymidine by a factor of 34. The antiviral mechanism of IdUrd is not well understood, but the phosphorylated derivatives interfere with the metabolic functions of various enzymes involved in DNA synthesis. The triphosphate of IdUrd (IdUrdTP) inhibits the DNA synthesis and is incorporated into viral and cellular DNA. Since at the physiologic pH the enolic form of IdUrd is far greater than the thymidine, altered DNA is more susceptible to strand breaks and incorrect genetic coding. In the presence of IdUrd, faulty transcription occurs and this in turn adversely affects the virion assembly.

**Untoward Effects.** Idoxuridine lacks selectivity. The compound is quite toxic to eukaryotic cells in cell cultures (Ayisi et al., 1980). Bone marrow depression, thrombocytopenia, alopecia and stomatitis are undesirable side effects reported on systemic administration of IdUrd, in patients (Kaufman, 1980). Preclinical studies have shown IdUrd to be mutagenic and teratogenic (Ito et al., 1975, De Clercq et al., 1978). Idoxuridine is available as a 0.1% solution for the treatment of epithelial infections caused by HSV-1. Adverse reactions include pain, pruritis, inflammation, or edema involving the eye (Kauffman, 1980).

**1.1.2.5 5-Ethyl-2'-deoxyuridine (EtdUrd)**

This deoxyuridine analog was synthesized by Swierkowski et al., (1969), and is a selective anti-herpes agent that is phosphorylated by herpes simplex virus encoded thymidine kinase (HSV-TK), but not by mammalian TK (Bernaerts and De Clercq, 1987). 5-Ethyl-2'-deoxyuridine has antiviral activity against several strains of HSV-1 and HSV-2.
(De Clercq and Shugar, 1975; Teh and Sacks, 1983) and is an effective non-mutagenic antiviral agent for the treatment of topical HSV infections (Swierkowski et al., 1969). It has been reported to increase the survival time of mice with HSV encephalitis (Davies et al., 1978) but is not approved for this use clinically. The nucleoside analog has some undesirable properties, including rapid elimination after i.v. administration, rapid enzymatic degradation to non-virostatic metabolites [5-ethyluracil and 5-(1-hydroxyethyl)uracil] (Buchelle et al., 1989) and low lipophilicity (Cheraghali et al., 1994) that may limit its passage across the blood-brain-barrier. Although studies in cell culture and mice have shown this compound to be nonimmunogenic and nonmutagenic (Shugar, 1964, Gauri et al., 1969), EtdUrd is fairly toxic to rapidly dividing cells (Ayisi et al., 1980). Recently, the cytotoxicity of EtdUrd was investigated by studying the incorporation of radiolabelled [U-14C]-L-leucine using seven human leukemia cell lines and human peripheral blood PHA-stimulated lymphocytes (Buchelle et al., 1989). Leukemia cells were more susceptible to EtdUrd (ED₅₀ 1.3-3.8 µM). Under similar assay conditions, the amount of EtdUrd required to inhibit PHA-stimulated lymphocytes growth was approximately 100-fold higher (ED₅₀ 130 µM to 380 µM). The toxicity of EtdUrd seemed to require active DNA synthesis, since the inhibition of leucine incorporation became obvious only after the first 24 hours in cultures.

1.1.2.6 Trifluorothymidine (trifluridine, TFT)

Trifluridine (5-trifluoromethyl-2'-deoxyuridine) is a fluorinated thymidine analog and was synthesized in early seventies as an anticancer drug (Heidelberger et al., 1964).
Trifluorothymidine is a potent inhibitor of herpes viruses (HSV-1, HSV-2, CMV) and vaccinia (ED₅₀ 0.05 to 0.6 µg/ml). Trifluridine is readily phosphorylated by cellular as well as HSV-induced thymidine kinase, to monohosphate (TFTMP). Trifluorothymidine monophosphate irreversibly inhibits thymidylate synthase, and the triphosphate (TFFTP) is a competitive inhibitor of thymidine triphosphate (dTTP) incorporation into DNA by cellular and viral DNA polymerases (Carmine et al., 1992). The incorporation of the TFTTP into the viral DNA results in faulty viral mRNA transcription leading to translation of proteins with altered morphology.

**Untoward Effects.** Trifluorothymidine lacks selectivity. The toxic manifestations are due to inhibition of enzyme thymidylate synthase and cellular DNA synthesis. Trifluorothymidine exhibits teratogenicity in a number of animal systems and is toxic to bone marrow and produces hematological abnormalities on systemic administration (Birch et al., 1992; Hiedelberger, 1975). In experiments using yeast, TFT was co-mutagenic without addition of an external metabolic activation system (Billimoria and Gupta, 1986). In the presence of activating S9-mix, the anti-recombinogenicity and co-mutagenicity was clearly demonstrated on exposure to TFT (Fahrig, 1996). The relative toxicity of antiviral agents approved for the treatment of herpes keratitis was investigated using monolayers of confluent rabbit corneal epithelial cell cultures (Birch et al., 1992). Significant dose, but not time-dependent, toxicity was observed at the clinical concentrations of IdUrd, TFT, and EtdUrd. In contrast, BrVdUrd did not exhibit toxicity. Trifluorothymidine (TFT) and IdUrd were the most toxic, and EtdUrd was of intermediate
toxicity. The results of this in vitro study are in agreement with the findings of previous in vivo corneal epithelial toxicity studies using TFT (Carmine et al., 1992).

1.1.2.7 5-Methoxymethyl-2'-deoxyuridine (MMdUrd)

This nucleoside analog was synthesized as part of a program to develop selective antiviral compounds in this laboratory (Bubbar and Gupta, 1970). This nucleoside analog is a potent and selective inhibitor of HSV-1 replication and has moderate activity against HSV-2 in cell cultures (Babiuk et al., 1975; Meldrum et al., 1980; Ayisi et al., 1983). When MMdUrd was used in combination with other antiviral agents, namely, Ara-A, TFT or foscarnet (PFA) synergistic activity was observed against HSV-1 and HSV-2 in cell cultures (Ayisi et al., 1983 and 1986). These studies were of considerable importance because they laid the foundation for the use of combination chemotherapy for the treatment of viral infections. 5-Methoxymethyl-2'-deoxyuridine was found to be effective when used alone or in combination with Ara-A in the treatment of herpes keratitis ((Meldrum et al., 1980) and genital herpes (Ayisi et al., 1983, 1985 and 1986).

Untoward Effects. Pre-clinical toxicology studies using mice and hamsters indicate that MMdUrd has low mammalian toxicity, devoid of embryotoxicity or teratogenicity in mice and mutagenicity in cell cultures (Meldrum et al., 1980; Aysi et al., 1983; Billimoria and Gupta, 1986). The nucleoside analog is preferentially phosphorylated by viral-induced thymidine kinase to the monophosphate (MMdUMP), which is further converted to the triphosphate (MMdUTP), the active form of the drug (Gupta et al., 1987). The active form of the drug, MMdUTP inhibits the viral DNA polymerase and is
also readily incorporated into the viral DNA (Aduma et al., 1992). Preferential phosphorylation of MMdUrd by HSV-induced kinase is the primary reason for the lack of host toxicity.

1.1.2.8 (E)-5-(2-bromovinyl)-2'-deoxyuridine (BrVdUrd)

(E)-5-(2-bromovinyl)-2'-deoxyuridine (BrVdUrd), and (E)-5-(2-iodovinyl) 2'-deoxyuridine (IVdUrd), are the most potent and selective inhibitor of HSV-1 (De Clercq et al., 1980). The model of gastrointestinal herpetic infection was used recently to compare the efficacy of selective antiherpes agents (Kodama et al., 1996). The inhibition of HSV-induced cytopathogenicity in gastric adenocarcinoma MKN-28 cells was monitored by MTT assay. From the various compounds that were evaluated for their activity against HSV-1 and HSV-2, BrVdUrd emerged as the most effective inhibitor of HSV-1 replication. The selectivity of BrVdUrd and IVdUrd toward HSV-1 and HSV-2 is largely dependent on their phosphorylation by the virus-induced deoxythymidine (Thd) kinase. The mechanism of action of BrVdUrd has been recently investigated using a 14C-radiolabeled compound (Ciucci et al., 1997). The [14C]BrVdUrd accumulated in greater quantities in HSV infected than in uninfected cells; and in infected cells it was phosphorylated intracellularly to its mono (BrVdUMP) and triphosphate (BrVdUTP) forms. Bromovinyl deoxyuridine triphosphate is a competitive inhibitor of viral DNA polymerase.

Untoward Effects. Preclinical studies have shown that BrVdUrd is non toxic to dividing cells and is devoid of any major mammalian toxicity (De Clercq et al., 1981) but
is a potential human clastogen (Oshiro et al., 1992). Catabolism of BVDU by thymidine phosphorylase to inactive bromovinyluracil reduces its antiviral potency (Desgranges et al., 1983).

1.1.2.9 9-β-D-Ribofuranosyladenine (Vidarabine, Ara-A)

9-β-D-Ribofuranosyladenine (Ara-A) is an adenosine analog with cytostatic and antiviral properties (Whitley et al., 1980). This compound is a moderate inhibitor of replication of herpes simplex virus, poxvirus, rhabdovirus, hepatoadenovirus and pseudorabies virus. The ED50 values in different cell lines vary from 1 to 3.0 µg/ml (Whitley et al., 1980). Thus Ara-A and its metabolite arabinofurosyl hypoxanthine inhibit viral DNA synthesis (Drach and Shipman, 1977; Whitley et al., 1980). Ara-A is phosphorylated by the cellular enzymes to the triphosphate (Ara-ATP), which is incorporated into both cellular and viral DNA and act as a chain terminator (Muller et al., 1977). The triphosphate of Ara-A (Ara-ATP) also acts as a competitive inhibitor of deoxyadenosine triphosphate for the cellular and viral DNA polymerase activity (Moore and Cohen, 1967).

Untoward Effects. Ara-A is cytotoxic, teratogenic to mice and rabbits and induces chromosomal breaks (Ayisi et al., 1980; Scharidin et al., 1977). Intravenous administration of vidarabine causes dose-related gastrointestinal toxicity, hypokalemia, anemia, leukopenia, thrombocytopenia, acute neurotoxicities and a syndrome of inappropriate antidiuretic hormone. Acute neurotoxicities including tremor and alterations in behavior have been reported in animals (Szczech and Tucker, 1992). Chronic Ara-A use is also associated with painful peripheral neuropathy.
1.1.2.10 5-Substituted analogs of deoxycytidines

The subject matter of this thesis deals with deoxycytidine analogs. Therefore, discussion on the topic is given in section 1.1.4.

1.1.2.11 Acyclic Nucleoside Phosphonates (ANPs)

Acyclic Nucleoside Phosphonates are broad-spectrum antiviral compounds, with very potent and selective antiviral activity in vitro and in vivo. The prototype compounds of most therapeutic interest are:) (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl cytosine (HPMC, Cidofovir), 9-(2-phosphonylmethoxyethyl) adenine (PMEA, adefovir) and (R)-9-(2 phosphonylmethoxypropyl) adenine (PMPA). These compounds were synthesized to circumvent the first phosphorylation step that is necessary for the 'activation' of nucleoside analogs such as acyclovir, ganciclovir and 5-substituted pyrimidines (MMdUrd, BrVdUrd). The lack of conversion of nucleoside analogs to monophosphosphate by HSV-induced viral kinase is an important mechanism responsible for the development resistance to these compounds (Naesens et al., 1997). These drugs do not inhibit virus strains, in which the phosphorylating enzymes are lacking or altered for example thymidine deficient (TK-) mutants.

The uptake of acyclic nucleoside phosphonates into the cells most likely occurs by an endocytosis-like process (Naesens et al., 1997). Once inside the cells the ANPs are activated by cellular enzymes to their active diposphoryl derivatives (Ho et al., 1992).

The antiviral action of the ANP analogs is based on a specific interaction of the active diposphorylated metabolite with viral DNA. These derivatives interact with viral DNA
polymerase either as competitive inhibitors with respect to natural substrates or as alternate substrates. Acyclic nucleoside phosphonates lacking a hydroxyl function on the acyclic side chain can act as chain terminators after incorporation into the DNA. The long intracellular half lives of the active diphosphoryl metabolites accounts for the prolonged antiviral effects seen in vitro and in vivo. The potential of HPMC as a broad-spectrum anti-DNA virus agent has been confirmed in clinical trials. Renal excretion is the primary route of excretion of ANPs with 70-90% of unchanged drug being recovered in the urine within 24 h after intravenous administration in mice or rhesus monkeys (Matsuo et al., 1998; Schwarz and Perez, 1998). Metabolism plays a very minor role in the clearance of these drugs. Apart from their mono and diphosphate forms, and the choline adduct HPMPCp-choline in the case of HPMPC, no other metabolite was found in tissues. More interesting is the fact that no signs of deamination at the nucleobase level could be found which would have resulted in antivirally inactive, HPMP-uracil in the case of HPMPC, or PME-hypoxanthine in the case of PMEA. The FDA has licensed both Cidofovir and Aciclovir for use as antiviral in 1997.

Untoward effects. Preclinical toxicity testing of HPMC in various animal species, mice, rats, guinea pigs, and monkeys has been carried out. This compound was found to produce nephrotoxicity in all species. Guinea pigs were found to be the most susceptible to the nephrotoxicity of HPMC and severe kidney damage was observed at doses greater than 5 mg/kg/day (Matsuo et al., 1998; Schwarz and Perez, 1998). The nephrotoxicity was manifested as acute nephritis with diffuse necrosis of the proximal tubular
epithelium. The clinical trial of HPMC in CMV-infected humans also produced dose-limiting nephrotoxicity at therapeutic doses (Snoeck et al., 1994). The rapid and active uptake of HPMC at the basolateral membrane of the proximal tubule, compared to the slow efflux of HPMC at the luminal side, results in accumulation of HPMPC (or its HPMPCp-choline metabolite) in the renal tubules and causes toxicity (Polis et al., 1995). The renal accumulation can be prevented by intravenous hydration, by decreasing the frequency of dosing and or by the administration of probencid, an inhibitor of organic ion transport, that interferes with the transporter-mediated tubular uptake of HPMPC (Cundy et al., 1996). The cyclic ester derivative (cHPMPC) has substantially lower nephrotoxicity. Once inside the cell, this ester derivative is converted to HPMPC, by cyclic CMP phosphodiesterase (Mendel et al., 1996).

Preclinical studies of PMEA in mice, cats and rhesus monkeys revealed severe bone marrow depression and dose limiting anemia (Tsai et al., 1995). Interestingly, the closely related analog PMPA showed no hematological toxicity and blood chemistry parameters were unchanged during treatment. Both compounds did not produce nephrotoxicity in experimental animals. Reproductive studies of PMEA in mice resulted in resorption of pregnancy, destruction of the thymus in the neonates and considerable death of the neonates (Mendel et al., 1996).

1.1.3. Antiviral Agents other than Nucleoside Analogs

1.1.3.1 Phosphonoformate (Foscarnet, PFA) and Phosphonoacetic acid (PAA)

Foscarnet and phosphoacetic acid are inorganic pyrophosphate analogs. These compounds
are moderate inhibitors of herpes viruses and retroviruses including HIV. The antiviral activity of PFA is due to specific inhibitory effects on viral induced DNA polymerase (Leinbach et al., 1976; Chrisp and Clissold, 1991). The affinity of PAA for herpes virus DNA polymerase is 100-fold greater than for cellular DNA polymerase. At physiologic pH, foscarnet is used as the trisodium salt and is approved for the treatment of CMV retinitis in AIDS patients.

**Untoward effects.** Foscarnet and PAA accumulate in bones and can produce symptomatic hypocalcemia (Schaeffer et al., 1978). Other toxic effects of PFA include nephrotoxicity characterized by acute tubular necrosis, crystalluria, and interstitial nephritis (Schwarz and Perez, 1998); central nervous system side effects; fever, nausea; hematological abnormalities including leukopenia; abnormal liver function tests, and painful ulceration of genitalia (Oberg, 1989). Preclinical studies in mice produced neurotoxicity at high concentrations (Lalezari, 1997; Matsuo et al., 1998).

### 1.1.3.2 Polyanionic compounds

The first event of virus cell interaction involves attachment and adsorption of viral particles to specific receptors on the cell. Penetration of the virus and synthesis of viral nucleic acid follows. If the viral attachment to cell receptors can be prevented, it can be used to therapeutic advantage. The adsorption of enveloped viruses like HSV, can be inhibited by polyanionic compounds such as dextran sulfate and poly-L-lysines (Mitsuya et al., 1988; Baba et al., 1990; Hosoya et al., 1991; Yang and Yang, 1997). Dextran sulphate was found to have lower ED$_{50}$ and CD$_{50}$ compared to poly-L-lysine in cell
Biological membranes contain charged particles like lipids and proteins, which produce an electrical potential and ionic double layer in the aqueous phase adjacent to the membrane. The adsorption of Polyanionic compounds to the cell membranes is likely to cause electrostatic and steric hindrance effects close to the cell membranes, which in turn affects, the adsorption of virions to the cell receptors. This effect in membrane potential is reflected by the difference in zeta potential of cellular membranes treated with these compounds (Yang and Yang, 1997).

1.1.3.3 Interferons

Interferons are a group of inducible cellular glycoproteins that interact with cells and render them resistant to infection by a wide variety of RNA-and DNA-containing viruses (Baron et al., 1992). In addition, interferons have numerous other effects on target cells, including a reduction in the rate of cell proliferation and alterations in the structure and function of the cell surface, the distribution of cytoskeletal elements, and the expression of several differentiated cellular functions (Hayden, 1995). Interferons induce the synthesis of new proteins that are responsible for the activation of cellular endonucleases that degrade viral mRNA (Sen and Ranshoff, 1993). Three major classes of naturally occurring interferons with antiviral activity are alpha, beta and gamma interferons. The clinically used interferon for antiviral action is the alpha form. The antiviral effects of these biological mediators are mediated through inhibition of viral penetration or uncoating, synthesis of messenger RNA, translation of viral proteins, and or viral assembly.
and release.

**Untoward Effects.** Interferon therapy has been shown to cause severe bone marrow suppression with granulocytopenia and thrombocytopenia in patients. Other side effects reported are: neurotoxicity, thyroid dysfunction, cardiotoxicity, elevations in liver enzymes, alopecia, proteinuria, and azotemia.

1.1.4 5-Substituted analogs of deoxycytidines

Prior to systemic investigations initiated in Dr. Gupta’s laboratory on 5-substituted deoxycytidine analogs as selective antiherpes agents, there was only cursory interest in this area. The information available on work carried out on substituted deoxycytidine compounds is therefore summarised under two sections. The background section summarizes earlier work (1975-1982) prior to initiation of studies in this laboratory. This is followed by a section summarizing studies carried out in this laboratory.

**Background**

Greer and his colleagues have shown that 5-halogenated analogs of deoxycytidine (dCyd) are more selective inhibitors of HSV than the corresponding analogs of deoxyuridine (dUrd) [Greer and Schildkrut, 1975; Fox et al., 1983]. The basis of selectivity is that halogenated deoxycytidines are selectively phosphorylated by the pyrimidine nucleoside kinase of HSV (Dobersen and Greer, 1978) and VZV (Jerkofsky et al., 1977) but are poor substrates for mammalian dCyd kinase and dThd kinase (Cooper and Geer, 1973).

Antiviral and antimetabolic properties of various 5-substituted-2'-deoxycytidine analogs were investigated in Professor De Clercq’s laboratory (De Clercq et al., 1981). These
authors also concluded that 5-substituted dCyd analogs were more selective inhibitors of HSV as compared to their corresponding dUrd compounds. The rationale proposed for selectivity was that 5-substituted dCyd monophosphates serve as substrates for virus-induced deoxycytidylate (dCMP) deaminase and their metabolism in virus-infected cells utilizes at least two HSV-1 induced enzymes, dThd/dCyd kinase and dCMP deaminase. Thus anabolism of these compounds could occur in HSV-infected cells by one of the following routes:

\[
5-X-dCyd \rightarrow 5-X-dCMP \rightarrow 5-X-dUMP \quad (i)
\]

OR

\[
5-X-dCyd \rightarrow 5-X-dUrd \rightarrow 5-X-dUMP \quad (ii)
\]

The resulting 5-X-dUMP metabolites are then converted to their corresponding 5'-triphosphates by cellular kinases and eventually incorporated into viral DNA. *This concept implies that nucleotide level deamination is essential for antiherpes activity.*

**Recent studies on 5-substituted dCyd analogs**

Approximately ten years back, Dr. Gupta and his colleagues hypothesized that administration of dCyd analogs endowed with antiherpes activity in combination with deaminase inhibitors should result in increased efficacy against HSV infections as compared to their corresponding deoxyuridine compounds and research program on the synthesis and study of biological properties of dCyd analogs was initiated.

These investigations led to the synthesis of a new antimetabolite 5-methoxymethyl-2'-deoxycytidine (MMdCyd) and served as prototype for investigations in support of this hypothesis (Aduma *et al.*, 1990a, 1992; Gupta *et al.*, 1991, 1992;
Aduma and Gupta, 1995). The salient points for the hypothesis were:

(i) MMdCyd co-administrated with a deaminase inhibitor (tetrahydrodeoxyuridine, H₄dUrd) will be anabolized exclusively through virus induced deoxycytidine-deoxycytidylate (dCyd/dCMP) kinase pathway in infected cells to its corresponding triphosphates which will inhibit HSV-induced DNA polymerase by competing with deoxycytidine triphosphate (dCTP) (Chart 1.1).

(ii) MMdCyd when maintained in the deoxycytidine form would be metabolically stable because dCyd and its analogs are not substrates of pyrimidine nucleoside phosphorylases.

(iii) dCyd analogs should also achieve better concentration in the CNS compared to their corresponding deoxyuridines because of their greater lipid solubility. This could be of considerable benefit particularly in the treatment of herpes encephalitis.

(iv) MMdCyd will lead to decreased formation of dCTP pools (competition with dCTP) and co-administration with a deaminase inhibitor will prevent synthesis of thymine nucleotides. This will lead to diminished deoxythymidine triphosphate (dTTP) pools. The limited supply of two pyrimidine nucleotide triphosphates (dCTP and dTTP) would seriously impair virus replication because of decreased synthesis of viral genome.

(v) 5-methoxymethyl-2'-deoxycytidine (MMdCyd) may also have desirable effect in preventing latency or emergence of HSV from latency in recurrent infections. This is based on the premise that viral DNA containing MMdCyd residues would behave like a hypermethylated DNA and therefore will be non-functional.

In this respect, the mode of action of deoxycytidine analogs is uniquely different from
presently available antiherpes drugs. Studies by my predecessors Drs Aduma and Zoghaib in this laboratory on MMdCyd and BrVdCyd have provided evidence in support of this hypothesis (Aduma et al., 1990a, 1990b and 1991; Gupta et al., 1991 and 1992; Zoghaib, 1996).
Chart 1.1 Proposed Pathways for metabolism of 5-substituted-2'-deoxycytidines (5XdCyd) in HSV-1 infected cells. Note: X= methoxymethyl or bromovinyl.

1: dThd/dCyd kinase (virus-induced);
2: dCyd deaminase;
3: dCMP deaminase;
4: dThd and or Urd phosphorylase;
5: dTMP synthase;
6: DNA Polymerase (virus-induced). H₄Urd (tetrahydrouridine) inhibits cytidine deaminase. H₄Urd (tetrahydrodeoxyuridine) inhibits both cytidine deaminase and deoxyctydylate deminase.

dCyd (deoxycytidine); dThd (deoxythymidine); dCMP (deoxycytidine monophosphate); dCDP (deoxycytidine diphosphate); and dCTP (deoxycytidine triphosphate); dUrd (deoxyuridine).
2. SCOPE OF THE PROBLEM

2.1 Herpes Virus Infection

The infections due to herpes viruses are among the oldest and the most common infections in humans. Herpes viruses are also source of number of infections in animals causing widespread morbidity and mortality. The members of the pathogenic viruses responsible for infection in humans include Herpes simplex virus (HSV) types 1 and 2, Varicella zoster virus (VZV), Cytomegalo virus (CMV) and the Epstein-Barr virus (EBV). One of the characteristics shared by members of herpes viruses is their tendency to become latent with periodic reactivation (recurrent infection). Primary infection is generally more severe than the recurrent infection. Immunocompromised hosts and the new born are particularly susceptible to herpes virus infection with varying degree of severity from discomfort (recurring labialis and genital herpes due to HSV and systemic CMV infection) to life-threatening diseases (HSV encephalitis and CMV retinitis). Herpes virus infections of humans have not proven amenable to control by immunization.

2.2 Rationale for Proposed Investigations.

In spite of the recent advances for the chemotherapy of herpes infections, there is a definite need of new drugs to treat HSV-associated diseases for the following reasons:

(i) Deoxyuridine analogs (BrVdUrd and EtdUrd) though potent antiherpes agents are metabolically unstable, and poorly cross the blood brain barrier. Thus these compounds are of limited use in the treatment of systemic HSV and VZV infections.

(ii) Currently approved drugs acyclovir (ACV), arabinosyl adenine (Ara-A), Cidofovir
(HPMPC) and Adeovir (PEMPA) are not effective in preventing latency or emergence of HSV from latency (recurrent HSV-infection).

(iii) Poor aqueous solubility and drug related host toxicities are other limitations of drugs currently approved for the treatment of HSV infections.

2.2.1 Deaminase-resistant deoxycytidines:

A major drawback for the therapeutic use of cytidine compounds is their tendency to undergo deamination in the presence of deaminating enzymes. These enzymes are usually present in blood and mammalian cells and catalyze the deamination of cytidine compounds to the corresponding uridine analogs, which are either less active (Camiener and Smith, 1965) or do not display selectivity towards HSV-infected cells (Fox et al., 1983). Deoxycytidines resistant to deamination will retain selectivity and more importantly would have the advantage of simplifying treatment regimens. Resistance to deamination can be attained by structural modifications of the molecule (Wang et al., 1973; Mancini and 1983; Chu et al., 1990).

In order to accomplish this goal, a systemic study was undertaken to correlate molecule structure of MMdCyd and BrVdCyd analogs and antiherpes activity. Based on conformational studies, it was concluded that the orientation of the N^4-substituent was critical for the activity (Gupta et al., 1992; Zoghaib, 1996). From these investigations it was concluded that amide derivatives (N^4-acyl analogs) are likely to be the most useful compounds because these compounds had the correct orientation of the N^4-substituent (proximal) to the 5-substituent on the pyrimidine ring. These conclusions were reached
based on the preliminary data on biological activity of compounds synthesised by Dr. Zoghaib (Zoghaib, 1996).

2.3 GOALS AND OBJECTIVES

Antiviral activity of MMdCyd, amides of MMdCyd and other N\(^4\)-substituted MMdCyd compounds against HSV-1 was determined by different assay methods. Acyclovir and BrVdCyd were used as control drugs. Cytotoxicity against VERO cells and lymphoblast cell lines, A-549 and CEM cells was investigated. Potential of delayed cytotoxicity was studied using CEM cells. Toxicity studies on butanoyl-MMdCyd (But-MMdCyd) the most active compound of this series were carried out in male CD-1 mice. Results of these studies are the subject matter of this thesis.
3. ANTIVIRAL ACTIVITY AND CYTOTOXICITY

Introduction

Several assay methods have been developed to determine the antiviral activity of inhibitors of HSV replication. The antiviral activity and cytotoxicity of antiherpes compounds has been shown to vary considerably depending on the cell line (different cell lines contain varying levels of nucleoside and nucleotide metabolizing enzyme) and the assay method used (De Clercq, 1981; Harmenberg et al., 1982; Aduma, 1989; Whalley et al., 1991). The merits and limitations of three assay methods currently used by researchers to evaluate activity of HSV inhibitors in situ are described below:

3.1 ANTIVIRAL ASSAYS

3.1.1 Cytopathic inhibition assay (MTT assay)

Herpes simplex virus replication results in lysis and cell death. Compounds capable of inhibiting the replication of HSV have been shown to protect the cells from the damage due to HSV (Inhibition of HSV-induced cell death and lysis). The viability of the cells can be determined by MTT assay or trypan blue exclusion method (Whalley et al., 1991; Shi, 1999).

(i) MTT assay: This assay method was originally developed for screening cytotoxic drugs. The spectrophotometric assay determines the cytopathic effects of infection on target cell metabolism and the assay method is based on the conversion of yellow coloured tertrazolium salt (MTT) by mitochondrial dehydrogenase of metabolically active cells to dark blue formazan product, which can be measured spectrophotometrically. The
cytopathic inhibition assay has been used by some investigators for the evaluation of HSV inhibitors because it is rapid and convenient for large-scale screening.

However, MTT assay can give variable results because the extent of cell damage is not identical in the presence of different strains of HSV. The major limitation of this assay method is that it can not detect the exact state of damage to cells caused by the virus. For example, cells, which are not dead but inhibited can give false results because the enzyme machinery of the cell is still viable (Shi, 1999).

(ii) Trypan blue-exclusion method: This assay is better than MTT method because the number of dead cells can be determined accurately. However, it is labour intensive, slow and therefore not suitable for the screening of large number of compounds.

3.1.2 Plaque reduction assay

This assay method is most valuable for assessing the activity of HSV inhibitors because it provides quantitative data. This opinion was supported by the FDA, which accepted that the gold standard measurement of the potency of an antiviral inhibitor, is an assay that measures virus production directly (De Clercq, 1981; Jones, 1998, Qui et al., 1997 and 1998). In this assay, cells grown to confluency are infected with virus and incubated for a short period to allow virus adsorption. After virus adsorption, known concentrations of the test compounds are added and plaques allowed to develop for 72-96 hours. The plaques are enumerated after magnification using a dissecting microscope. The percentage of inhibition (calculated from reduction in the number of plaques) at each concentration of the compound is determined. From the dose-response curves, the
concentration required to reduce the number of plaques by 50% (ED$_{50}$) is determined (Meldrum, 1978; Deluge et al., 1998). This assay is better than the cytopathic inhibition assay, because inhibition of the virus infectivity by compounds can be determined more accurately. However, it is labour intensive and slow.

### 3.1.3 Virus yield reduction assay

An alternate method of measuring the antiviral activity of compounds is a virus yield reduction assay (Ayisi et al., 1980, Aduma et al., 1990a; Qui et al., 1998). In these experiments, cells are exposed to virus so that all or a known proportion of them are infected. The infected cell monolayers are exposed to various concentrations of an antiviral compound. At periodic intervals, the infected cells are harvested, and the amount of virus produced is determined by virus titration (De Clercq, 1987; Sidwell and Huffman, 1971). These assay method enables the measurement of infectious virions produced in the media as well as the amount of virus present intracellularly. This assay can be used to determine the virucidal effect of a compound.

### 3.2 MATERIALS AND METHODS

**Tissue Culture Supplies, Cell Lines and Virus Strains**

Sterile plastic-tissue culture flasks, microtitre trays, test tubes and petri dishes were purchased from VWR Canlab, Ontario, Canada. Cell culture medium, RPMI 1640, RPMI 1640 DM (Dutch modification), Dulbecco’s Modified Eagle Media (DMEM), fetal bovine serum (FBS), L-glutamine, sodium carbonate, penicillin-streptomycin, fungizone and agarose powder were obtained from GIBCO, Burlington, Canada. Trypsin and versene
sterile solutions were obtained from the Department of Microbiology, University of Saskatchewan.

The cell lines used were: A-549 (Human lung cancer cells) and VERO Cells (African green monkey kidney cells). Herpes simplex virus type 1 (HSV-1) McIntyre strain was used. The stock cultures of A-54, VERO cells and virus strain were kindly provided by Mrs. J. Heis, Department of Microbiology, College of Medicine, University of Saskatchewan. Both cell lines were periodically tested for mycoplasma contamination by the Department of Veterinary Microbiology and were found free of mycoplasma.

Cell cultures

The cells were cultured in a 1:10 diluted Eagle's Minimum Essential Medium (MEM). Each litre of medium was supplemented with 2 mmol of L-glutamine, 10 ml of nonessential amino-acids, 54 ml of 4.4% sodium bicarbonate, 2.5 µg/ml fungizone, 100 µg/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). Cells were grown and maintained in sterile 75 cm² flasks. The cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere for two days. The monolayers were trypsinized by removing the medium from the flask and adding 2 ml of trypsin-versene to the flask (Schmidt, 1964; Meldrum, 1978). The flask was then incubated (Forma Scientific, Model 3326, Marietta, OH) at 37°C in a humidified 5% CO₂ atmosphere for 10 minutes and one fifth of the cells were transferred to another flask weekly or more often for propagation of cells.
Virus stocks

The virus was propagated and virus stock was prepared using a similar methodology described previously (Babiuk et al., 1975). VERO cells were grown to confluent monolayers in 75 cm² flasks. Cells seeded in the flask were from the same batch. After 24 hours, cells from one flask were trypsinized and counted using a hemocytometer (Coulter Electronics. Inc., Hialeah, FL) to give an estimate of cell concentration in the flask. Each confluent monolayer in the flask contained approximately 3 x 10⁷ cells. The media in the flasks was aspirated and 1.5 ml of virus inoculum in MEM (no serum) containing 10⁵ plaque-forming units (PFU) was added (Aduma, 1989). The stock virus at this concentration would cause low multiplicity of infection (0.01 PFU/cell). The flasks were incubated for one hour at 37°C and 5% CO₂ to allow for virus adsorption, unadsorbed virus was removed by washing with sterile phosphate buffered saline solution (FBS, 0.05 M, and pH 7.4). The media containing 10% heat inactivated FBS was added and incubated for 48 hours. At the end of incubation period, cells were examined for cytopathic effect (cell lysis and death). If the infection was not 100%, the media was removed and centrifuged at 1000 rpm at 5°C, for 5 minutes to pellet detached cells. The supernatant was removed, the cell pellet was resuspended in 20 ml of fresh MEM and overlaid on cells in the same flask. Additional media with 10% FBS was added and incubated for 48 hours until infection was 100% complete. At the end of this period, the cell culture fluid and cell debris were collected in a 30 ml sealed tube and subjected to four 30 second pulses of sonar boom lyse cells and release intracellular virus. The fluid
and the debris were centrifuged at 1000 rpm at 5°C for 7 minutes, the supernatant dispensed into vials (2 ml/vial) and stored at -70°C. Titre of stock virus was determined using the procedure of Reed and Muench (1938). Briefly, quadruplicate wells containing monolayers of VERO cells (2 x 10^5 cells/well) were inoculated with 50 µl of serial ten fold dilutions of the virus and allowed to adsorb for one hour at 37°C and 5% CO₂ in a humidified atmosphere. Unadsorbed virus was removed, 150 µl of MEM was added and the cultures were incubated for 72 hours at 37°C. The culture medium was removed. Cells were fixed and stained with 1% gentian violet in 70% ethanol and each well was examined for presence of viral cytopathogenic effect (CPE). The viral titre was computed using the method of Reed and Muench (1938).

**Drugs and chemicals**

5-Methoxymethyl-2'-deoxycytidine (MMdCyd, M. wt. 271) and amide derivatives, namely, 5-acetyl-MMdCyd (Ac-MMdCyd, M. wt. 313), 5-Propanoyl-MMdCyd (Prop-MMdCyd, M. wt. 327), 5-butanoyl-MMdCyd (But-MMdCyd, M. wt. 341) and 5-hexanoyl-MMdCyd (Hex-MMdCyd, M. wt. 368) were synthesised by Dr. Sashi Kumar in our laboratory using published procedures (Gupta *et al.*, 1991; Zoghaib, 1996). Structures of compounds are shown in chart 3.1. 5-bromovinyl-2'-deoxycytidine (BrVdCyd, M. wt. 332) and alkyl derivatives of MMdCyd, namely, N^4^-methyl-MMdCyd (Me-MMdCyd, M. wt. 285) and N^4^-phenyl-MMdCyd (Phe-MMdCyd, M. wt. 348) were also synthesised in our laboratory using published procedures (Zoghaib, 1996). Tetrahydrodeoxyuridine (H₄dUrd, M. wt. 234) was obtained from Terochem Laboratories,
Edmonton, Alberta. Acyclovir (ACV, M. wt. 225) was obtained from Sigma, Oakville, Ontario. The purity of all compounds was at least 95% and generally higher 98 – 99 % based on HPLC profiles.

For antiviral assays, stock solution of each compound (12.5 mg/ml) was prepared using PBS (0.05 M, pH 7.4). The sterile PBS solution was obtained from the Department of Veterinary Microbiology. The concentration of the stock solution of each compound was determined spectrophotometrically using Beckman DU-65 spectrophotometer. The stock solution was divided into aliquots, and used immediately or stored frozen at -20°C. Appropriate dilutions of the stock solutions were prepared in PBS immediately prior to antiviral assays.

3.3 ANTIVIRAL ASSAYS

3.3.1 Plaque reduction assay

Plaque reduction assays were carried out using published procedures (Babiuk et al., 1975; Ayisi et al., 1980; De Clercq et al., 1981; Aduma, 1989; Qui et al., 1998). VERO and A 549 cells were grown to confluency by seeding 5 x 10⁴ cells in 2 ml of media into each well of a 24 well microtitre plates. The monolayers were infected with 50 PFU of HSV-1. The virus was allowed to adsorb for one hour at 37°C and 5% CO₂ in a humidified incubator. Unadsorbed virus was removed by washing with sterile PBS (2 x 2 ml).

Graded concentrations of test compounds (2 x desired concentration) in MEM containing 10% FBS and antibiotics (1 % of penicillin, streptomycin and fungizone) were added to an equal volume of 2% agarose in MEM. The mixture was cooled (= 40-42°C) and
layered over the infected cell monolayer and allowed to harden. Culture plates were incubated at $37^\circ C$ in a humidified 5% CO$_2$ atmosphere for 72 hours. At the end of this period, the culture fluid was carefully removed, the monolayer were fixed with alcohol, stained with 1% crystal violet in 70% alcohol, air-dried and examined for virus plaques. The plaques were enumerated after magnification using a dissecting microscope (Olympus 40 x magnification). In each experiment, toxicity controls (cells containing the test compound and media), cell controls (media only), and virus control (cells containing virus and media) were run concurrently. The percentage of inhibition (calculated from reduction in the number of plaques) at each concentration of the test compound was determined. The data was used to construct dose-response curves for each compound. From dose response curves, the concentration required to reduce the number of plaques by 50% (ED$_{50}$) was determined. Each assay was run in quadruplicate and the experiment was repeated three times.

3.3.2 Virus yield reduction assay

The method used for evaluation of anti-HSV activity was according to recommendations of Ayisi et al., (1983) and Aduma (1989). Confluent monolayers of VERO cells in microtitre plates were infected with 10 PFU of HSV-1 and incubated in humidified 5% CO$_2$ atmosphere at $37^\circ C$. After infection, the monolayers were washed with sterile PBS (2 x 5 ml, 0.05M, pH 7.4) to remove the unadsorbed virus and immediately overlaid with graded concentrations of test compounds, MMdCyd or But-MMdCyd. Plates were incubated for 72 hours of at $37^\circ C$ in a 5% CO$_2$ humidified atmosphere. At the end of the
incubation period the percentage inhibition (reduction in infectivity) compared with 100%, infectivity (virus controls) on VERO cell monolayers was determined. The measures used for evaluation of infectivity were: rounding of cells, plaque formation, and the loss of monolayer structure. After this subjective score, the supernatant (extracellular fluid) from samples, that had received similar treatment, were pooled and 2 ml was used for the titration of the virus using VERO cells to determine the amount of virus present for each treatment (section 3.2). The remainder of the extracellular fluid was stored at -70°C.

The ability of virus present intracellularly to resume replication after the removal of test compounds was also determined. The monolayers in each well were washed with PBS (2 x 5 ml) to remove residual compound, metabolites and degradation products. Cells were overlaid with 0.2 ml of growth media (MEM supplemented with 10% heat inactivated FBS) and plates were further incubated for 72 hours at 37°C in a 5% CO₂ humidified atmosphere. The virus infectivity was scored after pooling of supernatant from samples, which had received similar treatment.

Total virus was harvested by two cycles of freezing (-70°C) and thawing (22°C) of cells plus supernatant. The fluid from each treatment group was pooled together and virus yield determined by titration on VERO monolayer as described previously. Virus control cultures (virus plus medium), and drug control cultures (drug plus medium) were tested concurrently.

The assay for each treatment was carried out in quadruplicate. If a compound is virucidal,
the treatment should result in inhibition of infectious virus particles, even after the removal of the compound from the media.

3.4 Cytotoxicity

Cytotoxicity is an important parameter to determine the selectivity of compounds. Therefore, these studies are an integral part of antiviral assays. Several methods have been used by investigators to determine cytotoxicity of compounds (Ayisi et al., 1983; Turk et al., 1987; Aduma, 1989; Daluge et al., 1997; Qui et al., 1998). Two different assays were used to determine cytotoxicity of compounds. Initially, the cytotoxicity of test the compound was determined along with drug inhibition assays of viral replication. In each antiviral assay, toxicity controls were run concurrently and the cytotoxicity of each compound was assessed microscopically by comparing the toxicity control culture (compound plus media) with the cell control cultures (media only) and the viral control cultures (virus plus media). Drug control cultures were scored as follows: increased granularity (1+), slight vacuolation (2+), large holes in the monolayer (3+) and destruction of the monolayer structure as (4+).

If no toxic manifestation was seen at the highest concentration of the test compound, the effects of higher concentrations on cell growth was evaluated using VERO cells (Ayisi et al., 1983; Turk et al., 1987; Qiu et al., 1998). Twenty-four hours prior to assay, VERO cells were seeded in five six-well plates (1045, Fisher Scientific) at a concentration of 2 x 10^4 cells per well in MEM containing 10% FBS (heat inactivated). On the day of the assay, test compounds were diluted serially in MEM containing 10% FBS at
concentrations of 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml and 1000 µg/ml. The media from the wells was then aspirated, and 2 ml of each drug concentration was added to each well. To the control wells (culture media only) 2 ml of growth media was added. The plates were incubated in a humidified CO₂ (5%) atmosphere at 37 °C for 72 hours. At the end of the incubation period, the media was removed, and monolayers were washed with PBS (2 x 5 ml). Trypsin-versene (1%, 1 ml) was added to each well and plates were incubated for 10 minutes to break monolayers and detach cells from plates. The cell-media mixture was then pipeted up and down vigorously to break up the cell suspension and 0.2 ml of the mixture added to 9.8 ml of Isoton III (Fisher Scientific, St. Louis, MO) and counted using Coulter counter (model Z1 Coulter electronics Ltd, Burlington, Ontario, Canada). Each sample was counted three times with six replicates per sample. The data was analyzed using one way ANOVA using Sigmastat computer program. The statistics were performed on the raw data, comparing cell control versus treatment groups with P <0.05 used as the level of significance.
Chart 3.1: Structures of 5-substituted-2'-deoxycytidines and acyclovir.

(A) 5-methoxymethyl-2'-deoxycytidine (MMdCyd): \( R^1 = -H \); N\(^4\)-acetyl-MMdCyd (Ac-MMdCyd): \( R^1 = -\text{CH}_3\text{-CO} \); N\(^4\)-propanoyl-MMdCyd (Prop-MMdCyd): \( R^1 = -\text{CH}_3\text{-CH}_2\text{-CO} \); N\(^4\)-butanoyl-MMdCyd (But-MMdCyd): \( R^1 = -\text{CH}_3\text{-}(-\text{CH}_2\text{)}_2\text{-CO} \); N\(^4\)-hexanoyl-MMdCyd (Hex-MMdCyd): \( R^1 = -\text{CH}_3\text{-}(-\text{CH}_2\text{)}_4\text{-CO} \); N\(^4\)-methyl-MMdCyd (Me-MMdCyd): \( R^1 = -\text{CH}_3 \); N\(^4\)-phenyl-MMdCyd (Phe-MMdCyd): \( R^1 = -\text{C}_6\text{H}_5 \);

(B) 5-bromovinyl-2'-deoxycytidine; and

(C) Acyclovir.
3.5 RESULTS

3.5.1 Antiherpes virus activity of MMdCyd, amides (Ac-MMdCyd, Prop-MMdCyd, But-MMdCyd, Hex-MMdCyd), alkyl derivatives (N⁴-methyl-MMdCyd, N⁴-phenyl-MMdCyd) and control drugs ACV and BrVdCyd

The ability of each compound to inhibit HSV-1 replication was determined in A-549 and VERO cells by plaque reduction assay. Butanoyl-MMdCyd (ED₅₀ 0.87 ± 0.54 µM) in A549 cells was the most potent compound of this series. Propanoyl-MMdCyd and acetyl-MMdCyd (ED₅₀ range 3 to 5 µM) were less active than butanoyl-MMdCyd but more active than MMdCyd (ED₅₀ 6.69 ± 0.70 µM). Essentially similar pattern of herpes activity was observed for amide derivatives of MMdCyd in VERO cells except that the potency was lower (approximately 3 to 4 fold) as compared to A 549 cells. Results of studies on MMdCyd (positive control) in Vero cells (ED₅₀ 25.8 ± 0.55 µM) are within the range reported previously (Aduma et al., 1990). Interestingly, results of anti-HSV activity assays of MMdCyd in A549 cells (ED₅₀ 6.69 ± 0.70 µM) are similar to results reported previously in rabbit kidney (RK-13) cells (Aduma et al., 1990).

Alkyl derivatives, N⁴-methyl-MMdCyd and N⁴-phenyl-MMdCyd were devoid of antiherpes activity. These results are in agreement with studies reported previously on methyl-MMdCyd (Gupta et al., 1991). Ayclovir (ACV) and 5-bromovinyl-2'-deoxycytidine (positive controls) were potent inhibitors with ED₅₀ values of 3 ± 0.23 µM and 1.51 ± 0.23 µM respectively in VERO cells. Results are summarized in Tables 3.1 and 3.2 and Figures 3.1 to 3.3.
3.5.2 Effect of MMdCyd and butanoyl-MMdCyd on HSV-1 replication

Antiviral screen data on \( N^4 \)-substituted analogs of MMdCyd indicated that butanoyl-MMdCyd (But-MMdCyd) was the most potent inhibitor of HSV-replication. To confirm the findings of PRA (section 3.5.1), the ability of MMdCyd and But-MMdCyd to inhibit cytopathogenic effects (cell morphology changes) of HSV and the production of infectious virus particles was determined by the virus yield reduction assay in VERO cells. Butanoyl-MMdCyd was able to prevent virus-induced cytopathogenic effect to a substantial degree (> 50%) at 1.5 µM and completely (> 99%) at 24 µM (Table 3.3). Under similar assay conditions, inhibition of virus cytopathogenicity greater than 50% and 99% in the presence of MMdCyd was observed at 48 µM and 192 µM, respectively. After washing of monolayers and incubation of cells in fresh drug-free maintenance medium, no decrease in virus infectivity was observed in cells previously treated with less than 48 µM MMdCyd. However, in infected cells previously exposed to But-MMdCyd, a decrease in virus cytopathogenicity was observed at a concentration of 3 µM (Table 3.3).

To substantiate these findings, the production of infectious virus particles was determined by the virus yield reduction assay in VERO cells. Both compounds inhibited the replication and extracellular release of HSV-1. The IC \( 99 \) of MMdCyd (concentration required to reduce the yield of infectious virus obtained after 72 hours infection by 99% relative to control cultures) derived from the data for MMdCyd was 48 µM. The IC \( 99 \) for But-MMdCyd was less than 3 µM (Fig 3.4 A). These results indicate that production of
infectious virus particles was prevented by MMdCyd, and much more effectively by But-MMdCyd.

The ability of residual intracellular virus to resume replication after drug removal was also studied by the virus yield assay. Total virus yield was determined by releasing intracellular virus particles by two cycles of freeze thawing and virus titer was determined using VERO cell monolayers. For infected cells treated with But-MMdCyd and MMdCyd, decrease in virus yield was observed at a concentration of 3 µM and 24 µM, respectively (Fig 3.4B). These results further substantiate the findings that But-MMdCyd is a more potent inhibitor of HSV-1 replication, compared to MMdCyd.

3.5.3 Antiherpes activity of MMdCyd, Ac-MMdCyd, But-MMdCyd and Hex-MMdCyd in combination with tetrahydrodeoxyuridine (H₄dUrd)

Previous studies from this laboratory have shown that the optimum concentration of a deaminase inhibitors (H₄dUrd) for cell culture experiments is 540 µM and H₄dUrd was devoid of antiviral activity up to 2.15 mM (Aduma et al., 1990a). Therefore, initially, the effect of H₄dUrd on HSV-1 replication was investigated. Tetrahydrodeoxyuridine was devoid of antiviral activity up to a concentration of 2160 µM (highest concentration tested). These results are in agreement with earlier findings (Aduma et al., 1990a). The potency of MMdCyd was increased approximately 10 fold against HSV-1 with ED₅₀ value of 2.6 ± 0.4 µM in the presence of H₄dUrd in VERO cells. Interestingly and unexpectedly, the potency of Ac-MMdCyd, But-MMdCyd, and Hex-MMdCyd was also increased seven to ten fold in combination with H₄dUrd. The results are summarized in Table 3.4. These
results indicate that anti-HSV activity of amide derivatives of MMdCyd is influenced by the Cyd/dCyd deaminase (EC 3.5.4.5) and deoxycytidylate/dCMP deaminase (EC 3.5.4.12) content of the cell line. VERO cells used for antiviral assays contain both Cyd/dCyd deaminase and dCMP deaminase (Fox et al., 1983).

3.5.4 Cytotoxicity of MMdCyd, Ac-MMdCyd, Prop-MMdCyd, But-MMdCyd and Hex-MMdCyd in VERO cells

Cytotoxicity is an important parameter for evaluation of the selectivity of anti-herpes agents. To ascertain that the antiviral effect of a compound was not due to toxic effects on cells, the effects of increasing concentrations of MMdCyd and amide derivatives (Ac-MMdCyd, Prop-MMdCyd, But-MMdCyd and Hex-MMdCyd) on monolayers (VERO and A549 cells) and rapidly growing VERO cells were examined.

The results of acute cytotoxicity studies on MMdCyd and amide derivatives against VERO and A549 cells are summarized in Tables 3.1 and 3.5. The acute toxicity of each compound was determined using two parameters: MCC, the minimum cytotoxic concentration required to cause a microscopically detectable alteration of cell morphology and CC₅₀, the concentration required to reduce the cell growth by 50%. The MCC and CC₅₀ values for MMdCyd and amide derivatives were greater than 200 μg and 1000 μg respectively (highest concentrations tested).
Table 3.1 Antiviral Activity of 5-methoxymethyl-2'-deoxycytidine (MMdCyd), amides (Ac-MMdCyd, Prop-MMdCyd, But-MMdCyd, Hex-MMdCyd), alkyl derivatives (Me-MMdCyd, Phe-MMdCyd) and control drug 5-bromovinyl-2'-deoxycytidine (BrVdCyd) against Herpes simplex virus in A549 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral activity ED₅₀ (µM)</th>
<th>ED₉₀ (µM)</th>
<th>Cytotoxicity Cell Morphology (MCC) µM</th>
<th>Cytotoxicity Cell Growth (CC₅₀) µM</th>
<th>Selectivity Index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMdCyd</td>
<td>6.69 ± 0.70</td>
<td>29 ± 3.69</td>
<td>&gt;738</td>
<td>&gt;3690</td>
<td>&gt;551</td>
</tr>
<tr>
<td>Ac-MMdCyd</td>
<td>4.79 ± 0.35</td>
<td>20 ± 3.2</td>
<td>&gt;640</td>
<td>&gt;3195</td>
<td>&gt;667</td>
</tr>
<tr>
<td>Prop-MMdCyd</td>
<td>3.06 ± 0.76</td>
<td>28 ± 3.06</td>
<td>&gt;612</td>
<td>&gt;3060</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>But-MMdCyd</td>
<td>0.87 ± 0.54</td>
<td>3.8 ± 2.82</td>
<td>&gt;586</td>
<td>&gt;2933</td>
<td>&gt;3371</td>
</tr>
<tr>
<td>Hex-MMdCyd</td>
<td>4.89 ± 0.16</td>
<td>22 ± 2.72</td>
<td>&gt;544</td>
<td>&gt;2717</td>
<td>&gt;555</td>
</tr>
<tr>
<td>BrVdCyd</td>
<td>0.61 ± 0.27</td>
<td>12 ± 2.45</td>
<td>&gt;604</td>
<td>&gt;3020</td>
<td>&gt;4950</td>
</tr>
<tr>
<td>Me-MMdCyd</td>
<td>&gt;1200⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe-MMdCyd</td>
<td>&gt;1200⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. HSV-1 McIntyre strain was used. Virus input was 50 PFU.
2. MMdCyd, 5-methoxymethyl-2'-deoxycytidine; Ac-MMdCyd, N-acetyl-MMdCyd; Prop-MMdCyd, N⁴-propanoyl-MMdCyd; But-MMdCyd, N⁴-butanoyl-MMdCyd; Hex-MMdCyd, N⁴-hexanoyl-MMdCyd; Me-MMdCyd, N⁴-methyl-MMdCyd; Phe-MMdCyd, N⁴-phenyl-MMdCyd; BrVdCyd, 5-bromovinyl-2'-deoxycytidine.
3. ED₅₀ and ED₉₀: Inhibitory concentration required to reduce viral plaques by 50% and 90%, respectively (mean ± SD, n=12)
4. MCC: Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
5. Cytotoxic concentration required to reduce cell growth by 50%.
6. SI: Ratio of CC₅₀/ED₉₀.
7. Highest concentration tested
Table 3.2 Antiviral Activity of 5-methoxymethyl-2'-deoxycytidine (MMdCyd), amides (Ac-MMdCyd, Prop-MMdCyd, But-MMdCyd, Hex-MMdCyd), alkyl derivatives (Me-MMdCyd, Phe-MMdCyd) and control drugs acyclovir (ACV) and 5-bromovinyl-2'-deoxycytidine (BrVdCyd) against Herpes simplex virus in VERO cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiviral activity</th>
<th>Cytotoxicity</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED$_{50}$ (µM)</td>
<td>ED$_{90}$ (µM)</td>
<td>Cell Morphology (MCC) µM</td>
</tr>
<tr>
<td>MMdCyd</td>
<td>25.8 ± 55 ± 0.55</td>
<td>55 ± 3.69</td>
<td>&gt;738</td>
</tr>
<tr>
<td>Ac-MMdCyd</td>
<td>19.14 ± 80 ± 0.99</td>
<td>80 ± 3.2</td>
<td>&gt;640</td>
</tr>
<tr>
<td>Prop-MMdCyd</td>
<td>12.24 ± 55 ± 0.76</td>
<td>55 ± 3.06</td>
<td>&gt;612</td>
</tr>
<tr>
<td>But-MMdCyd</td>
<td>3.5 ± 15 ± 0.54</td>
<td>15 ± 2.82</td>
<td>&gt;586</td>
</tr>
<tr>
<td>Hex-MMdCyd</td>
<td>17.6 ± 100 ± 2.1</td>
<td>100 ± 2.72</td>
<td>&gt;544</td>
</tr>
<tr>
<td>BrVdCyd</td>
<td>1.51 ± 18 ± 0.27</td>
<td>18 ± 2.45</td>
<td>&gt;604</td>
</tr>
<tr>
<td>ACV</td>
<td>3 ± 22 ± 0.23</td>
<td>22 ± 1.8</td>
<td>&gt;640</td>
</tr>
<tr>
<td>Me-MMdCyd</td>
<td>&gt;1200$^7$</td>
<td>&gt;1200</td>
<td></td>
</tr>
<tr>
<td>Phe-MMdCyd</td>
<td>&gt;1200$^7$</td>
<td>&gt;1200</td>
<td></td>
</tr>
</tbody>
</table>

1. HSV-1 McIntyre strain was used. Virus input was 50 PFU.
2. MMdCyd, 5-methoxymethyl-2'-deoxycytidine; Ac-MMdCyd, N$^4$-acetyl-MMdCyd; Prop-MMdCyd, N$^4$-propanoyl-MMdCyd; But-MMdCyd, N$^4$-butanoyl-MMdCyd; Hex-MMdCyd, N$^4$-hexanoyl-MMdCyd; Me-MMdCyd, N$^4$-methyl-MMdCyd; Phe-MMdCyd, N$^4$-phenyl-MMdCyd; BrVdCyd, 5-bromovinyl-2'-deoxycytidine; ACV, Acyclovir.
3. ED$_{50}$ and ED$_{90}$: Inhibitory concentration required to reduce viral plaques by 50% and 90%, respectively (mean ± SD, n=12)
4. MCC: Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
5. Cytotoxic concentration required to reduce cell growth by 50%.
6. SI: Ratio of CC$_{50}$/ED$_{50}$.
7. Highest concentration tested

55
Table 3.3 Effect of MMdCyd and But-MMdCyd on HSV-1 replication.

<table>
<thead>
<tr>
<th>Concentration (in µM)</th>
<th>Cytopathogenicity (presence of compounds)</th>
<th>Cytopathogenicity (absence of compounds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMdCyd</td>
<td>Bu-MMdCyd</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1.5</td>
<td>85</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>65</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>24</td>
<td>54</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>192</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>384</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. MMdCyd: 5-methoxymethyl-2'-deoxycytidine; But-MMdCyd: butanoyl-5-methoxymethyl-2'-deoxycytidine
2. Cytopathogenic effect was subjectively scored using following parameters: rounding of cells, plaque formation, and loss of monolayers. The values (%) given are relative to control cultures (virus + media only).
3. Infected VERO cells were incubated for 72 h in presence of increasing amounts of MMdCyd or Bu-MMdCyd. Media from samples which had received similar treatment were pooled and assayed for virus yield (Fig. 3.4A)
4. After 72 h incubation, media was aspirated, monolayers were washed twice with MEM, overlaid with growth medium and incubated for 72 h. virus was harvested by two cycles of freezing and thawing. Samples from similar treatments were pooled and assayed for total virus yield (Fig. 3.4B)
Table 3.4 Antiviral Activity of methoxymethyl deoxycytidine (MMdCyd) and amides (Ac-MMdCyd, But-MMdCyd and Hex-MMdCyd) against Herpes simplex virus alone and in combination with tetrahydrodeoxyuridine (H₄dUrd) in VERO cells

<table>
<thead>
<tr>
<th>Compound²</th>
<th>ED₅₀ (µM)⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMdCyd</td>
<td>25.8 ± 0.6</td>
</tr>
<tr>
<td>MMdCyd</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>H₄dUrd³</td>
<td></td>
</tr>
<tr>
<td>Ac-MMdCyd</td>
<td>19.2 ± 0.9</td>
</tr>
<tr>
<td>Ac-MMdCyd</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>H₄dUrd</td>
<td></td>
</tr>
<tr>
<td>But-MMdCyd</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>But-MMdCyd</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>H₄dUrd</td>
<td></td>
</tr>
<tr>
<td>Hex-MMdCyd</td>
<td>17.6 ± 2.1</td>
</tr>
<tr>
<td>Hex-MMdCyd</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>H₄dUrd</td>
<td></td>
</tr>
</tbody>
</table>

1. HSV-1 McIntyre strain was used. Virus input was 50 PFU.
2. MMdCyd, 5-methoxymethyl-2'-deoxycytidine; Ac-MMdCyd, N₄-acetyl-MMdCyd; But-MMdCyd, N₄-butanoyl-MMdCyd; Hex-MMdCyd, N₄-hexanoyl-MMdCyd.
3. Tetrahydrodeoxyuridine (H₄dUrd) was used at a concentration 540 µM in each assay.
4. ED₅₀: Inhibitory concentration required to reduce viral plaques by 50% (mean ± SD, n =12).
Table 3.5 Cytotoxicity of 5-methoxymethyl-2'-deoxycytidine (MMdCyd) and four amides (Ac-MMdCyd, Prop-MMdCyd, But-MMdCyd and Hex-MMdCyd) derivatives measured by cell proliferation assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell number° (x10⁵)</th>
<th>Concentration (µg)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>MMdCyd</td>
<td>3.13 ± 0.30</td>
<td>2.98 ± 0.30</td>
<td>3.41 ± 0.30</td>
</tr>
<tr>
<td>Ac-MMdCyd</td>
<td>3.13 ± 0.31</td>
<td>3.18 ± 0.28</td>
<td>2.95 ± 0.28</td>
</tr>
<tr>
<td>Prop-MMdCyd</td>
<td>3.23 ± 0.36</td>
<td>2.91 ± 0.44</td>
<td>3.06 ± 0.44</td>
</tr>
<tr>
<td>But-MMdCyd</td>
<td>3.06 ± 0.34</td>
<td>3.29 ± 0.42</td>
<td>2.63 ± 0.41</td>
</tr>
<tr>
<td>Hex-MMdCyd</td>
<td>3.05 ± 0.34</td>
<td>3.21 ± 0.42</td>
<td>2.74 ± 0.43</td>
</tr>
</tbody>
</table>

1. VERO cells (2 x 10⁵) were incubated with increasing concentrations of the test compounds for 72 hours. At the end of the incubation period, total cell number was determined for each compound.

2. MMdCyd, 5-methoxymethyl-2'-deoxycytidine; Ac-MMdCyd, N⁴-acetyl-MMdCyd; Prop-MMdCyd, N⁴-propanoyl-MMdCyd; But-MMdCyd, N⁴-butanoyl-MMdCyd; Hex-MMdCyd, N⁴-hexanoyl-MMdCyd; Me-MMdCyd.

3. Values shown are mean ± standard deviation (SD), n=6 for all experiments. The data was analyzed using one way Analysis of variance comparing cell controls versus treatment groups with P <0.05 used as the level of significance.
Fig. 3.1: Relative antiviral activity of (A) MMdCyd (5-methoxymethyl-2'-deoxycytidine), (B) Ac-MMdCyd (acetyl-MMdCyd) and (C) Prop-MMdCyd (propanoyl-MMdCyd) against HSV-1 (McIntyre strain) in VERO Q, and A-549 ■, cells by plaque reduction assay. In panels A and B, MMdCyd and Ac-MMdCyd •, with tetrahydrodeoxuridine (H4dUrd) in Vero cells. The assay for each compound was carried out in triplicate and each point represents an average of four independent determinations.
Fig. 3.2: Relative antiviral activity of But-MMdCyd (butanoyl-5-methoxymethyl-2'-deoxycytidine) and Hex-MMdCyd (hexanoyl-MMdCyd) against HSV-1 (McIntyre strain) in VERO □, and A-549 ■, cells by plaque reduction assay. In both panels ●, But-MMdCyd and Hex-MMdCyd with tetrahydrodeoxyuridine (H₄dUrd) in Vero cells. The assay for each compound was carried out in triplicate and each point represents an average of four independent determinations. The values shown represent the average percentage of viral control ± SD. Virus input 50 PFU (plaque forming units).
Fig. 3.3: Relative antiviral activity of (A) acyclovir and (B) BrVdCyd (5-bromovinyl-2'-deoxycytidine) against HSV-1 (McIntyre strain) in VERO □ and A-549 ■ cells by plaque reduction assay. The assay for each compound was carried out in triplicate and each point represents an average of four independent determinations. The values shown represent the average percentage of viral control ± SD. Virus input 50 PFU (plaque forming units).
Fig. 3.4: Effect of MMdCyd and But-MMdCyd on yield of infectious virus particles. (A) Release of virus in extracellular fluid, (B) total yield (extracellular and intracellular virus), after further incubation of cells for 72 hrs in the absence of the compounds. Symbols: □, MMdCyd (5-methoxymethyl-2'-deoxycytidine); ■, But-MMdCyd (butanoyl-5-methoxymethyl-2'-deoxycytidine).
3.6 Discussion

Amides (N\textsuperscript{4}-acyl analogs) and alkyl derivatives of MMdCyd were synthesized to improve the cell penetration of the antimetabolite MMdCyd. These compounds should also be resistant to ubiquitous deaminases present in blood and mammalian tissues because N\textsuperscript{4}-substituted compounds are not likely to serve as substrates for dCyd deaminase and dCMP deaminase. It was reasoned that amides of MMdCyd would serve as pro-drugs and thus prolong the sojourn of MMdCyd and its metabolites ("phosphorylated compounds") in HSV-infected cells.

The ability of two classes of N\textsuperscript{4}-substituted MMdCyd derivatives to inhibit HSV-1 replication in VERO cells and A-549 cells was determined by the plaque reduction assay. The infectious virus yield assay was used to substantiate the findings of antiviral activity. The results of antiviral screen data for amide deviates indicate that these compounds are selective inhibitors of HSV-1 replication and were more potent than MMdCyd. Butanoyl-MMdCyd was the most potent compound of this series. The data indicate that the optimum chain length of alkyl residues at N\textsuperscript{4}-position for anti-HSV activity is four carbon atoms, when the substituent at C (5) position of the pyrimidine ring is methoxymethyl.

In contrast, the alkyl derivatives, N\textsuperscript{4}-methyl and N\textsuperscript{4}-phenyl-MMdCyd were found to be completely devoid of activity. The amide derivatives of MMdCyd are more lipophilic than MMdCyd and it is logical to assume that these compounds will penetrate HSV-infected cells more rapidly than MMdCyd and thus higher levels of theses compounds should be achieved intracellularly. The antiherpes activity of amide derivatives of
MMdCyd may be due to intrinsic activity of these molecules. This is based on the assumption that these compounds are substrates for pyrimidine kinase (dThd/dCyd kinase) and are activated (converted to monophosphate) and eventually to triphosphate by the dCyd/dCMP kinase pathway (Chart 1.1). The other possibility is that amide derivatives get slowly hydrolyzed to the active compound MMdCyd intracellularly and the enhanced potency of these compounds is due to increased levels of MMdCTP formed intracellularly and its longer sojourn inside the HSV-infected cell. Further studies using radiolabelled butanoyl-MMdCyd should be undertaken to have a better understanding of mechanism responsible for its increased potency.

Previous studies from this laboratory on the conformation of nucleoside analogs (active) as well as inactive have shown that drug receptor interaction with receptor HSV-induced pyrimidine kinase (dThd/dCyd kinase) plays a central role in determining biological activity of 5-substituted deoxyuridine and deoxycytidine derivatives (Gupta et al., 1987; Jia et al., 1990a and 1990b; Gupta et al., 1991 and 1992). Dr. Zoghaib carried out systemic investigations on conformation of MMdCyd, amide derivatives of MMdCyd and alkyl derivatives of MMdCyd in solution by NMR spectroscopy (Zoghaib, 1996). These studies have shown that the conformation of amides in solution is different than other alkyl substituted MMdCyd derivatives (Chart 3.2). For N4-acyl analogs (amides), the preferred conformation about the glycosidic bond is anti and the N4-substituent proximal to C(5) of the pyrimidine ring. This appears to the preferred conformation for anti-HSV activity when N4-position is substituted with an acyl residue. In contrast, for all
compounds, which were inactive, namely, N⁴- (methyl, phenyl, benzyl, and methoxy)-MMdCyd, the preferred conformation about the glycosidic bond is syn and the N⁴-substituent is proximal to N(3) of the pyrimidine ring. Based on these investigations it is logical to conclude that molecular conformation of N⁴-substituted compounds plays an important role in determining biological activity.

Interestingly, in contrast to 5-substituted pyrimidine derivatives, the purine analogs with the side chain substitution at the N⁷-position of the purine ring or alkyl substitution at the N⁶-position of the amino group of the guanine residue have been found to be potent inhibitors of cytomegalovirus (HCMV) and human immunodeficiency virus (HIV) replication, respectively (Chu et al., 1990; Neyts et al., 1998). The metabolism of the acyclic nucleoside analog [2-amino-7-(1-3-dihydroxy-2-propoxymethyl)purine, code name S2242] was studied in HCMV-infected cells (Neyts et al., 1998). The uptake of this compound was linearly correlated with its extracellular concentration and was blocked by inhibitors of nucleoside transport. The compound S2242 was readily phosphorylated in infected cells. In HCMV-infected human embryonic lung cells, a 25-fold increase in S2242 metabolite formation was observed compared with the non-infected cells suggesting that an HCMV-encoded or induced enzyme is responsible for the specific phosphorylation of S2242. Exogenously added dCyd had little effect on the activity of S2242 against HCMV and on the phosphorylation of the compound in HCMV-infected cells. The compound S2242 is a good substrate for purified human deoxyguanosine kinase and the later enzyme is stimulated 3-4 fold in HCMV-infected cells. These studies
are interesting and indicate that alkyl substituted purines can serve as substrates for deoxyguanosine kinase. This enzyme obviously has broader substrate specificity as compared to HSV-induced viral pyrimidine kinase.

To study the structure activity-relationships of 2',3'-dideoxypurine nucleosides as potential anti-HIV compounds, various 6-substituted purine analogs were synthesized and examined in virus-infected human peripheral blood mononuclear cells (Chu et al., 1990). Among these compounds, N-6-methyl-2',3'-dideoxyadenosine proved to be the most potent antiviral agent and was resistant to adenosine deaminase.

Antiviral activity of Ac-MMdCyd, Prop-MMdCyd and But-MMdCyd is cell dependent (Tables 3.1 and 3.2, Figs. 3.1 and 3.2) and was potentiated (approx. 10 fold) in the presence of tetrahydrodeoxyuridine (H₄dUrd) in HSV- infected cells (Tables 3.4, Figs. 3.1 and 3.2). H₄dUrd is an inhibitor of both Cyd/dCyd deaminase and dCMP deaminase. This transient state analog in anabolized by a phosphotransferase to its corresponding nucleotide, H₄dUMP, which is a potent inhibitor of dCMP deaminase (Maley and Maley, 1971). On the basis of these results, the following conclusions can be drawn: (i) the introduction of acyl residues at N⁴-position of MMdCyd confers only partial refractoriness to deaminase and (ii) the anti-herpes activity of acyl analogs of MMdCyd is influenced by the Cyd/dCyd deaminase (EC 3.5.4.5) and deoxycytidylate (dCMP) deaminase (EC 3.5.4.12). The increase in activity observed for these compounds in combination with H₄dUrd could also be due to greatly diminished levels of deoxythymidine triphosphate (dTTP) levels. H₄dUrd prevents synthesis of dUMP from
dCyd pools and this leads to negligible levels of dTTP formed in uninfected and HSV-infected cells VERO cells (Aduma et al., 1991). Further studies using butanoyl-MMdCyd (most active compound) in various cell lines and in the presence of tetrahydrouridine (H₄Urd, inhibits only Cyd/dCyd deaminase) as well as H₄dUrd are needed to validate these findings. Studies on metabolism of butanoyl-MMdCyd in normal and HSV-infected cells should also be undertaken to define the active molecule moiety responsible for antitherpes activity.

5-Methoxymethyl 2'-deoxycytidine (MMdCyd), amide derivatives (N⁴-acyl analogs) of MMdCyd and alkyl derivatives (N⁴-methyl and N⁴-phenyl) are non-toxic to monolayers and rapidly dividing A-549 and VERO cells. The low cytotoxicity of these compounds towards rapidly dividing cells is of considerable relevance for use of these compounds for therapeutic purposes. These compounds should have minimal effects on tissues such as the gastrointestinal tract, bone marrow or skin which have rapid cell turnover (Jones, 1998). In addition, it is logical to assume that in uninfected cells, these compounds would diffuse freely in and out, because of the lack of catabolism or anabolism. This has practical implications in determining the host toxicity and further descriptive toxicity studies should be undertaken to determine the effects of butanoyl-MMdCyd (most active compound) on host metabolism.

The cytotoxicity profile of butanoyl-MMdCyd compares favourably with antiviral drugs currently approved for the treatment of HSV- infection. For example, mammalian cell growth is unaffected by acyclovir up to concentrations of 50 µg/ml (Wagstaff et al., 1998).
1994). Penciclovir and famciclovir are devoid of cytotoxicity up to a concentration of 80 µg/ml for rapidly growing mammalian cells (Boyd et al., 1993). Butanoyl-MMdCyd is considerably less toxic than other agents, such as, foscarnet, ganciclovir, idoxuridin and trifluridine. For example, the ED$_{50}$ of foscarnet against herpes viruses is around 80 µg/ml but concentrations of over 300 µg/ml inhibit the proliferation and DNA synthesis of uninfected cells (Oberg, 1989; Chrisp and Clissold, 1991). Ganciclovir licensed for the treatment of cytomegalovirus infections causes inhibition of human lymphocyte proliferation at clinically achievable concentrations of 1 to 10 µg/ml (Sommadossi et al., 1987). Idoxuridine inhibits the growth of uninfected cells around 2 to 10 µg/ml (Prusoff, 1980) and trifluridine inhibits cellular DNA synthesis at relatively low concentrations (5 to 10 µg/ml) [Birch et al., 1992].

The most likely reason for the lack of cytotoxicity of these compounds is that in uninfected cells, MMdCyd, amide derivatives of MMdCyd and alkyl derivatives of MMdCyd are not converted to the triphosphate form in significant amounts. Therefore, little (or nil) amount is incorporated into the cellular DNA in uninfected cells. Thus, the reason for the selectivity of action of these compounds is that they are only 'anabolized' to the active form (triphosphate) in HSV-infected cells and this anabolism is mediated by the HSV-induced pyrimidine kinase and subsequently by the enzymes of dCyd/dCMP kinase pathway (Chart 1.1). Previous studies on the utilization of MMdCTP (the active moiety responsible for aniherpes activity of MMdCyd) by viral and cellular DNA polymerases provide a rationale basis for the selectivity of this novel antimetabolite
Earlier investigations on N⁴-Me-MMdCyd are of relevance (Gupta et al., 1991). This compound was found to be completely devoid of biological activity at high concentrations in the presence and absence of deaminase inhibitors (up to 1796 µM) and was non-toxic to VERO cells. To address issues regarding the substrate specificity with respect to two essential enzymes (viral kinase and DNA Polymerase), the monophosphate (N⁴-Me-MMdCMP) and triphosphate (N⁴-Me-MMdCTP) were synthesized. The monophosphate was found to be devoid of antiviral activity as well as cellular toxicity, and N⁴-Me-MMdCTP was neither a substrate nor an inhibitor of viral DNA polymerase, bacterial DNA polymerase or human DNA polymerase α. To explore further, reasons for the complete refractoriness of this molecule, molecular conformation studies on MMdCyd (active) and N⁴-methyl-MMdCyd (inactive) by X-ray crystallography were undertaken (Jia et al., 1990 and 1990a). These studies revealed that conformation of the exocyclic (C5') side chain was altered by substitution of methyl group at N⁴-position of the pyrimidine moiety and the change in conformation was likely responsible for the lack of biological activity (Chart 3.2). As alluded previously, the conformation of N⁴-substituted acyl analogs (amides) is obviously favourable for binding to two essential enzymes and this confers potential antiviral activity and selectivity (lack of cytotoxicity) towards host cell for these compounds.

In summary, the antiviral potency of butanoyl-MMdCyd and its cytotoxicity profile compares favourably with currently approved drugs for the treatment of viral infections.
Pre-clinical toxicity studies were undertaken to determine its therapeutic potential and results of these studies are described in chapter 4.
Chart 3.2: Representative conformations

N^4 substituent proximal to N3

N^4 substituent proximal to C5
4. TOXICITY STUDIES

4.1 Introduction

At present, nucleoside analogs as a class of compounds are most useful therapeutic agents for the treatment of systemic viral infections caused by herpes viruses (HSV, CMV, VZV); human immunodeficiency virus (HIV) and hepatitis B virus (Jones, 1998). Antiviral nucleoside analogs (ANA) elicit antiviral activity by inhibiting DNA synthesis of susceptible viruses. Besides inhibiting viral DNA synthesis, ANA triphosphates (active form of drugs) also interfere with the nuclear DNA synthesis or mitochondrial DNA synthesis of host cell by serving as a competitive alternate substrate and incorporating into DNA or by terminating the nascent DNA chain (chain termination) [Haefeli et al., 1993; Saltzman et al., 1994; Agarwal and Olivero, 1997; Parker et al., 1997; Shi et al., 1997; Sawyer et al., 1988]. Therefore, antiviral nucleoside analogs have the potential of causing serious toxicity problems especially when these drugs are used for long term in patients.

Toxicities manifested by antiviral nucleoside analogs approved for clinical use in humans have been well-documented (Hayden, 1995; Jones, 1998). Drugs, such as, 2', 3'-dideoxycytidines (ddC), 5-trifluoromethyl-2'-deoxyuridines (trifluridine) and 5-iodo-2'-deoxyuridine are readily incorporated into nuclear DNA and produce serious toxic manifestations when give systemically (Parker et al., 1997, Shi et al., 1999). The dose limiting toxicities seen with the use of nucleoside analogs include nephrotoxicity, CNS disturbances and hematopoietic toxicity manifested as anemia, and thrombocytopenia (Tucker et al., 1983; Arnaudo et al., 1991; Berger et al., 1993; Goldberg et al., 1993;
The long term use of dideoxynucleoside analogs, such as azidothymidine (AZT), ddC (low dose) and 2', 3'-dideoxyinosine (ddl) for the treatment of HIV infection in AIDS patients and fluorinated analogs, namely, 2-fluoro-2'-deoxy-D-arabinofuranosyl uracil (FIAU) and 2-fluoro-2'-deoxy-D-arabinofuranosyl cytosine (FIAC) for the treatment of viral hepatitis in humans have been shown to produce unique multiorgan toxic effects (Hayden, 1995; Tennant et al., 1998). The manifestation of toxicity exhibited by these drugs in different tissues appears to be due to defective mitochondrial DNA synthesis due to inhibition preferentially of mitochondrial DNA polymerase gamma (γ) [Agarwal and Olivero, 1997; Shi et al., 1997; Parker et al., 1997]. This type of toxicity by ANA is generally referred to as delayed cytotoxicity.

The toxicity manifestations exhibited by fluorinated analogs of uracil (FIAU) and cytosine are of particular relevance because these compounds are potent inhibitors of HSV (McKenzie et al., 1995) and hepatitis virus (Tennant et al., 1998). These compounds require metabolic activation for antiviral activity and are preferentially phosphorylated in infected cells. Based on these results, clinical trials with FIAU for the treatment of hepatitis were initiated in human patients. This trial had to be terminated prematurely because of the sudden development of severe “multi-system toxicity” (hepatic failure, lactic acidosis and pancreatitis), which resulted in the deaths of five patients (McKenzie, 1995; Kliener et al., 1997). Severe hepatotoxicity from FIAU was characterized by hepatomegaly with diffuse, predominantly microvesicular steatosis, hepatocellular
glycogen depletion, marked bile duct proliferation, and cholestasis. The microscopic and ultrastructural patterns of injury and systemic symptoms in patients with FIAU toxicity are consistent with severe mitochondrial and metabolic derangement (Kleiner et al., 1997).

Several investigators have shown that in vitro assays of delayed cytotoxicity are adequate models for predicting the delayed toxicities of antiviral drugs (Chen and Cheng, 1989; Chen et al., 1991; Parker et al., 1997). Therefore, it was decided to evaluate the potential of delayed toxicity of MMdCyd and But-MMdCyd using CEM cells. After completion of acute and delayed toxicity studies using in vitro assays, pre-clinical toxicity studies on But-MMdCyd in mice were undertaken. The toxicological parameters investigated include acute toxicity, subacute toxicity, gross and histopathology, hematology, clinical chemistry and urinalysis. The protocols for pre-clinical studies were according to the recommendations of Gad and Chengelis (1998). The results of toxicity studies are discussed in this chapter.

4.2 MATERIALS AND METHODS

4.2.1 Compounds, Chemicals, Cells and Reagents

MMdCyd and But-MMdCyd were prepared as described previously (section 3.2.2). Dideoxycytidine (ddC) was purchased from Royalo chemicals, Edmonton. The sources of media, fetal bovine serum and tissue culture supplies have been described previously (section 3.2). Isoton III was purchased from Fischer Scientific, St. Louis, Mo. The stock solution of each compound was made using PBS and concentration determined
spectrophotometrically. The stock solution was used immediately or divided into small aliquots (1 to 2 ml) for storage at −20°C. CEM cells, (a human T lymphoblastic cell line) was used for acute and delayed cytotoxicity studies. The stock culture of CEM cells was initially obtained from Dr. Warrington, Department of Biochemistry, University of Saskatchewan. For tissue fixation, 10% phosphate buffered-formalin was used. For staining, Lillie’s hematoxylin (0.5%) and eosin (1%) solution was used. Procedures for fixation and staining are described in Appendix A Table 2. Formalin and staining solutions were supplied by the Department of Pathology, College of Medicine, University of Saskatchewan. Plasma separator tubes (Microtainer, Becton Dickson) and heparin were obtained from Source Medical, Saskatoon, Saskatchewan. Coulter counter, Model Z, Coulter electronics, Burlington, ON, Canada was used for counting cells.

4.2.2. Animals

White Swiss male CD-1 mice weighing 20-22g were obtained from the Animal Resources Centre, University of Saskatchewan. The mice were allowed to acclimatize for a period of one week. Mice were housed in groups of five in clear, autoclavable, polycarbonate cages, provided with hardwood shavings for bedding. Mice used for different treatment groups were housed in separate cages, as desired for a completely randomised trial. Identification of the mouse was done by tail marking. Commercially prepared mouse pellets (Prolab, PMI Feeds Inc, St. Louis, MO) and tap water in bottles fitted with stainless steel sipper tubes were available ad libitum. The animal facility was maintained at 21±2°C and 50±10% relative humidity. Lighting was controlled to provide 12 hr
light and 12 hr darkness per day. The care and treatment of the mice were in accordance with the Canadian Council on Animal Care (1993).

4.3 EXPERIMENTAL PROTOCOLS

4.3.1 Acute and Delayed Cytotoxicity Studies

CEM cells were grown in RPMI 1640 medium supplemented with 5% FBS. The cells were grown for many generations and the doubling time was determined every 48 hours. On alternate days, the viability of the cells was determined using the trypan blue-exclusion method (Parker et al., 1997). When the doubling time of CEM cells was constant (range 21 to 24 hours); these cell cultures were used for cytotoxicity studies. Experimental protocols used for assessing the acute and delayed toxicity of nucleoside analogs But-MMdCyd, MMdCyd (negative control), and ddC (positive control) were similar as described previously by Shi et al (1997). Briefly CEM cells (5 x 10^5 cells/ml per well) were seeded in growth medium in six-well plates and incubated with various concentrations of test compound or ddC. The concentration was adjusted so that 2 ml of the diluted drug was added to each well. To cell controls (media plus cells) 2 ml of media was added. The plates were incubated at 37 °C in 5% CO₂ humidified atmosphere for 48 hours. Every two days 0.2 ml of the cell media mixture was added to 9.8 ml of Isoton III and the cell number was determined using the Coulter counter. The cells were centrifuged at 400g for seven minutes, and the cell pellet was re-suspended in fresh media containing the compound at a density of 5 x 10^5 cells/ml. This cycle was repeated several times to complete 14 days of continuous exposure. The doubling time was calculated.
using the formula: number of population doubling $n = \frac{\log_{10} \left( \frac{N}{N_0} \right)}{3.33}$: Doubling time $= \frac{T_N - T_{N0}}{n}$ where, $N_0 =$ cell number at the beginning of the period: $N =$ cell number at the end of the period; $T_{N0} =$ time at the beginning of the period; $T_N =$ time when cells were counted (Shi et al., 1997). The assays were carried out in quadruplicates. The viability of cells was checked daily by trypan blue exclusion method (Shi et al., 1997).

4.4 DESCRIPTIVE ANIMAL TOXICITY STUDIES.

4.4.1 Acute toxicity studies

Forty male white Swiss mice weighing 20 to 22 g were divided into four groups ($n = 10$) and housed in-groups of five in each cage. Butanoyl-MMdCyd was administered as a single intraperitoneal (i.p.) injection at dosages of 100 mg/kg, 200 mg/kg and 1000 mg/Kg. The concentration of But-MMdCyd was adjusted so that the total volume injected into any animal was not more than 0.2ml. Control mice were treated in an identical manner (a single i.p. injection of 0.2 ml sterile PBS). The protocols recommended for minimal acute toxicity studies by Gad and Chenglis (1998) were adhered to for monitoring of all animals. All mice were examined daily for any signs of intoxication, including respiratory and cardiovascular distress, lethargy, motor and behavioural modifications, and morbidity. The parameters used for clinical observation are summarized in Table 4.3. Body weights (each animal), feed and water consumption (for each group) was determined on day 1 (prior to dosing), day 7 and day 15. The animals were killed by CO$_2$ over dose inhalation on day 15 and monitored for gross and histopathological, hematological and clinical chemistry parameters. Urine was collected
from all animals on day 7 and day 15 and urine analysis was carried out according to recommendations of Rousseaux (1998). The parameters determined were: specific gravity (SG), protein, heme, glucose and casts.

4.4.2. Subacute-toxicity

Following completion of acute toxicity studies, subacute toxicity studies were undertaken using But-MMdCyd (100 mg/Kg). This dose was selected to determine whether repeated administration of one tenth the maximum dose used for the acute toxicity study would produce any drug-induced toxicity (Meldrum, 1978). The protocol used for subacute toxicity studies was according to the guidelines of Gad and Chenglis (1998). Fifty white Swiss male mice weighing 20-22 g were used for these studies. Butanoyl-MMdCyd (100 mg/kg), i.p., was administered to a group of mice (n = 25) as a single daily injection for 14 days. Control mice (n = 25) were treated in an identical manner with an equal volume of sterile PBS (0.2ml). All animals were examined clinically once daily for 15 days post drug administration for any signs of intoxication, including respiratory and cardiovascular distress, lethargy, motor and behavioural modifications, and morbidity using parameters outlined in Table 4.3. All mice were weighed on day 1 (prior to dosing) and on days 7, 15, 22 and 30 post drug administration. Feed and water consumption was recorded for days 7, 15, 22 and 30. Five mice from each group (control and treatment) were killed on day four. Ten mice were killed from each group on day 15. This sacrifice schedule allowed monitoring of any changes that might have occurred during treatment and emphasised the time at which maximal drug exposure had occurred (treatment of full
duration). In order to follow drug-induced changes after cessation of drug administration, the remaining mice were allowed to recover. Following the recovery period, five mice from each group were killed on days 22 and 30. Urine was collected from all animals on days 7, 15, 22 and 30. Urine samples were analyzed for specific gravity (SG), protein, heme, glucose and casts as suggested by Rousseaux and Haschek (1998). The objective of this study was to determine whether drug-induced injuries, if any, could be repaired during the study period.

4.4.3. Pathological studies

All animals were sacrificed in an identical manner. Each mouse was placed in a jar connected to a carbon-dioxide gas cylinder and the jar was saturated with the gas to the point when breathing stopped. The animal was immediately removed from the jar, and the abdominal muscle and the rib cage were incised, the xyphoid cartilage of sternum was grasped with rat tooth forceps and the diaphragm muscles were incised. The ribs were quickly incised on both sides lateral to the internal mammary artery and the flap containing the sternum reflected cranially. This provided good exposure of the heart. In order to prevent severe hemorrhage due to severing of brachial artery, care was taken not to incise too far cranially on the ribs. The blood was collected using a tuberculin syringe fitted with a 3/8 inch, 25 G needle. The dead space of syringe was filled with heparin (1:1000). The right ventricle of the heart was penetrated and blood slowly withdrawn. Gentle pressure on the abdomen aided exsanguination and blood, 0.5 to 1.0 ml could be removed without hemolysis.
At this point, each animal was carefully examined for the presence of grossly visible lesions as described by Feldman and Seely (1998). The greater curvature of stomach was grasped with serrated forceps and the filmy membrane between the stomach and the caudate lobe of liver was severed. The oesophagus was severed approximately 0.5 cm above the liver allowing the stomach to be loosened from its attachments. The oesophagus was grasped with fine forceps close to the stomach and while maintaining tension, the bile duct and remaining connective tissue between the liver and stomach was severed. The mesentery between the intestines and the dorsal body wall was cut, without cutting the large blood vessels in the area. This freed the spleen and the entire gut except for its termination at the anus. The intestine was cut around the anus and the intestinal tract was examined by stretching it to its full length by severing the mesentery, which was bound to the coils of the small intestine. The entire gut was laid out in a serpentine fashion.

The spleen was separated by cutting between the spleen and stomach. The liver was excised intact by grasping the tough connective tissue under the median lobe with fine tissue forceps and while exerting tension posteriorly, the oesophagus and blood vessels passing through the diaphragm were severed. All remaining connections were cut to free the liver. Then the kidneys (exposed when the liver was removed) were collected. The attachments of the mandible to the body were cut and the mandible was grasped with small forceps and pulled downward. The soft palate and remaining pharyngeal attachments behind the oesophagus was cut using a sharp scissors. The trachea and oesophagus together was dissected away from the body and at the level of the first ribs the
attachments to the dorsal chest wall was severed. All the remaining attachments of the lungs as far as the diaphragm were cut to free trachea, heart and lungs. The brain was removed in the following manner. The mouse was turned over to its ventral side and the fur on the head was saturated with alcohol. An incision was made between the eyes to the back of the head and the skin was reflected away from the cranium. The mouse's head was dissected through the dorsal muscles of the neck until the spinal cord was exposed. One blade of a small pointed scissors was inserted into the opening of the skull (Foramen magnum) and the bones were cut just above the opening of the ear canal. This procedure was repeated on the other side of the skull. The bone incision was then extended anteriorly to the level of the nose and the frontal bones covering the olfactory lobes were removed. The posterior cranium was lifted upward away from the head, exposing the cerebellum and cerebral hemispheres. The dura mater was then removed.

The olfactory lobes were severed with fine scissors and head was then tilted upward and backward. All remaining attachments to the brain were severed. The brain stem (medulla oblongata) was then cut transversely posterior to the cerebellum to free the brain. All organs removed were examined extensively for any gross lesions and selected tissues were placed in 10% phosphate buffered-formalin (PBF) for fixation for 48 hours (Appendix A, Table 1). Marrow was removed from the right femur. After fixation (except blood and marrow), tissues were trimmed to the appropriate size and processed through a series of alcohol in an Autotechnicon (Model 2A, Technicon Co., Chauncey, New York) in preparation for embedding. The tissues were embedded in paraffin
(Paraplast) and sectioned at five microns using rotary microtome (American Optical Instrument Co., Buffalo, New York) at the Department of Pathology, College of Medicine. Tissue sections were stained with Lillie’s hematoxylin (0.5%) and eosin (1%) and evaluated for histopathological lesions by conventional light microscopy by the investigator and Dr. A. Saxena in a blind fashion. The scoring of lesions and protocols used for histopathological examination were according to the guidelines of Rousseaux (1998). The extent of lesions was scored using a scale of 0 to 4: Nil = 0; Minimal (< 1%) = 1; Mild (1-10%) = 2; Moderate (10-25%) = 3; and Severe (> 75%) = 4.

4.4.4 Hematological Studies

Blood from each mouse was collected in a 2 ml plasma separator tube coated with heparin and mixed gently but thoroughly. The blood samples were submitted for both hematological and clinical chemistry parameters to the Department of Pathology, University of Saskatchewan. The hematological parameters evaluated were: total red blood cells (RBC) count (cells x $10^{12}$/L), total white blood cell (WBC) count (cells x $10^9$/L), hemoglobin concentrations [Hb, (g/L)], hematocrit [HCT, (L/L)], platelet count (x $10^9$/L) and mean corpuscular volume (MCV). Mean corpuscular volume is expressed as femtoliter (fL). Hematological profiles were obtained using a hematology analyzer Coulter Counter (STKS Coulter, Hialeah, FL, USA).

The right femur of each mouse was dissected and split longitudinally with a scalpel. Sufficient marrow was scooped out to make two marrow smears. The marrow smears were air dried and stained with Wright-Giemsa stain (Appendix A Table 3). Each slide
was then evaluated microscopically to ensure that all precursor cells were present.

4.4.5 Clinical Chemistry Studies

Nucleoside analogs manifest toxicity for organs of high metabolic activity or where they are metabolized and excreted (Hayden, 1995; Jacobson et al., 1995; Gad and Chengelis, 1998). Since But-MMdCyd is a nucleoside analog, the parameters were studied which would reflect changes in the liver and kidney. The parameters chosen for these studies were according to the recommendations of Frith et al (1988) and are summarized in Table 4.3. The parameters evaluated were: serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum gamma-glutamyltransferase (GGT), glucose, creatinine, blood urea nitrogen (BUN), potassium, sodium and total protein (g/L).

Determination of these parameters was carried out using microanalytical techniques using a Chemistry Analyzer (Kodak Ektachem, 500 Analyzer, Rochester, MN). Enzyme activities are expressed as U/L. Glucose, sodium, potassium, BUN and creatinine are expressed as mmol/L.

4.4.6 Urinalysis

From each mouse, a 24-hour urine sample was collected using a metabolic cage (Access Technologies, Skokie, IL) and transferred to a 5-ml vial. Semiquantitative evaluation of glucose, protein, heme and pH was carried out using Reagent Strip (Chemstrip®, BMC, Laval, Quebec, Canada) according to manufacture's instructions. For analysis of the sediment, each urine sample was centrifuged at low speed (1200 x g) for 5 minutes and the supernatant was discarded. The sediment was resuspended in 0.5ml of distilled water.
and examined microscopically (100 X and 400 X magnification) for the presence of RBC, WBC, casts and crystals. Urinalysis was performed at the Department of Pathology, College of Medicine, University of Saskatchewan.

4.5 Statistical analysis of the data

The data of hematological and clinical chemistry parameters were analysed using one way ANOVA using Sigmastat computer program. The statistics were performed on the raw data, comparing control versus experimental groups with P <0.05 used as the level of significance.
Table 4.1 Organs examined and the histopathological parameters evaluated in the acute and subacute toxicity study

<table>
<thead>
<tr>
<th>Organs</th>
<th>Subtopography</th>
<th>Lesions</th>
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<tr>
<td>Liver</td>
<td>Portal tracts</td>
<td>Vasculitis, Bile duct proliferation</td>
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<td></td>
<td>Parenchyma</td>
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<td>Cell necrosis, Steatosis, lobular inflammation, Kupffer cell hyperplasia</td>
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<td>Glomeruli</td>
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<td></td>
<td>Tubules</td>
<td>Tubulitis, Tubular casts</td>
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<td>Spleen</td>
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<td>Red pulp</td>
<td>Hemosiderosis, Congestion</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Necrosis, Lymphocytic infiltration</td>
</tr>
<tr>
<td>Brain</td>
<td>Cerebellum</td>
<td>Vacuolization, necrosis inflammation</td>
</tr>
<tr>
<td></td>
<td>Cerebrum</td>
<td>Necrosis, vacuolization, cellular inflammatory cell infiltrate</td>
</tr>
</tbody>
</table>

Table 4.2 Clinical Observation in Acute and Subacute Toxicity Tests

<table>
<thead>
<tr>
<th>Organ system</th>
<th>Parameters investigated</th>
<th>Overt signs of toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS and somato-motor</td>
<td>Behavior</td>
<td>Unusual aggressiveness, unusual vocalization, restlessness, sedation.</td>
</tr>
<tr>
<td></td>
<td>Movements</td>
<td>Twitch, tremor, ataxia, catatonia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paralysis, convulsion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Irritability, passivity, anesthesia, hyperesthesia.</td>
</tr>
<tr>
<td></td>
<td>Reactivity to cerebral and spinal reflexes</td>
<td>Sluggishness, absence.</td>
</tr>
<tr>
<td>Autonomic nervous system</td>
<td>Muscle tone</td>
<td>Rigidity, flaccidity</td>
</tr>
<tr>
<td></td>
<td>Pupil size</td>
<td>Myosis, mydriasis</td>
</tr>
<tr>
<td>Respiratory</td>
<td>Nostrils</td>
<td>Discharge (color Vs uncolored)</td>
</tr>
<tr>
<td></td>
<td>Character and rate</td>
<td>Bradypnoea, dyspnoea</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Palpation of cardiac region</td>
<td>Thrill, bradycardia, stronger or weaker beat</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Feces consistency and color</td>
<td>Unformed, black or clay colored</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>Perineal region</td>
<td>Swelling</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prolapse</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soiled</td>
</tr>
<tr>
<td>Skin and fur</td>
<td>Color, turgor, integrity</td>
<td>Reddening, eruptions, piloerection</td>
</tr>
<tr>
<td>Mucous membranes</td>
<td>Conjunctiva, mouth</td>
<td>Discharge, congestion, hemorrhage, cyanosis, jaundice</td>
</tr>
<tr>
<td>Eye</td>
<td>Eyelids and Eyeballs</td>
<td>Ptosis, exophthalmus, nystagmus</td>
</tr>
<tr>
<td>Others</td>
<td>General condition</td>
<td>Abnormal posture, emaciation</td>
</tr>
</tbody>
</table>

1. According to recommendations of Gad and Chenglis (1998)
Table 4.3 Biochemical Parameters Evaluated in this Study and their indications

<table>
<thead>
<tr>
<th>Parameter evaluated</th>
<th>Indication of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine amino transferase (ALT)</td>
<td>Damage to hepatocytes</td>
</tr>
<tr>
<td>Aspartate amino transferase (AST)</td>
<td>Damage to hepatocytes, muscles</td>
</tr>
<tr>
<td>Gamma-glutamyl transferase (GGT)</td>
<td>Biliary disease</td>
</tr>
<tr>
<td>Blood Urea Nitrogen (BUN)</td>
<td>Altered Kidney function</td>
</tr>
<tr>
<td>Creatinine</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endocrine or Kidney dysfunction</td>
</tr>
</tbody>
</table>

1. (Adapted from Frith et al., 1988 with modifications)
4.6 RESULTS

4.6.1 Acute and delayed cytotoxicity studies on the antiviral agents 5-methoxy-2'-deoxycytidine (MMdCyd) and of But-MMdCyd

Generally, if the cell growth is inhibited within 3 to 4 days of exposure to a compound it is considered to be due to acute toxicity. However, if changes in cellular growth pattern, (cellular multiplication time or viability) starts to occur after 6 days of exposure, and the toxic effect is amplified on longer duration of exposure, to the test compound then it is considered that toxic effects are due to delayed cytotoxicity. Previous studies have shown that dideoxycytidine (ddC) at low concentrations produced delayed cytotoxicity in CEM cells (Chen et al., 1991; Parker et al., 1997; Shi et al., 1999). Therefore, ddC was used as a positive control drug. MMdCyd was used as negative control because this nucleoside analog did not exhibit acute or delayed cytotoxicity (Shi et al., 1997). The results of ddC and 5-substituted nucleoside analogs, MMdCyd and But-MMdCyd at various doses on the doubling time of CEM cells are summarized in Table 4.4 and Fig. 4.1. Butanoyl-MMdCyd was tested at concentrations of 250 µM, 500 µM, 1000 µM and 2000 µM. The cells not exposed to nucleoside analogs had a relatively stable doubling time (21.8 ± 0.5 to 24 ± 0.4 hrs) throughout the testing period (16 days incubation). In contrast, in the presence of ddC 0.5 µM, the doubling time of CEM cells started to increase from the fourth day onwards. Exposure to higher concentration of ddC (1µM) resulted in increase of doubling time within 2 days (Fig. 4.1, panel A). These results are in agreement with previously reported studies on the delayed cytotoxic effects of ddC in lymphoblast cell
lines (Chen et al., 1991; Parker et al., 1997; Shi et al., 1997). Both MMdCyd and But-MMdCyd did not alter the doubling time of the cells at the highest concentration tested (Fig. 4.1, panels B and C).

4.6.2 TOXICOLOGICAL STUDIES IN MICE

4.6.2.1 Acute toxicity

No mortality or morbidity was observed in mice from any of the treatment or the control groups. No difference was noticed in the food and water consumption between the treatment group and the control group (Table 4.5). There were no overt signs of toxicity on clinical examination in any of the treatment group. Based on clinical observations all animals appeared healthy. No significant dose-dependent reduction in body weight gain was noticed in any of the treatment group, when compared to the control group (Fig. 4.2, P = 0.35).

4.6.2.2 Subacute toxicity studies

The results of the subacute toxicity studies were similar to those observed in the acute toxicity study. No morbidity or mortality was observed in the control or treated animals receiving 100 mg/kg of But-MMdCyd for 14 days. No statistically significant difference in body weight gain was observed between the control and treated animals (Figs. 4.3, panels A and B; P = 0.64 & 0.67 respectively). No difference was documented in the food and water consumption between the treatment group and the control group (Table 4.6). There were no overt signs of toxicity in the treatment group on clinical examination using various parameters (Table 4.2).
4.6.3. Gross and Histopathological findings

4.6.3.1 Acute

Following the killing of each mouse, a thorough necropsy examination of the carcass was completed in a systematic manner to determine the presence of grossly visible lesions. Three mice from the control group and two from the treatment group (100 mg/kg), had spleens enlarged two to three times the normal size. This enlargement was due to splenic congestion observed in histological sections. In all other mice of the treatment group the internal organs were unremarkable. Histopathological examination of the sampled tissues revealed no lesions in the kidney, liver, spleen and brain. The hemopoietic and lymphoid tissues in the spleen were similar in control and treated groups. Liver sections of the treated and control mice did not reveal any lesions. Prominent Kupffer cells were common in all the groups. The histologic appearance of examined organs was similar (i.e., no histological abnormalities) in both control and treatment groups (Table 4.7). Representative sections of tissues examined in treated and control groups are shown in Figures 4.4 to 4.7.

4.6.3.2 Subacute

A detailed necropsy was performed on each mouse.

Gross appearance: Four of the treated and two of the control mice (killed on day 15) had spleens 2-3 times the normal size. This was due to splenic congestion. In other mice, the internal organs were unremarkable.

Microscopic findings: Splenic congestion was observed in four treated and two control
mice (killed on day 15). Multifocal lymphocytic infiltration of glomeruli was seen in three mice (two control and one treated) killed on day 30. The histological appearance of all other tissue examined was similar (i.e., no histologic abnormalities) in both control and treated groups (Table 4.8). Representative sections of tissues examined in treated groups are shown in Figures 4.4 to 4.7.

4.6.4 HEMATOLOGICAL STUDIES

4.6.4.1 Acute

The effects of But-MMdCyd after administration of a single dose of 100 mg/kg, 200 mg/kg and 1000 mg/kg, i.p., to male white Swiss CD-1 mice and killed on day 15 post administration on the hematological parameters investigated are summarised in Table 4.9. The values listed for each dose are mean ± standard deviation (SD) of samples data from animals in each group (n = 10). There were no statistically significant differences (P < 0.05) between the control and treated animals in any of the parameters examined.

4.6.4.2 Subacute

Mice were sacrificed serially to obtain a progressive picture of any changes that may occur during treatment with But-MMdCyd as well as during the post recovery period. The effects of But-MMdCyd (100 mg/kg/day for 15 days) administration on the hematological parameters are shown in Tables 4.10 and 4.11. The values listed are mean ± SD of samples data obtained from animals killed on days 5, 15, 22 and 30. The sample size for animals killed on day 15 was ten. On days, 5, 22 and 30 only five mice were killed for the collection of blood samples. The number of animals in each group killed on
days 5, 22 and 30 was small due to the limited availability of the test compound. As in the acute toxicity study, few changes in any of the parameters examined were observed. There were no statistically significant differences (P < 0.05) between the control and treated animals in any of the parameters examined.

4.6.5 CLINICAL CHEMISTRY STUDIES

4.6.5.1 Acute

The effects of But-MMdCyd after administration of a single dose of 100 mg/kg, 200 mg/kg and 1000 mg/kg, intraperitoneally to male white Swiss CD-1 mice and killed on day 15 post But-MMdCyd administration on the clinical chemistry parameters investigated are summarised in Table 4.12. The values listed for each dose are mean ± standard deviation (SD) of samples data from animals in each group (n = 10). There were no statistically significant differences (P < 0.05) between the control and treated animals in any of the parameters examined.

4.6.5.2 Subacute

The effects of administration of But-MMdCyd (100mg/kg) daily for 15 days on clinical chemistry parameters of mice are summarized in Tables 4.13 and 4.14. The values of various parameters examined were very similar for control and treated animals during the treatment duration or the follow-up period. No consistent pattern of change or trend in toxicity for liver and kidney functions was evident from the examination of the data.

4.6.6 Urinalysis.

The urine sample collected from each animal was analysed for specific gravity, pH, the presence of protein, crystals, cast, heme, red blood cells (RBC), white blood cells (WBC)
and glucose, and the data is summarized in Tables 4.15 and 4.16. The values of all parameters of urine samples from treatment groups were similar to the control group.
Table 4.4 Effect of Methoxymethyl deoxycytidine (MMdCyd), butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) and didoxycytidine (ddC) on the doubling time of CEM cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doubling Time (hours)²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMdCyd (1000 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.9 ± 0.6</td>
<td>23.8 ± 0.6</td>
<td>23.9 ± 0.4</td>
<td>22.5 ± 0.5</td>
<td>23.5 ± 0.3</td>
<td>22.6 ± 0.5</td>
<td>23.4 ± 0.3</td>
<td>22.5 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>But-MMdCyd (1000 µM)</td>
<td>23.5 ± 1</td>
<td>22.8 ± 0.4</td>
<td>22.2 ± 0.6</td>
<td>21.8 ± 0.7</td>
<td>22.5 ± 0.9</td>
<td>23 ± 0.8</td>
<td>22.7 ± 1</td>
<td>23.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>But-MMdCyd (2000 µM)</td>
<td>23.4 ± 1.8</td>
<td>23.2 ± 0.8</td>
<td>21.5 ± 1.2</td>
<td>23.2 ± 0.6</td>
<td>22.8 ± 0.9</td>
<td>21.9 ± 0.6</td>
<td>22.2 ± 0.8</td>
<td>23 ± 1</td>
<td></td>
</tr>
<tr>
<td>ddC (0.5µM)</td>
<td>23.6 ± 4.5</td>
<td>44.3 ± 12.2</td>
<td>48.5 ± 8.3</td>
<td>54.2 ± 22</td>
<td>64.7 ± 12.7</td>
<td>75 ± 8.5</td>
<td>84.5 ± 17.4</td>
<td>83 ± 14.4</td>
<td></td>
</tr>
<tr>
<td>ddC (1 µM)</td>
<td>44.3 ± 7.1</td>
<td>53.8 ± 10.7</td>
<td>56.6 ± 11</td>
<td>83.4 ± 8.2</td>
<td>85.9 ± 11.1</td>
<td>83.7 ± 10.4</td>
<td>90.1 ± 4.1</td>
<td>86.6 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>Cell control</td>
<td>21.8 ± 0.5</td>
<td>23 ± 1.3</td>
<td>22.4 ± 0.5</td>
<td>22.9 ± 0.4</td>
<td>22.3 ± 0.6</td>
<td>21.9 ± 0.2</td>
<td>22.8 ± 0.5</td>
<td>24 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

1. CEM cells were incubated with the test compounds and drug ddC. The cell numbers were determined every two days and the doubling time was calculated.

2. The values shown are mean ± standard deviation (SD). Each assay was run in quadruplicate (n = 4)

3. Statistical analysis was performed using two way analysis of variance with P<0.05 chosen as the level of significance. The P values were 0.00045 (column) and 0.54 (rows) respectively.

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Fig. 4.1: Delayed cytotoxicity of dideoxycytidine (ddC), MMdCyd (5-methoxymethyl-2'-deoxycytidine) and But-MMdCyd (butanoyl-MMdCyd). Symbols: Panel A ddC: •, cell control; □, 0.5 µM; and ■, 1.0 µM; Panel B MMdCyd: ■, cell control; and □, 1000 µM; Panel C Bu-MMdCyd: •, cell control; □, 1000 µM; and ■, 2000 µM. The values shown are average of four independent assays ± SD. CEM cells were incubated with the drug and the doubling time was calculated.
Table 4.5 Acute Toxicity study. Effects of administration of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) on feed and water consumption in mice.

<table>
<thead>
<tr>
<th>Treatment schedule 2</th>
<th>Parameters evaluated 3</th>
<th>Feed consumption</th>
<th>Water consumption</th>
</tr>
</thead>
<tbody>
<tr>
<td>But-MMdCyd (mg/kg)</td>
<td></td>
<td>Week 1</td>
<td>Week 2</td>
</tr>
<tr>
<td>100</td>
<td>142.5</td>
<td>139.2</td>
<td>194.5</td>
</tr>
<tr>
<td>200</td>
<td>138.5</td>
<td>144.4</td>
<td>207.3</td>
</tr>
<tr>
<td>1000</td>
<td>147.5</td>
<td>138.4</td>
<td>198.6</td>
</tr>
<tr>
<td>Control 4</td>
<td>136.5</td>
<td>128.3</td>
<td>196.2</td>
</tr>
</tbody>
</table>

1. Male White Swiss CD-1 mice (weight 18-20g) were used. Group size n=10.

2. But-MMdCyd was administered in PBS by i.p. route. Control mice were given equivalent amount of PBS.

3. The data for feed consumption (g) and water consumption (ml) per 100 g body weight per week. The values shown are the average of the observations.

4. The normal values for feed and water consumption per 100 g body weight per week in mice are in the range (130-165 g) and (200-220 ml), respectively (Collins, 1979).
Table 4.6 Subacute Toxicity Study. Effects of administration of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) on feed and water consumption in mice.

<table>
<thead>
<tr>
<th>Parameters evaluated</th>
<th>Days</th>
<th>Control</th>
<th>But-MMdCyd</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed consumption</td>
<td>7</td>
<td>141.2 ± 2.6</td>
<td>135.0 ± 3.0</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>132.7 ± 2.7</td>
<td>140.4 ± 2.7</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>136.5 ± 3.5</td>
<td>139.5 ± 6.3</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>142.5 ± 4.8</td>
<td>135.2 ± 8.4</td>
<td>0.64</td>
</tr>
<tr>
<td>Water consumption</td>
<td>7</td>
<td>202.5 ± 4.2</td>
<td>212.0 ± 2.3</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>207.0 ± 7.0</td>
<td>203.0 ± 6.3</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>200.5 ± 3.1</td>
<td>207.0 ± 6.3</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>215.0 ± 6.3</td>
<td>211.0 ± 5.6</td>
<td>0.68</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (weight 18-20g) were used. Group size: day 7, n=25, day 15, n=20, day 22, n =10 and day 30, n=5.
2. The data for feed consumption (g) and water consumption (ml) per 100 g body weight per week. The values shown are mean ± standard deviation (SD).
3. The normal values for feed and water consumption per 100 g body weight per week in mice are in the range (130-175 g) and (200-220 ml) respectively (Collins, 1979).
4. But-MMdCyd was administered in PBS by i.p. route. Control mice were given equivalent amount of PBS.
5. The data was analyzed using one way analysis of variance (P < 0.05).
Table 4.7 Acute Toxicity study: Number of mice with histopathological lesions killed on day fifteen post administration of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd)\(^1\)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesions and extent(^2)</th>
<th>Control</th>
<th>But-MMdCyd (mg/kg)(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Spleen</td>
<td>Congestion (2)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>Nil (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
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<td></td>
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<td>0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>Nil (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200</td>
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<td>0</td>
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<tr>
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<td></td>
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<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>Nil (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

1. Male White Swiss CD-1 mice (weight 18-20g) were used. Group size n=10.

2. The tissues were evaluated for histopathological lesions by conventional light microscopy. The scoring system used for lesions was: nil = 0; Minimal 1% = 1; Mild (1-10%) = 2; Moderate (10-25%) = 3; and Severe (> 75%) = 4. The extent of lesions is shown in brackets.

3. Butanoyl-MMdCyd was administered in PBS i.p. Control mice were given equivalent amount of PBS.
4.8 Subacute Toxicity Study: Number of mice with histopathological lesions treated with 100 mg/kg of butanoyl-5-methoxymethy-2'-deoxycytidine (But-MMdCyd) for fifteen days

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesions and extent</th>
<th>But-MMdCyd Days</th>
<th>Control Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Spleen</td>
<td>Congestion (2)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Kidney</td>
<td>Multi focal lymphocytic infiltration (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>Nil (0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>Nil (0)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (wt 18-20 g) were used. Group size: day 7, n=25, day 15, n=20, day 22, n =10 and day 30, n=5. At periodic intervals, equal number of mice from control and drug-treated was killed by CO$_2$ inhalation overdose to determine the drug-related toxic effects during treatment as well as during post recovery period.

2. The tissues were evaluated for histopathological lesions by conventional light microscopy. The scoring system used for lesions was nil = 0; Minimal 1%= 1; Mild (1-10%)= 2; Moderate (10-25%)= 3; and Severe (> 75%)= 4. The extent of lesions is shown in brackets.

3. Butanoyl-MMdCyd (100 mg/kg) was administered daily in PBS i.p. Control mice were given equivalent amount of PBS.
Fig. 4.2: Acute toxicity trial. Weight gain of mice given different dosages of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) on day 15 after But-MMdCyd administration. Data (mean ± SD, n = 10 for all groups) indicates no effect using ANOVA (P = 0.35).
Fig. 4.3. Subacute toxicity study. Weight gain of control mice and mice treated with 100 mg/Kg of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) on day 14 (A) and day 30 (B). Data (mean + SD, n = 20 on day 14 and n = 5 on day 30) indicates no effect of But-MMdCyd using ANOVA (P = 0.64 and 0.67 respectively on days 14 and 30)
Fig. 4.4 Liver of mice treated intraperitoneally with butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) in the toxicity study (10X). (A) Acute toxicity study (1000 mg/kg of But-MMdCyd, killed on day 15 post drug administration). (B) Subacute toxicity study (100 mg/kg of But-MMdCyd daily for 15 days, killed on day 30). (C) Control (0.2 ml of PBS administrated daily for 15 days, killed on day 15). The liver has a normal architecture and vasculitis, bile duct proliferation, cell necrosis, steatosis, lobular inflammation, and Kupffer cell hyperplasia are absent in all the sections.
Fig. 4.5 Kidney of mice treated intraperitoneally with butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) in the toxicity study (10X). (A) Acute toxicity study (1000 mg/kg of But-MMdCyd, killed on day 15 post drug administration). (B) Subacute toxicity study (100 mg/kg of But-MMdCyd daily for 15 days, killed on day 30). (C) Control (0.2 ml of PBS administrated daily for 15 days, killed on day 15). The kidney has normal architecture and glomerulitis, tubulitis, tubular casts, vasculitis, inflammation and infiltration are absent.
Fig. 4.6 Spleen of mice treated intraperitoneally with butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) in the toxicity study (10X). (A) Acute toxicity study (1000 mg/kg of But-MMdCyd, killed on day 15 post drug administration). (B) Subacute toxicity study (100 mg/kg of But-MMdCyd daily for 15 days, killed on day 30). (C) Control (0.2 ml of PBS administered daily for 15 days, killed on day 15. The spleen has a normal architecture and necrosis, hemosiderosis, and lymphocytic infiltration are absent.
Fig. 4.7 Brain of mice treated intraperitoneally with butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) in the toxicity study (10X). (A) Acute toxicity study (1000 mg/kg of But-MMdCyd, killed on day 15 post drug administration). (B) Subacute toxicity study (100 mg/kg of But-MMdCyd daily for 15 days, killed on day 30). (C) Control (0.2 ml of PBS administrated daily for 15 days, killed on day 15). The brain has a normal architecture and vacuolization, necrosis, inflammation and cellular infiltration are absent.
Table 4.9 Acute Toxicity Study. Hematological parameters of control mice\(^1\) and mice treated with butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd)\(^2\).

<table>
<thead>
<tr>
<th>Parameters(^3)</th>
<th>Range(^4)</th>
<th>Control(^5)</th>
<th>But-MMdCyd (mg/kg)</th>
<th>P value(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10(^{12})/L)</td>
<td>7-9.5</td>
<td>7.25 ± 0.69</td>
<td>7.05 ± 0.74</td>
<td>7.09 ± 0.67</td>
</tr>
<tr>
<td>WBC (10(^9)/L)</td>
<td>2-12</td>
<td>7.42 ± 1.66</td>
<td>8.10 ± 1.69</td>
<td>8.22 ± 2.45</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>120-170</td>
<td>125.5 ± 7.4</td>
<td>123.6 ± 12.1</td>
<td>125.2 ± 8.3</td>
</tr>
<tr>
<td>HCT (L/L)</td>
<td>0.35-0.45</td>
<td>0.37 ± 0.02</td>
<td>0.367 ± 0.014</td>
<td>0.365 ± 0.014</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>45-55</td>
<td>57.6 ± 1.9</td>
<td>56.5 ± 1.2</td>
<td>55.6 ± 2.5</td>
</tr>
<tr>
<td>Platelets (10(^9)/L)</td>
<td>900-1800</td>
<td>1592 ± 270</td>
<td>1631 ± 257</td>
<td>1508 ± 248</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (weight 18-20g) were used. Group size, n=10.
2. A single dose of But-MMdCyd was administrated i.p.; mice were sacrificed on day 15.
3. The values obtained for each parameter are mean ± standard deviation (SD): RBC – Red blood cells; WBC -- white blood cells; Hb -- Hemoglobin; HCT – hematocrit; MCV – mean corpuscular volume.
4. The values indicated are taken from Hall (1998).
5. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance.
Table 4.10 Subacute Toxicity Study. Hematological parameters of control mice and mice given 100 mg/kg of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) daily for 5 and 15 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Sacrifice time (days)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>P value</td>
<td>Control</td>
<td>Treated</td>
<td>P value</td>
</tr>
<tr>
<td>RBC ((10^{12}/L))</td>
<td>7-9.5</td>
<td>± 6.92± 0.38</td>
<td>± 7.22± 0.68</td>
<td>0.4</td>
<td>± 6.95± 0.58</td>
<td>± 7.05± 0.63</td>
<td>0.72</td>
</tr>
<tr>
<td>WBC ((10^9/L))</td>
<td>2-12</td>
<td>± 5.90± 2.58</td>
<td>± 8.60± 2.23</td>
<td>0.22</td>
<td>± 8.21± 2.25</td>
<td>± 7.02± 2.03</td>
<td>0.25</td>
</tr>
<tr>
<td>Hb ((g/L))</td>
<td>120-170</td>
<td>± 130.0± 8.3</td>
<td>± 128.0± 7.1</td>
<td>0.69</td>
<td>± 127.0± 9.9</td>
<td>± 125.9± 13.3</td>
<td>0.83</td>
</tr>
<tr>
<td>HCT ((L/L))</td>
<td>0.35-0.45</td>
<td>± 0.36± 0.01</td>
<td>± 0.36± 0.02</td>
<td>0.72</td>
<td>± 0.37± 0.01</td>
<td>± 0.36± 0.02</td>
<td>0.5</td>
</tr>
<tr>
<td>Platelets ((10^9/L))</td>
<td>900-1800</td>
<td>± 1560± 256</td>
<td>± 1666± 250</td>
<td>0.52</td>
<td>± 1550± 370</td>
<td>± 1614± 286</td>
<td>0.82</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (wt 18-20 g) were used. Group size: day 7, n=5, day 15 n=10. On days 5 and 15 equal number of mice from control and drug-treated were killed by CO2 inhalation overdose.

2. Butanoyl-MMdCyd (100 mg/kg) in 0.2 ml PBS was administered daily i.p. Control mice were given equivalent amount of PBS.

3. The values obtained for each parameter is mean ± standard deviation (SD): RBC -- Red blood cells; WBC -- white blood cells; Hb -- Hemoglobin; HCT -- hematocrit.

4. The values indicated are taken from Hall (1998).

5. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance.
Table 4.11. Subacute Toxicity. Hematological parameters of control mice and mice given 100 mg/kg of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) daily for 15 days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>22</th>
<th>30</th>
<th>22</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10¹²/L)</td>
<td>7-9.5</td>
<td>7.36 ± 0.61</td>
<td>7.18 ± 1.05</td>
<td>0.75</td>
<td>7.14 ± 0.79</td>
</tr>
<tr>
<td>WBC (10⁹/L)</td>
<td>2-12</td>
<td>8.7 ± 2.47</td>
<td>8.33 ± 3.03</td>
<td>0.75</td>
<td>8.98 ± 2.88</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>120-170</td>
<td>126.2 ± 5.8</td>
<td>133.9 ± 12.1</td>
<td>0.3</td>
<td>122.2 ± 1.4</td>
</tr>
<tr>
<td>HCT (L/L)</td>
<td>0.35-0.45</td>
<td>0.37 ± 0.01</td>
<td>0.360 ± 0.021</td>
<td>0.73</td>
<td>0.366 ± 0.014</td>
</tr>
<tr>
<td>Platelets (10⁹/L)</td>
<td>900-1800</td>
<td>1520 ± 251</td>
<td>1558 ± 263</td>
<td>0.82</td>
<td>1462 ± 300</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (wt 18-20 g) were used. Group size, n=5. On day 22 and day 30 equal number of mice from control and drug-treated were killed by CO₂ inhalation overdose.

2. Butanoyl-MMdCyd (100 mg/kg) in 0.2 ml PBS was administered daily i.p. Control mice were given equivalent amount of PBS.

3. The values obtained for each parameter is mean ± standard deviation (SD): RBC – Red blood cells; WBC – White blood cells; Hb – Hemoglobin; HCT – Hematocrit.

4. The values indicated are taken from Hall (1998).

5. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance.
Table 4.12 Acute Toxicity Study. Clinical chemistry parameters of control mice and mice treated with butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Range</th>
<th>Control</th>
<th>But-MMdCyd (mg/kg)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>12-16</td>
<td>± 14.9</td>
<td>± 15.3</td>
<td>± 14.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 2.3</td>
<td>± 1.6</td>
<td>± 2.5</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>25-200</td>
<td>± 107.5</td>
<td>± 102.3</td>
<td>± 103.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 16.8</td>
<td>± 12.9</td>
<td>± 14.5</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>70-400</td>
<td>± 364.6</td>
<td>± 382.0</td>
<td>± 388.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 40.6</td>
<td>± 42.09</td>
<td>± 44.3</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>15-45</td>
<td>± 24.5</td>
<td>± 24.4</td>
<td>± 24.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.8</td>
<td>± 1.3</td>
<td>± 1.3</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>0.3-1</td>
<td>± 0.63</td>
<td>± 1.10</td>
<td>± 0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.24</td>
<td>± 1.07</td>
<td>± 0.31</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>135-167</td>
<td>± 137.9</td>
<td>± 137.4</td>
<td>± 138.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 2.9</td>
<td>± 1.8</td>
<td>± 2.7</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>5-8.5</td>
<td>± 5.2</td>
<td>± 5.21</td>
<td>± 5.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.1</td>
<td>± 0.06</td>
<td>± 0.05</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>48-70</td>
<td>± 62.4</td>
<td>± 61.1</td>
<td>± 63.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 33.7</td>
<td>± 27.7</td>
<td>± 17.1</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (weight 18-20g) were used. Group size, n=10.
2. A single dose of But-MMdCyd was administrated i.p.; mice were sacrificed on day 15.
3. The values obtained for each parameter is mean ± standard deviation (SD): ALT -- Alanine aminotransferase; AST -- Aspartate aminotransferase; BUN -- Blood urea nitrogen.
4. The values indicated are taken from Hall (1998).
5. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance.
Table 4.13 Subacute Toxicity Study. Clinical Chemistry parameters of control mice and mice given 100 mg/kg of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) daily for 5 and 15 days.

<table>
<thead>
<tr>
<th>Parameter (mmol/L)</th>
<th>12-16</th>
<th>25-200</th>
<th>70-400</th>
<th>15-45</th>
<th>0.3-1</th>
<th>135-167</th>
<th>5-8.5</th>
<th>48-70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>14.9 ± 2.3</td>
<td>104.5 ± 14.5</td>
<td>374.4 ± 36.3</td>
<td>25.08 ± 2.14</td>
<td>0.74 ± 0.33</td>
<td>137.6 ± 6.3</td>
<td>5.17 ± 0.12</td>
<td>63.4 ± 36.5</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>15.3 ± 1.6</td>
<td>101.3 ± 7.9</td>
<td>373.0 ± 58.6</td>
<td>23.24 ± 1.20</td>
<td>0.68 ± 0.25</td>
<td>137.8 ± 6.4</td>
<td>5.25 ± 0.10</td>
<td>62.2 ± 14.8</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>14.4 ± 1.1</td>
<td>104.8 ± 17.1</td>
<td>374.4 ± 36.0</td>
<td>24.3 ± 1.4</td>
<td>0.96 ± 1.00</td>
<td>136.0 ± 4.4</td>
<td>5.3 ± 0.26</td>
<td>69.2 ± 7.6</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>15.1 ± 1.8</td>
<td>104.4 ± 15.1</td>
<td>373.0 ± 58.6</td>
<td>23.6 ± 1.2</td>
<td>1.01 ± 0.99</td>
<td>137.6 ± 4.8</td>
<td>5.35 ± 0.31</td>
<td>73.6 ± 6.11</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>14.4 ± 1.8</td>
<td>104.4 ± 15.1</td>
<td>373.0 ± 58.6</td>
<td>23.6 ± 1.2</td>
<td>1.01 ± 0.99</td>
<td>137.6 ± 4.8</td>
<td>5.35 ± 0.31</td>
<td>73.6 ± 6.11</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>136.0 ± 4.4</td>
<td>137.6 ± 4.8</td>
<td>137.6 ± 4.8</td>
<td>137.6 ± 4.8</td>
<td>137.6 ± 4.8</td>
<td>137.6 ± 4.8</td>
<td>137.6 ± 4.8</td>
<td>137.6 ± 4.8</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>5.3 ± 0.26</td>
<td>5.35 ± 0.31</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>69.2 ± 7.6</td>
<td>73.6 ± 6.11</td>
<td>69.2 ± 7.6</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
<td>73.6 ± 6.11</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (wt 18-20 g) were used. Group size: day 7, n=5, day 15 n=10. At periodic intervals equal number of mice from control and drug-treated were killed by CO₂ inhalation overdose.
2. Butanoyl-MMdCyd (100 mg/kg) in 0.2 ml PBS was administered daily i.p. Control mice were given equivalent amount of PBS.
3. The values obtained for each parameter is mean ± standard deviation (SD): RBC — Red blood cells; WBC — white blood cells; Hb — Hemoglobin; HCT — hematocrit.
4. The values indicated are taken from Hall (1998).
5. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance.
Table 4.14 Subacute Toxicity Study. Clinical Chemistry parameters of control mice and mice given 100 mg/kg of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd).

| Parameter  | Range | 22 Sacrifice time | 30 Sacrifice time | P value
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>12-16</td>
<td>13.8 ± 1.3</td>
<td>14.3 ± 1.6</td>
<td>0.72</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>25-200</td>
<td>110.8 ± 16.2</td>
<td>103.4 ± 16.7</td>
<td>0.54</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>70-400</td>
<td>382.0 ± 61.3</td>
<td>394.0 ± 47.6</td>
<td>0.75</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>15-45</td>
<td>25.1 ± 1.2</td>
<td>24.3 ± 1.2</td>
<td>0.30</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>0.3-1</td>
<td>1.00 ± 0.31</td>
<td>0.96 ± 0.32</td>
<td>0.85</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>135-167</td>
<td>137.0 ± 3.4</td>
<td>139.2 ± 4.2</td>
<td>0.52</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>5-8.5</td>
<td>5.27 ± 0.24</td>
<td>5.23 ± 0.14</td>
<td>0.75</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>48-70</td>
<td>62.4 ± 28.4</td>
<td>65.1 ± 40.7</td>
<td>0.35</td>
</tr>
</tbody>
</table>

1. Male white Swiss CD-1 mice (wt 18-20 g) were used. Group size: day 7, n=5, day 15 n=10. At periodic intervals equal number of mice from control and drug-treated were killed by CO₂ inhalation overdose.
2. Butanoyl-MMdCyd (100 mg/kg) in 0.2 ml PBS was administered daily i.p. Control mice were given equivalent amount of PBS.
3. The values obtained for each parameter is mean ± standard deviation (SD): RBC -- Red blood cells; WBC -- white blood cells; Hb -- Hemoglobin; HCT -- hematocrit.
4. The values indicated are taken from Hall (1998).
5. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance.
Table 4.15 Acute Toxicity Study. Parameters of urine analysis of mice\textsuperscript{1} and mice given butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd)\textsuperscript{2}

<table>
<thead>
<tr>
<th>Parameter\textsuperscript{a3}</th>
<th>Day</th>
<th>Control</th>
<th>But-MMdCyd (mg/kg)</th>
<th></th>
<th></th>
<th></th>
<th>100</th>
<th>200</th>
<th>1000</th>
<th>P value\textsuperscript{4}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific gravity</td>
<td>7</td>
<td>1.020 ± 0.002</td>
<td>1.017 ± 0.006</td>
<td>1.023 ± 0.004</td>
<td>1.022 ± 0.003</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.023 ± 0.005</td>
<td>1.019 ± 0.003</td>
<td>1.024 ± 0.008</td>
<td>1.021 ± 0.006</td>
<td>0.64</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>7</td>
<td>7.88 ± 0.15</td>
<td>5.78 ± 0.26</td>
<td>6.04 ± 0.25</td>
<td>6.98 ± 0.37</td>
<td>0.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.86 ± 0.36</td>
<td>5.86 ± 0.36</td>
<td>5.92 ± 0.15</td>
<td>6.07 ± 0.26</td>
<td>0.71</td>
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<td>Protein</td>
<td>7</td>
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</table>

\textsuperscript{a} All urine samples were negative for RBC, WBC, casts and crystals.

1. Male white Swiss CD-1 mice (weight 18-20g) were used. Group size, n=10.

2. A single dose of But-MMdCyd was administrated i.p.; mice were sacrificed on day 15.

3. The values obtained for each parameter is mean ± standard deviation (SD).

4. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance.
Table 4.16 Subacute Toxicity Study. Parameters of urine analysis of mice and mice given 100 mg/kg of butanoyl-5-methoxymethyl-2'-deoxycytidine (But-MMdCyd) daily for 15 days.

<table>
<thead>
<tr>
<th>Parameters&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day</th>
<th>Control&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Treated</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Specific gravity</td>
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<tr>
<td></td>
<td>7</td>
<td>1.016 ± 0.044</td>
<td>1.120 ± 0.003</td>
<td>0.11</td>
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<tr>
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<td>15</td>
<td>1.017 ± 0.003</td>
<td>1.019 ± 0.003</td>
<td>0.49</td>
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<tr>
<td></td>
<td>22</td>
<td>1.020 ± 0.003</td>
<td>1.022 ± 0.012</td>
<td>0.92</td>
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<tr>
<td></td>
<td>30</td>
<td>1.017 ± 0.003</td>
<td>1.022 ± 0.002</td>
<td>0.09</td>
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<td>PH</td>
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<td>6.10 ± 0.39</td>
<td>5.80 ± 0.44</td>
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<td>15</td>
<td>5.98 ± 0.38</td>
<td>5.66 ± 0.43</td>
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<td>22</td>
<td>5.86 ± 0.45</td>
<td>6.26 ± 0.11</td>
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<td>30</td>
<td>6.12 ± 0.26</td>
<td>6.14 ± 0.29</td>
<td>0.91</td>
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<td>Glucose</td>
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<sup>a</sup>All urine samples were negative for RBC, WBC, casts and crystals.

1. Male white Swiss CD-1 mice (wt 18-20 g) were used. Group size: day 7, n=25; day 15, n=20; day 22, n=10 and day 30, n=5. At periodic intervals, equal number of mice from control and drug-treated was killed by CO<sub>2</sub> inhalation overdose to determine the drug-related toxic effects during treatment as well as during post recovery period.
2. Butanoyl-MMdCyd (100 mg/kg) in 0.2 ml PBS was administered PBS i.p. Control mice were given equivalent amount of PBS.
3. The values obtained for each parameter is mean ± standard deviation (SD).
4. Statistical analysis was performed using one way analysis of variance with P <0.05 chosen as the level of significance
Nucleoside analogues used for treatment of cancer and viral infections are structurally related to natural substrates utilized by the host cell for the synthesis of DNA. Thus, some of these drugs have been found to exhibit unique multi-organ toxicosis on long term therapy. The manifestations of toxicity suggest defective cellular multiplication due to inhibition of polymerases required for the synthesis of nuclear DNA or due to defective mitochondrial DNA (mtDNA) as a result of the inhibition of mitochondrial DNA polymerase gamma (Haefeli et al., 1993; Saltzman et al., 1994; Tsai et al., 1994; Agarwal and Olivero, 1997; Parker et al., 1997; Shi et al., 1997; Sawyer et al., 1988).

Manifestations of toxicity due to long term use of antiviral nucleoside analogs (ANA) in selected tissues appears be due to the combined effect of four principal factors: (i) subcellular availability of ANA in the target tissue; (ii) anabolism of ANA by cellular kinases (thymidine and deoxycytidine kinases) to monophosphate (activation) and the ability of cellular enzymes to convert ANA monophosphate to ANA triphosphate (active form of the drug); (iii) the ability of ANA triphosphate to inhibit DNA polymerases by serving as competitive alternate substrates and incorporation into DNA by terminating the DNA chain (chain termination) and (iv) the metabolic requirements in the target tissue for oxidative phosphorylation. Thus drugs which have the ability to inhibit nuclear DNA synthesis are more toxic, e.g., arabinofuranosyl cytosine (Ara-C) and to 2-fluoro-5-iodo arabinofuranosyl uracil [fialuridine, (FIAU)]. In contrast, drugs like dideoxycytidine (ddC) and azidothymidine (AZT) appear to cause multi-organ toxicosis due to defective
mitochondrial DNA synthesis (Parker et al., 1997; Chen et al., 1991; Chen and Cheng, 1989). According to DNA polymerase γ (gamma) hypothesis, the cellular enzymes play a critical role in mt DNA toxicity because these enzymes phosphorylate the nucleoside analogs to their corresponding monophosphates (Tsai et al., 1994; Parker et al., 1997; Shi et al., 1997).

Several investigations have shown that the potential of nuclear and mitochondrial DNA toxicity of a nucleoside analog can be estimated in situ using lymphoblast cell lines (Colacino et al., 1997; Shi et al., 1997; Temant et al., 1998). There appears to be good correlation between delayed cytotoxicity and mitochondrial DNA levels. High concentrations of drugs like ddC produce acute toxicity in CEM cells and this is due to incorporation of ddC into nuclear DNA. In contrast, low concentrations of ddC induced delayed toxicity in cell cultures and resulted in depletion of mt DNA (Chen et al., 1991; Lewis et al., 1994; Lewis and Dalakas, 1996). Dideoxycytidine lacks the 3′-OH group necessary for DNA chain extension and therefore incorporation of ddC into DNA results in obligatory chain termination and thus inhibition of DNA synthesis (Martin et al., 1998). Considerable effort has been devoted to understand the multi-organ toxic manifestations exhibited by fialuridine (FIAU). Metabolic studies on FIAU using human lymphoblast cell lines suggest that its toxicity is associated with its phosphorylation by the cellular enzymes and incorporation into DNA (Klecker et al., 1994; Colacino et al., 1996; Lewis et al., 1996; Martin et al., 1998). However, the delayed cytotoxicity (retardation of cell growth after long term exposure at low concentration) of FIAU has been correlated with
mitochondrial injury (Colacino et al., 1994; Parker and Cheng, 1994). Recent studies have shown that the hepatic injury seen in patients and experimental animals treated with fialuridine is due to marked decrease in hepatic mitochondrial DNA (Tennant et al., 1998). The delayed onset of the mitochondrial toxicity might be due the presence of multiple copies of mt DNA in the cell.

In summary, the antiviral nucleoside analogs (ANA's) phosphorylated by the cellular kinases (non-selective phosphorylation) are generally more toxic to host. The in vitro assays of delayed cytotoxicity by monitoring the doubling time are useful in predicting the potential of delayed clinical toxicity of ANA's (Chen et al., 1991; Parker and Cheng, 1994; Lewis and Dalakas, 1995; Parker et al., 1997).

The result of cytotoxicity studies indicate that ddC (control drug) at high concentrations produced acute toxicity (cell growth inhibited within four days) and at very low concentrations induced delayed cytotoxicity (cell growth retarded after long term exposure). In contrast, MMdCyd and But-MMdCyd did not exhibit either acute or delayed cytotoxicity in the assay system. The compounds MMdCyd and But-MMdCyd are potent antiherpes agents with low cytotoxicity (section 3.5.1 to 3.5.4; pages 50 to 53). Earlier studies have shown the selectivity of MMdCyd is due to its anabolism by the viral-induced dThd kinase (deoxythymidine/deoxycytidine kinase) to its monophosphate (MMdCMP) primarily in HSV-infected cells (Aduma et al., 1991). Further studies have shown that MMdCTP (active drug) is a better substrate for DNA polymerase induced by HSV-1. Interestingly, MMdCTP is also a good substrate for human DNA polymerase.
alpha (α) and gamma (γ) (Aduma et al., 1992; Shi et al., 1997). Thus based on these results, one would expect that MMdCyd should exhibit acute and possibly delayed cytotoxicity in CEM cells. However, the lack of cytotoxicity against rapidly dividing cells indicates that MMdCyd is not anabolized in uninfected cells because most likely MMdCyd does not serve as a substrate for cellular deoxycytidine kinases. These results are in agreement with earlier findings from this laboratory (Shi et al., 1997). The lack of cytotoxicity of But-MMdCyd in rapidly dividing cells also indicates that But-MMdCyd is also most likely not anabolized in uninfected cells, because it does not serve as a substrate for cellular deoxycytidine kinase and therefore the monophosphate (But-MMdCMP) is not formed in adequate amounts. Alternatively, it is conceivable that But-MMdCMP, if formed, does not serve, as a substrate for cellular enzymes deoxycytidylate kinases and thus triphosphate (But-MMdCTP) is not generated in significant amounts in uninfected cells. Consequently little or no But-MMdCTP is available for incorporation either into the nuclear or mitochondrial DNA and thus neither acute or delayed cytotoxicity is observed when CEM cells are exposed even to massive concentrations of But-MMdCyd. However, to clearly delineate the basis of lack of cytotoxicity of these compounds, further studies on metabolic fate of MMdCyd and But-MMdCyd in infected and uninfected cells need to be undertaken. These studies will be greatly facilitated by the use of radiolabelled compounds.

The aims of acute and subacute toxicity trials are to determine the dose limiting toxicities of the compound, to identify the target organ, and to determine whether the drug induced
injuries, if any, can be repaired. Histological, hematological and clinical chemistry parameters, are useful to identify the target organ(s) for toxicity of test substance. During pre-clinical and early clinical phases of the pharmaceutical discovery and development process, an important aspect of drug safety assessment involves monitoring for drug induced hepatic and renal injury (Amacher, 1998). Drug induced hepatic injuries vary in nature from direct, intrinsic effects that are observed in most animals and more than one species to rare ‘idiosyncratic’ responses seen only in few animals. Histological type of injury varies from hepatocellular to hepatobiliary with multiple cellular characteristics of each type. Of the various clinical laboratory markers for hepatic injury, ALT and AST are the most sensitive indicators of toxic damage in mice (Gad and Chengelis, 1998).

Decreased ability to concentrate urine is one of the most sensitive indicators of renal injury in subacute toxicity studies (Kluwe, 1981). Of the various clinical laboratory markers for renal injury, BUN and creatinine are the most sensitive indicators of toxic damage in mice (Gad and Chengelis, 1998). Studies with antiviral nucleoside analogs indicate that low platelet count and anemia is correlated with bone marrow toxicity (Tucker et al., 1983).

The systemic toxicity of antiviral nucleoside analogs currently approved or under development has been well documented in animal species and humans (Chan et al., 1982; Ayers, 1988; Lindstrom et al., 1990; Philips et al., 1991; Hayden, 1995). Nucleoside analogs generally have low toxicity after oral administration. The LD$_{50}$ of AZT, ddC, ddI, d4T, ACV and Ara-A after oral administration in mice were: >3000 mg/kg, >4000 mg/kg.
>3000mg/kg, >2000mg/kg, >5000 mg/kg and 600 mg/kg respectively (Tucker et al., 1983; Szczech and Tucker, 1992). The LD₅₀ of AZT was greater than 750 mg/kg in mice when it was administered by i.v. (Ayers, 1988). The LD₅₀ values of ACV and Ara-A were 6000 mg/kg and 5000 mg/kg, respectively, in mice when the compounds were administered I.V. (Tucker et al., 1983; Szczech and Tucker, 1992). A study conducted in mice using a 3-consecutive-day treatment protocol (dose 100-500 mg/kg) has shown that with the exception of ddI, all other ddNs (AZT, d4T, ddA and ddC) induced hematological toxicities characterized by anemia, and thrombocytopenia (Ayers, 1988). The subacute and subchronic studies of AZT and d4T at doses of 100 mg/kg carried out in mice, dogs and monkeys revealed gastrointestinal, hepatic and renal abnormalities (Ayers, 1988; Schilling et al., 1995). Toxicity of acyclovir in animals has been well documented. Tucker et al. (1983) reported signs of toxicosis consisting of ataxia and depression in mice receiving greater than 600 mg/kg of acyclovir i.v. in acute toxicity study. Sedation occurred in mice and rats after i.p. administration at the same dose. In a one-month oral toxicity study in mice, given 450 mg/kg of acyclovir daily for 33 days produced a slight decrease in leukocyte count. The most serious toxicity associated with repeated dose administration of acyclovir was the development of obstructive nephropathy seen at doses of 20 mg/kg/day or larger for 21 days. Renal damage was caused by the precipitation of drug crystals, which produced acute interstitial nephritis (Tucker et al., 1983). In these studies, increases in water consumption, urine output with low urine specific gravity (<1.012), elevated BUN and creatinine were observed.
A phase II trial of the nucleoside analog, fialuridine (FIAU) was halted because of the sudden development of severe multi-system toxicity (hepatic failure, lactic acidosis and pancreatitis), which resulted in the deaths of five patients (McKenzie, 1995; Kliener et al., 1998). Severe hepatotoxicity from FIAU was characterized by hepatomegaly with diffuse, predominantly microvesicular steatosis, hepatocellular glycogen depletion, marked bile duct proliferation, and cholestasis. The microscopic and ultrastructural patterns of injury and systemic symptoms in patients with FIAU toxicity are consistent with severe mitochondrial and metabolic derangement (Kleiner et al., 1998). At a dose of 1.5 mg/kg, daily for 14 days Tennant et al. (1998) noticed similar hepatic pathologic findings, in woodchucks treated with FIAU orally.

Kurtz (1975) reported lymphopenia, increased values for serum alkaline phosphatase and transaminases and hepatic megalocytosis in mice given arabinosyl adenine (Ara-A) 50 mg/kg, i.p., for 21 days. These alterations in clinical pathology tests did not improve during the two-week post dose drug free recovery period. Biro and Goldenberg (1971) reported that administration of cytosine arabinoside (Ara-C, a nucleoside analog approved for cancer treatment) at 100 mg/kg for 15 days orally, produced cessation of normoblast production and other hematological effects characterized by non-regenerative anemia, and low platelet count in mice.

Ribavarin, a triazole nucleoside having broad-spectrum antiviral activity, was relatively well tolerated when given orally (100 mg/kg for 3 days) to monkeys, rats and mice (Sidwell et al., 1978 and 1979). However, oral dose levels of 120 mg/kg/day or
larger for 15 days in monkeys and 100 mg/kg/day or larger doses for 15 days in mice caused anemia. Reno et al. (1982) reported non-regenerative anemia and lymphoid depletion in mice treated with 60 mg/kg/day for 28 days with ribavarin.

Preclinical toxicity studies on the nucleoside analog, 2'-valeryl-6-methoxypurine arabinoside, at doses of 25, 50 and 100 mg/kg given daily orally for 14 days produced severe toxicosis including decrease in food consumption, decreased values of RBC and WBC counts, and behavioural changes (body tremors, incoordination, reduced motility, sleepiness, stupor and lack of eye tracking) in monkeys (Szczech and Tucker, 1992). These signs were first observed during the first week of dosing. In rats given single daily doses of this compound at 150, 300 and 600 mg/kg orally produced decrease in body weights and food consumption and alterations of hematological parameters (RBC, WBC and platelet numbers), hemoglobin concentration and hematocrit and serum chemistry parameters (glucose, serum protein, ALT and AST). Liver lesions consisting of necrosis of individual hepatocytes, megalocytosis, and biliary stasis was observed for animals given 600 mg/kg. Clinical signs of central nervous system toxicity (ataxia and altered reflexes) were also reported in rats at these doses. Histopathological examination of the brain revealed groups of small vacuoles in cerebellar white matter.

In summary, from the foregoing discussion it is clear that toxic manifestations exhibited by ANAs are drug-related. The route of administration, duration of treatment, and the total dose administered are other factors, which determine the toxicity of nucleoside analogs.
The toxicity studies presented in this chapter suggest that But-MMdCyd have low mammalian toxicity. In this respect, the behavior of But-MMdCyd is similar to other selective antiherpes agents, methoxymethyldeoxyuridine (MMdUrd), bromovinyl deoxyuridine (BrVdUrd) and acyclovir (Meldrum et al., 1980; Tucker et al., 1983; Ayisi et al., 1986; Machida and Sakata, 1994; Griffiths, 1995). The acute parenteral toxicity of But-MMdCyd appears to be in the same order of magnitude as that of MMdUrd (LD$_{0}$, i.p., > 4000 mg/kg) and Ara-A (LD$_{50}$, i.p., > 5000 mg/kg) and compares favorably with that of acyclovir (LD$_{50}$ = 999 mg/kg, i.p.) in mice ((Meldrum et al., 1980; Tucker et al., 1983). Since no adverse effects in mice have been observed at the highest dosage tested (1000 mg/kg), further studies at higher dosages and in other species are indicated to clearly define the toxicity of But-MMdCyd.

There were no behavioural or functional changes in any of the animals in either the acute study or the subacute study. This information was corroborated by the absence of any histopathological changes, in liver and kidney. The findings of focal lymphocytic infiltration is an incidental finding and are unrelated to drug administration because this was seen in both the control and treatment group (2 animals from a group of 25 in the control group and 1 animal from a group of 25 in the treatment group) and no consistent pattern could be established. The reasons for these findings are not clear at this time. The congestion of spleen observed in few animals in both the acute study and subacute study may be due to the euthanasia procedure. But-MMdCyd did not cause any histological changes in the kidney. In addition, BUN and creatinine were within the normal values,
which indicate that renal system also is not a target tissue for But-MMdCyd. The dose level of 100 mg/kg in the subacute toxicity study was a no effect level in all respects. The routine urinalysis further supported this finding.

The absence of any significant changes in ALT and AST values suggest that liver is not a target organ for But-MMdCyd, in mice. Previous studies indicate that liver is a target organ for acute toxic manifestations of nucleoside analogs stavudine and FIAU (Schiling et al., 1995; Tennant et al., 1998) In both studies, there were histological changes in liver specimens. The ALT and AST values were elevated and corroborated with pharmaco-toxic changes manifested by these compounds. Comparison of data from hematological examination indicates the lack of toxicity to the hemopoietic system. The platelet counts were normal. One of the weaknesses of this study was the poor quality of the bone marrow slides, which made the subjective evaluation and interpretation of these slides impossible. The most logical explanation for the lack of toxicity of But-MMdCyd in vivo is that this compound is not anabolized to the phosphorylated products, in uninfected cells, and hence the lack of target organ toxicity.

In summary, the lack of any toxicity as evidenced by normal weight gain, absence of behavioural changes, normal hematological and clinical chemistry parameters, rules out the possibility of target organ toxicity by But-MMdCyd after the administration of a single dose (1000 mg/kg) or repeated daily dose of 100 mg/kg for 15 days. Considering the fact that absorption after i.p. administration is rapid and the distribution of the drug by i.p. route parallels that by iv. route, it is reasonable to conclude that But-MMdCyd has very
low mammalian toxicity and in this respect, the behaviour of this nucleoside analog is uniquely different in comparison to other drugs approved for the treatment of systemic herpes infections. Toxicokinetic studies should be carried out to address the question of target organ toxicity more extensively.

Some of the variables which are not addressed in this toxicology study are species, strain, variation within species, sex difference, diurnal variations, age differences and dosage, route, frequency and duration of administration of drug. It is well established that, different species differ in their susceptibility to toxic effects of nucleoside analogs (Tucker et al., 1983; Tucker, 1983). Sample size of this study was also small. To completely understand the dose related response in organs and tissues, the drug should be administered for prolonged periods by two or more routes and in more than one species. A longer period of follow up to monitor the changes would also be desirable. Antiviral nucleoside analogs are known mutagens (Clive et al., 1983; Tucker et al., 1983). The mutagenic potential of this compound should be investigated by different assays. The potential of teratogenicity and embryotoxicity of But-MMdCyd also should be determined.

In conclusion, the toxicity studies reported in the thesis were aimed at determining the target organ toxicity of But-MMdCyd after administration of a large single dose (1000 mg/kg) and repeated doses (100 mg/kg/day) for 15 days. The results generated from these investigations are encouraging and suggest more extensive battery of in vitro and interspecies toxicology studies for this compound are warranted, if But-MMdCyd is chosen for the development as an antiherpes agent.
5.0 General Discussion

The impact of viral diseases has been profound on human and animal health. There are 350 million carriers of herpes virus worldwide and two percent of the world's population is infected with hepatitis C virus (HCV). Herpes infection of the human genital tract is usually caused by herpes simplex virus type 2 (HSV-2) and based on the number of cases that have been observed, it now represents most common venereal disease in this continent and the world. It has been estimated that among the sexually active population, in the western world 25-30% are infected with genital herpes (Johnson et al., 1989).

Acquired immunodeficiency syndrome is a lethal "multi-system" disease that has become a major health problem world-wide since its recognition in 1981. On a world-wide basis, the world health organization estimates that there are approximately 30.6 million cases of AIDS and by the end of the century, the total number of infected people with human immunodeficiency virus (HIV) will rise to an estimated 40 million. Thus, the global epidemic of AIDS continues unabated. With the spread of AIDS epidemic and the frequency of transplantation surgery, the number of immunosuppressed patients is growing. Therefore, the incidence of herpes virus infections is growing rapidly. Thus, it is obvious that the impact of viral diseases in the human population in 21st century is expected to grow at a rapid pace.

The infections due to herpes simplex virus (HSV) are among the oldest and the common infections in humans. The virus persists in latent form and causes recurrent
disease. It has been estimated that ten to fifteen percent of the population on this continent over the age of 18 years have recurrent herpes infection three or more times every year. Herpes simplex virus type 1 (HSV-1) is a source of frequent infection in humans with varying degree of severity from mild discomfort (recurrent HSV labialis) to serious ocular infection including impairment of vision (herpes keratitis) and life threatening disease (herpes encephalitis). The immunocompromised and immunosuppressed patients are particularly prone to herpes virus infections. The discovery of drugs that have the ability to inhibit virus replication by targeting the unique functions of HSV, namely, viral dThd kinase and DNA polymerase have been successfully utilized to combat HSV infection in humans. However, the rebound of HSV replication following withdrawal of the drug is a common phenomenon seen in-patient (e.g. acyclovir for the treatment of genital herpes). The emergence of drug resistant strains of virus, toxicity, and lack of efficacy for the treatment of recurrent infections are additional limitations for the clinical use of presently approved drugs for the treatment of herpes virus infections (Griffiths, 1995; Whitley, 1996; Jones, 1998). Anti-herpes agents currently approved for the treatment of herpes keratitis (ocular herpes) have been shown to inhibit stromal repair and slow wound healing (Gasset and Katzin, 1975; Prusoff, 1980; Faulds et al., 1990; Meyer et al., 1996). Therefore, there is a need for the development of drugs with greater selectivity for the treatment of HSV infection, to minimize toxicity to the host and the search for these compounds is being actively pursued around the world.
The rational for the synthesis of deoxycytidine analogs as selective antihepres agents was discussed earlier (section 2.1). Amide derivatives of MMdCyd were synthesized to improve delivery of MMdCyd intracellularly. The hypothesis was that these compounds would serve as 'prodrugs' of MMdCyd. It was reasoned that these compounds might also be refractory to deaminase(s) because amide derivatives are not likely to serve as substrate for deaminase. Result presented in this thesis provides evidence to support this hypothesis.

The results presented in this thesis indicate that N^4-acyl derivatives of MMdCyd are selective inhibitors of HSV-1 replication (Table 3.1, page 54). Butanoyl-MMdCyd (ED_{50} = 1 to 2µM) was the most potent compound of this series. Interestingly, all amide derivatives were more active than the parent compound MMdCyd. Another unique property of amide derivatives of MMdCyd is that these compounds have low cytotoxicity. The selectivity index (SI) of But-MMdCyd (SI > 3371) is considerably better than MMdCyd (SI > 551) when A-549 cells were used for the antiviral assays. Interestingly, the selectivity index of But-MMdCyd (SI > 838) was also considerably better than acyclovir (SI > 333) and MMdCyd (SI > 143) when VERO cells were used. The results of virus infectivity studies by cell morphology changes and virus yield experiments substantiated the observations of antiviral activity of MMdCyd and But-MMdCyd by the plaque reduction assay. The virus yield experiments also clearly demonstrate that production of infectious virus particles was much more effectively prevented in the presence of But-MMdCyd (Fig.3.4, page 62). The findings that exposure to But-MMdCyd
prevented the ability of residual intracellular virus to resume replication have implicit significance in its value as a potential antiviral drug.

Dideoxycytidine (ddC) and filauridine (FIAU) have been shown to cause multi-organ toxicity in-patients (Fischl et al., 1993; Tennant et al., 1998). The molecular basis for multi-organ toxicosis has been suggested to be inhibition of gamma (γ) DNA polymerase which results in defective mitochondrial DNA synthesis (Chen and Cheng, 1989; Chen et al., 1991; Parker et al., 1997; Shi et al., 1997). In contrast the acute toxicity manifested by nucleoside analogs is believed to be due to inhibition of α and β DNA polymerases leading to defective nuclear DNA synthesis (Chen and Cheng, 1989; Chen et al., 1991; Shi et al., 1997). Several investigators have proposed that potential adverse effects of a drug (specifically nucleoside analogs) for the host can be predicted to some extent by undertaking acute and delayed cytotoxicity studies using human lymphoblast cell lines in situ (Faraj et al., 1994; Parker et al., 1997; Shi, 1999). The studies carried out using CEM cells indicated that MMdCyd and But-MMdCyd did not produce any dose related acute or delayed cytotoxicity in CEM cells up to a concentration of 2000 µM. Compared to ddC these compounds were at least 800-8000 folds less toxic to CEM cells (Figures 4.1, page 95). These results are interesting and suggest that the probability of delayed cytotoxicity arising from MMdCyd and But-MMdCyd are lower, if these compounds are given for the treatment of HSV infection. The most likely reason for these observations is that neither MMdCyd nor But-MMdCyd is converted to the triphosphate form (“active drug”) in significant amounts in uninfected cells. As result,
little or no MMdCTP or But-MMdCTP builds up in the nucleus or mitochondria and thus acute or delayed cytotoxicity is not observed. This is based on the premise that But-MMdCyd is stable intracellularly and But-MMdCTP is the 'active form' of the drug. Further studies using human DNA polymerases should be undertaken to determine substrate properties of But-MMdCTP.

Pre-clinical toxicity studies in animals are a pre-requisite for a compound with therapeutic potential. To determine the acute and subacute toxicities, descriptive animal toxicity studies were undertaken. The main principle of all descriptive animal studies is that effects produced by a compound in laboratory animals when properly quantified are applicable to humans (Eaton and Klassen, 1996; Gad and Chengelis, 1998). This premise applies to all experimental biology and medicine. Based on dose per unit body surface, toxic effects in humans are usually in the same range as those in experimental animals. Toxicological studies on But-MMdCyd indicate that this compound has low systemic toxicity in mice (LD₀ > 1000 mg/kg). A single dose of But-MMdCyd up to 1000 mg/kg did not result in mortality nor were any pathological, hematological or clinical chemistry changes observed. The results of subacute toxicity studies (100 mg/kg/day for 15 days) were essentially similar to acute toxicity studies, except that multifocal lymphocytic infiltration of glomeruli was observed in one animal. Except for these minor histopathological changes, no other functional disturbances could be detected. At this stage, however, one should be very careful to make any conclusions from this preliminary study, regarding the potential of mammalian toxicity potential of But-MMdCyd.
Nonetheless, these results are encouraging and suggest that But-MMdCyd may have a favourable toxicity profile for \textit{in vivo} administration on long term basis.

The new generation of selective antiviral agents, such as acyclovir (ACV), 5-substituted deoxyuridines (BrVdUrd, MMdUrd) and deoxycytidines (MMdCyd, BrVdCyd) elicit antiviral activity by selectively utilizing or inhibiting virus specified functions to a greater degree than host cells (Babiuk \textit{et al.}, 1975; Meldrum \textit{et al.}, 1980; De Clercq, 1980; De Clercq \textit{et al.}, 1981; Aduma \textit{et al.}, 1990a; Aduma \textit{et al.}, 1991; Gupta \textit{et al.}, 1991; Hayden, 1995). These nucleoside analogs are selectively phosphorylated to their corresponding monophosphates (‘anabolism’) by virus-induced pyrimidine deoxyribonucleoside kinase in HSV-infected cells. After conversion to the triphosphates, they specifically inhibit viral DNA polymerase and are incorporated into the viral DNA (Allaudeen \textit{et al.}, 1982; Gupta \textit{et al.}, 1991; Hayden, 1995). At present, the mechanism(s) by which But-MMdCyd elicits selective and potent antiviral activity remains unknown. However, several pieces of evidence suggest that this antiviral activity is selectively directed at a viral specific function and is not due to alteration of the metabolic functions of the cells. Thus, it has been shown that But-MMdCyd has no effect on confluent or rapidly growing cells \textit{in vitro} at concentrations far in excess (> 800-1000 times) of those inhibitory for virus replication (ED$_{50}$ 1-4 µM). The results of delayed cytotoxicity studies \textit{in vitro} and \textit{in vivo} toxicity studies in mice also favour the view that selective toxicity of But-MMdCyd is possibly due to its effect on a virus specified event.

We suggest the following mechanisms for the anti-herpes activity of But-
MMdCyd. The nucleoside analogue penetrates HSV-infected cells and is converted to butanoyl-5-methoxymethyl-2'-deoxycytidine triphosphate (But-MMdCTP) and inhibits HSV- induced DNA polymerase by competing with deoxycytidine triphosphated (dCTP) and is incorporated into viral DNA or alternatively, But-MMdCyd serves as a 'pro-drug'.

After penetration into the infected cells, But-MMdCyd is hydrolyzed to MMdCyd. Methoxymethyl-2'-deoxycytidine is phosphorylated to MMdCMP by viral- induced pyrimidine kinase (dThd/dCyd kinase) which after conversion by deoxycytidylate kinase to the end product (MMdCTP) is responsible for inhibiting replication of HSV-1. The marked increase in potency of But-MMdCyd (ED50 1-4 µM) compared to MMdCyd (ED50 7-26 µM) favours the hypothesis that the antiherpes activity is most likely due to intrinsic activity of But-MMdCyd. Further studies on the cellular and molecular pharmacology of But-MMdCyd should be undertaken to understand fully the molecular mechanism for selective anti-herpes activity.

In summary, results of biological studies presented in this thesis have shown that amide derivatives of MMdCyd are selective anti-herpes agents. The potency of But-MMdCyd (ED50 3.5 ± 0.54 µM) is comparable to acyclovir (ED50 3 ± 0.23 µM), presently approved drug for the treatment of HSV infection. Butanoyl-MMdCyd has low mammalian toxicity.

Concluding Remarks

The clue to successful antiviral drug development elicits from understanding the viral functions at the molecular level and formulating strategies to circumvent these viral functions to therapeutic advantage so that cytotoxic actions of the compound affects
only the virus sparing the host from the toxicity.

To determine further therapeutic potential of But-MMdCyd following studies should be undertaken:

1. The possibility of development of resistance to But-MMdCyd in cell cultures should be investigated.

2. The metabolism of But-MMdCyd in uninfected and HSV-infected cells should be studied using radiolabelled compounds.

3. Studies on mechanism of But-MMdCyd in situ should be carried out using purified DNA polymerases.

4. Pharmacokinetic properties of But-MMdCyd, especially its metabolic fate in blood, plasma and tissues of animals should be investigated.

5. Toxicological studies: subacute (medium and high dosage administration) and chronic in other animal species, as well as mutagenicity and reproductive studies (embryotoxicity and teratogenicity) should be undertaken to evaluate further the safety profile of But-MMdCyd.

The data available from these investigations should be adequate to undertake clinical development of this compound by forging a strategic alliance with a pharmaceutical company.
5.0 References


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Desgranges, C., Razaka, G., Raboud, M., Bricaud, H., Balzarini, J., and De Clercq, E., (1983) Phosphorolysis of (E)-5- (bromovinyl)-2'- deoxurydine (BrVdUrd) and other 5- substituted- 2'- deoxuridines by purified human thymidine phosphorylase and intact blood platelets. *Biochem Pharmacol* 32: 3583-359


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APPENDIX A

Table 1 Fixative solution for tissues

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH$_2$PO$_4$ · 2 H$_2$O</td>
<td>95 gm</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$ · 12 H$_2$O</td>
<td>360 gm</td>
</tr>
<tr>
<td>Formaldehyde (37%)</td>
<td>-two litres</td>
</tr>
<tr>
<td>Q.S. with double distilled water</td>
<td>to 20 litres</td>
</tr>
</tbody>
</table>

pH of the solution was between 6.7 and 6.8
### Table 2

**Solutions and methodology for staining of histological sections**

**A. Lillie’s haematoxylin**
- ammonium alum: 50 gm
- Haematoxylin: 5 gm
- Sodium iodate: 1 gm
- Distilled water: 700 ml
- Glycerol: 300 ml
- Glacial acetic acid: 200 ml

Dissolve hematoxylin in water with warming and add sodium iodate and alum when dissolved. Then add glycerol and acetic acid. Mix well.

**B. Acid-Alcohol**
- Hydrochloric acid: 20 ml
- Distilled water: 600 ml
- Absolute alcohol: 1400 ml

**C. Eosin**
- Saturated aqueous picric acid: 200 ml
- Eosin y or yx: 30 gm
- Potassium dichromate: 10 gm
- Absolute alcohol: 100 ml
- Distilled water: 1600 ml

**Haematoxylin and eosin method**
1. Sections to water: 5-10 min
2. Haematoxylin (Soln. A)
3. Wash in water
4. Differentiate (Soln. B): 5-10 sec
5. Blue in tap: 5-10 min
7. Rinse in water
8. Dehydrate, clear and mount
Table 3  Solutions for staining of blood and marrow smears

A. Wright-Giemsa stain
1. Wright's stain - 12 gm
2. Giemsa stain - 1.6 gm
3. Q.S. with absolute methanol to four times
   Add stain to small amount of methanol and mix into a paste. Mix paste with
   the rest of methanol and allow to stir for one hour.

B. Phosphate buffer
1. Sodium phosphate (dibasic) Na$_2$HPO$_4$ - 9.4 gm/L
2. Potassium phosphate (monobasic) KH$_2$PO$_4$ - 9.08 gm/L
   To 1000 ml of KH$_2$PO$_4$, add 967 ml of Na$_2$HPO$_4$ to obtain a pH of 6.8.

C. Staining method
1. Wright-Giemsa stain - 4 min
2. Phosphate buffer (pH 6.8) - 4 min
3. Blow on slide gently to mix stain with buffer.
4. Wash off with distilled water.