BIOLOGICAL STUDIES ON INHIBITORS OF HUMAN IMMUNODEFICIENCY VIRUS

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Veterinary Physiological Sciences
Western College of Veterinary Medicine
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. By
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Spring 1999

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UNIVERSITY OF SASKATCHEWAN
College of Graduate Studies and Research

SUMMARY OF DISSERTATION
Submitted in partial fulfillment
of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY
by
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ABSTRACT

The toxicities induced by dideoxynucleoside analogs (ddNs, RT inhibitors) are due to the incorporation of ddNs triphosphates into the cellular or mitochondrial DNA of the host cells. Our hypothesis was that carboxyphosphonyl (CP) analogs of nucleosides should have better toxicity profile because these pharmacophores would not require "activation" by host enzymes for anti-HIV activity.

The antiviral activity of CP analogs against HIV and AZT-resistant variants was determined by focal immunoassay (FIA), reverse transcriptase (RT) assay and cytopathic inhibition (MTT) assay. The antiviral activity of CP analogs is greatly influenced by the assay methodologies, virus strains and cell lines used. CP-deoxyuridine (CP-dUrd), CP-deoxyinosine (CP-dIno) and CP-deoxythymidine (CP-dThd) were selective inhibitors of HIV replication. The concentrations required to inhibit HIV-1 strain IIIB and AZT-resistant variants replication by 50% (ED_{50}) was in the range of 0.15 μM to 0.55 μM by RT assay. These compounds were found to have moderate activity against HIV strains when tested using FIA and MTT assays. The antiviral activity of CP-ddNs was determined by different assay methods. CP analogs in general were less potent than ddNs. CP-AZT was found to have good antiviral activity. Foscarnet, AZT, ddC, ddI and d4T were used as drug controls. CP-ddNs have low acute cytotoxicity. The cytotoxicity of CP-ddNs was 4 to 85 folds lower than the parent ddNs for different cell lines.

The uptake and efflux of [6-^{3}H] CP-dUrd was studied using CEM cells. The highest intracellular concentration of CP-dUrd attained was 1.8 pmole/10^6 cells after 72
hours of incubation with 50 μM of compound. The half-life of CP-dUrd in CEM cell was 25 hours. CP-dUrd was stable during the incubation period. No binding of CP-dUrd to plasma proteins occurred. The pharmacokinetic studies of [6-\(^{3}\)H] CP-dUrd (i.p. administration) in mice indicated that CP-dUrd was slowly absorbed, homogeneously distributed in body tissues and slowly excreted after extensive metabolism in the body.

Acute and delayed cytotoxicities of active CP analogs were evaluated using CEM cells. CP-dUrd, CP-dIno and CP-AZT did not exhibit acute or delayed cytotoxicity. Control drug ddC exhibited acute and delayed cytotoxicity in CEM cells at 1 μM and 0.125 μM, respectively. The concentrations of AZT, CP-dUrd and CP-dIno required to inhibit 50% colony formation (IC\(_{50}\)) of murine granulocyte-macrophage (CFU-GM) were 2.5 μM, 250 μM and 160 μM, respectively. CP-dUrd has low mammalian toxicity (LD\(_{50}\): \(\approx 1000\) mg/kg, i. p.).

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ABSTRACT

The toxicities induced by dideoxynucleoside analogs (ddNs, RT inhibitors) are due to the incorporation of ddNs triphosphates into the cellular or mitochondrial DNA of the host cells. Our hypothesis was that carboxyphosphonyl (CP) analogs of nucleosides should have a better toxicity profile because these pharmacophores would not require "activation" by host enzymes for anti-HIV activity.

The antiviral activity of CP analogs against HIV and AZT-resistant variants was determined by focal immunoassay (FIA), reverse transcriptase (RT) assay and cytopathic inhibition (MTT) assay. The antiviral activity of CP analogs is greatly influenced by the assay methodologies, virus strains and cell lines used. CP-deoxyuridine (CP-dUrd), CP-deoxyinosine (CP-dIno) and CP-deoxythymidine (CP-dThd) were selective inhibitors of HIV replication. The concentrations required to inhibit HIV-1 strain IIIB and AZT-resistant variants replication by 50% (ED$_{50}$) was in the range of 0.15 µM to 0.55 µM by RT assay. These compounds were found to have moderate activity against HIV strains when tested using FIA and MTT assays. The antiviral activity of CP-ddNs was determined by different assay methods. CP analogs in general were less potent than ddNs. CP-AZT was found to have good antiviral activity. Foscarnet, AZT, ddC, ddI and d4T were used as drug controls. CP-dNs have low acute cytotoxicity. The cytotoxicity of CP-ddNs was 4 to 85 fold lower than the parent ddNs for different cell lines.
The uptake and efflux of [6-³H]CP-dUrd was studied using CEM cells. The highest intracellular concentration of CP-dUrd attained was 1.8 pmole/10⁶ cells after 72 hours of incubation with 50 μM of compound. The half-life of CP-dUrd in CEM cells was 25 hours. CP-dUrd was stable during the incubation period. No binding of CP-dUrd to plasma proteins occurred. The pharmacokinetic studies of [6-³H]CP-dUrd (i.p. administration) in mice indicated that CP-dUrd was slowly absorbed, homogeneously distributed in body tissues and slowly excreted after extensive metabolism in the body.

Acute and delayed cytotoxicities of active CP analogs were evaluated using CEM cells. CP-dUrd, CP-dIno and CP-AZT did not exhibit acute or delayed cytotoxicity. Control drug ddC exhibited acute and delayed cytotoxicity in CEM cells at 1 μM and 0.125 μM, respectively. The concentrations of AZT, CP-dUrd and CP-dIno required to inhibit 50% colony formation (IC₅₀) of murine granulocyte-macrophage (CFU-GM) were 2.5 μM, 250 μM and 160 μM, respectively. CP-dUrd has low mammalian toxicity [LD₅₀ (mouse) ≈ 1000 mg/kg, i. p.].
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DEDICATION

To my father, Chengbin Shi and mother, Lisheng Wang

my husband Junbao Yang and daughter Wendy Yang

for their love, encouragement and support.
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LIST OF ABBREVIATIONS

3TC  (-)2'-deoxy-3'-thiacytidine, Lamivudine, Epivir
AIDS  Acquired immunodeficiency syndrome
ANOVA  Analysis of variance
ANP  Acyclic nucleoside phosphonate
AZHMddUrd  3'-azido-2',3'-dideoxy-5-hydroxymethyluridine
AZT  3'-Azido-2',3'-dideoxythymidine, Zidovudine, Retrovir
BFU-E  Burst-forming unit of erythroid
CBMC  Cord blood mononuclear cells
CC50 and CC90  Cytotoxic concentration required to reduce cell growth by 50% and 90%
CFU-GM  Colony forming unit of granulocyte-macrophages
Cmax  Maximum concentration
Cmin  Minimum concentration
CP  Carboxyphosphonyl group
CP-AZT  5'-carboxyphosphonylazidothymidine
CP-d4T  5'-carboxyphosphonyldidehydrodideoxythymidine
CP-ddC  5'-carboxyphosphonyldideoxyctydine
CP-ddI  5'-carboxyphosphonyldideoxyinosine
CP-ddN  CP derivatives of dideoxynucleoside
CP-dIno  5'-carboxyphosphonyl-deoxyinosine
CP-dMMdCyd  5'-carboxyphosphonyl-5'-methoxymethyldeoxyctydine
CP-dN  CP derivatives of deoxyribonucleoside
CP-dThd  5'-carboxyphosphonyldeoxythymidine
CP-dUrd  5'-carboxyphosphonyldeoxyuridine
CP-EtdUrd  5'-carboxyphosphonyl-5'-ethyldeoxyuridine
CP-Ino  5'-carboxyphosphonylninosine
CP-MMdUrd  5'-carboxyphosphonyl-5'-methoxymethyldeoxyuridine
d4T  Didehydrodideoxythymidine , Stavudine, Zerit
dCyd \hspace{1em} 2'-deoxycytidine
ddC \hspace{1em} Dideoxycytidine, Zalcitabine, Hivid
ddI \hspace{1em} Dideoxynosine, Didanosine, Videx
DP \hspace{1em} Diphosphate
DAPI \hspace{1em} Diaminophenylindole
dThd \hspace{1em} Deoxythymidine
dUrd \hspace{1em} 2’-deoxyuridine

ED\textsubscript{50} and ED\textsubscript{90} Concentration required to inhibit virus replication by 50% and 90%.

FDA \hspace{1em} Food and Drug Administration

FHMddUrd \hspace{1em} 3’-fluoro-2’,3’-dideoxy-5-hydroxymethyluridine
FIA \hspace{1em} Focal immunoassay
HIV \hspace{1em} Human immunodeficiency virus
HMd4Urd \hspace{1em} 2’,3’-dideoxy-2’,3’-didehydro-5-hydroxymethyluridine
HMddUrd \hspace{1em} 2’,3’-dideoxy-5-hydroxymethyluridine
HMdUrd \hspace{1em} 5-Hydroxymethyldeoxyuridine

IC\textsubscript{50} and IC\textsubscript{90} Concentration required to inhibit colony formation by 50% and 90% or concentration required to inhibit enzyme activity by 50% and 90%

i.p. \hspace{1em} Intraperitoneal administration
i.v. \hspace{1em} Intravenous administration

K\textsubscript{i} \hspace{1em} The dissociation constant of the enzyme-inhibitor complex
K\textsubscript{m} \hspace{1em} The Michaelis constant
MCC \hspace{1em} Minimum cytotoxic concentration
MP \hspace{1em} Monophosphate

MTT assay \hspace{1em} Cytopathic inhibition assay

NNRTI \hspace{1em} Non-nucleoside reverse transcriptase inhibitors
PBMC \hspace{1em} Human peripheral blood monocyte-macrophage
PFA \hspace{1em} Phosphonoformic acid, phosphonoformate, foscarnet
PFU \hspace{1em} Plaque forming unit
PR \hspace{1em} Protease
RT \hspace{1em} Reverse transcriptase
SI \hspace{1em} Ratio of CC\textsubscript{50}/ED\textsubscript{50}
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPIA</td>
<td>Suspension-phase infectivity assay</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Tissue culture infective dose</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TP</td>
<td>Triphosphate</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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1.0 INTRODUCTION

1.1 ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) - ETIOLOGY AND INFECTION

Two major groups of human retroviruses have been isolated: the human immune deficiency virus type 1 (HIV-1) and type 2 (HIV-2) which belong to the lentivirus subfamily (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Popovic et al., 1984a), and the human T-cell lymphotropic virus type 1 (HTLV-I) and type 2 (HTLV-II) which belong to oncovirus subfamily (Levy, 1986). HIV-1 and HIV-2 are etiological agents of Acquired Immunodeficiency Syndrome (AIDS), a lethal "multi-system" disease that has become a major health problem since its recognition in 1981. HIV-1 has nine subtypes and is more virulent than HIV-2. In Europe and North America, infection is predominantly reported from HIV-1. AIDS is primarily a sexually transmitted disease caused by the HIV. The disease has spread rapidly throughout the world, and the highest number of cases is currently in countries of the Third World. On a worldwide basis, the World Health Organization estimates that in 1997, there were 5.8 million people newly infected with HIV and the total number of cases is approximately 30.6 million worldwide (WHO, 1998). By the year 2000, the total figure of infected people will rise to an estimated 40 million. Since the global epidemic of AIDS, approximately 11.7 million people have died. "The impact of AIDS death which rose an estimated 50 percent this year, is only just beginning". Despite extensive research around world, HIV infection, at present remains incurable and only modestly treatable.
The mechanisms of AIDS pathogenesis are complex and not completely understood. The virus primarily infects the cells bearing CD4 receptors, particularly T cells and monocytes/macrophages of the immune system. The interaction of HIV with the immune system of an individual results in selective destruction (either by direct effect of the virus replication or as a result of a cytotoxic immune response) and consequently, there is a marked decrease in the number of CD4 lymphocytes. Since the CD4 lymphocytes play an important role in regulating the cellular and humoral immune responses, therefore, selective depletion of the T-helper/inducer lymphocytes is responsible, at least in part, for the profound immunodeficiency in patients (Ho et al., 1987). The immunodeficient state makes the patient highly susceptible to opportunistic life-threatening protozoal, bacterial, fungal and viral infections, as well as unusual forms of malignancies, such as Kaposi's sarcoma or malignant (non-Hodgkin's) lymphoma. In addition, HIV is neurotropic and it has been suggested that the central nervous system serves as a sanctuary site for the virus. Neurological dysfunctions occur in approximately 60% of the patients and in the brain the cells predominantly infected by HIV appear to be monocytes and macrophages. Thus another principal sequel of HIV infection is subacute encephalitis (AIDS encephalopathy or AIDS dementia complex) (Ho et al., 1987; Michaels et al., 1988; Price, 1996).

According to Pantaleo, the course of HIV infection can be divided into three phases (Pantaleo et al., 1993a). The early phase of HIV infection is characterized by a burst of viremia and large number of infected CD4 lymphocytes in blood. The viremia then falls due to the induction of the cellular and humoral immune responses to the viral infection. Following this, there is a prolonged latency phase. During this period, there is
a very low but constant amount of virus and infected cells in the blood and there is a very gradual decline in the numbers of CD4 lymphocytes. The final phase of HIV infection is characterized by a sharp decrease of CD4 lymphocytes and an increase of virus and infected cells in the circulation. The clinical symptoms of AIDS appear at this stage of the infection.

The ultimate collapse of the immune system despite the apparent low level of virus in the blood is the central paradox of AIDS. Interestingly, recent studies have shown that the clinical latency phase is not a period of viral inactivity. The viral load in circulation only reflects the spillover from HIV replication in lymphoid tissues because the predominant site of infection are the lymphoid tissues. During the entire course of HIV infection, tremendous amounts of virus are present in the lymphoid tissues, such as lymph nodes, spleen, adenoid glands and tonsils. The studies done by Embretson and Pantaleo indicate that at an early stage of infection, there are more viruses in lymphoid tissues than in blood. In the late stage of infection, the viral burden is equal in these two compartments (Embretson et al., 1993; Pantaleo et al., 1993b). There are about $10^9$ viral particles produced and cleared from the blood of an infected individual everyday. The average life span of the virus and infected cells in the circulation is very short, approximately 1-2 days (Ho et al., 1995). The rapid replication of the HIV results in the rapid turnover of the CD4 lymphocytes to be about $2 \times 10^9$ CD4 lymphocytes dying and replaced everyday (Ho et al., 1995). On the basis of these results, it has been suggested that there exists a steady-state model of HIV replication in the infected individual, that is, the apparent latent period between infection with HIV and the overt symptoms of AIDS is not a period of viral inactivity, but an extraordinarily dynamic process in which
infection, cell death and replacement are kept in balance (Coffin, 1995a; Ho et al., 1995). The final dramatic drop of the CD4 lymphocytes appears to be due to the exhaustion of the regenerative capacity of the immune system after years of HIV infection. In contrast, however, studies done on T cell telomere length do not support the theory of increased CD4 lymphocytes turnover in the HIV infected individual (Wolthers et al., 1996). These authors found that the length of the terminal restriction fraction of CD4 lymphocytes, which reflects the replicative history of the cells, is stable during the course of HIV-1 infection. These results indicate that the bulk of the CD4 lymphocytes in HIV infected individuals is turning over at the same rate as in healthy persons. From these investigations, it was deduced that HIV may damage the undefined precursor source of CD4 lymphocyte so that the regenerative ability of CD4 lymphocyte is damaged during the entire course of infection and not only at the late stage of infection as proposed by Ho et al.

Whichever theory for pathogenesis of HIV infection is correct, it is obvious that the continuous high-level replication of HIV is the driving force responsible for progression of the disease. The increased viral load correlates with the CD4 lymphocyte depletion, and this in turns, decides the progress of HIV infection ultimately manifested as AIDS. From the steady-state model, it was deduced that a 2-fold permanent reduction of the viral load might lead to a 2-fold increase in the mean clinical latency and a 10-fold permanent reduction might prolong latency indefinitely (Coffin, 1995a). Therefore, the critical point for treatment of AIDS is to kill the virus or alternatively reduce the virus load to a level where manifestations of disease are kept under control. This can be accomplished by the use of chemotherapeutic agents capable of inhibiting HIV
replication. Thus, chemotherapy plays an important role in slowing down progression of the disease, even though the use of drugs can not completely eradicate the HIV infection. Therefore, this model of HIV replication is also useful for the development of better therapeutic strategies for the treatment of AIDS.

1.2 HIV REPLICATIVE CYCLE

HIV is a lentivirus, which belongs to retroviridae. HIV is one of the most complex retroviruses studied. The structure of human retroviruses and replicative cycle of HIV is shown in Figure 1.1.

HIV is an enveloped virus, which contains a diploid, single-stranded RNA genome. The RNA genome consists of three major genes, gag, pol, env and other adjust genes, namely, tat, rev, vif, vpr, vpu, vpx and nef. The env gene codes for the envelope glycoproteins gp120 and gp41, which are responsible for viral adsorption and fusion process. The pol and some gag genes encode the reverse transcriptase, integrase and protease. These enzymes are required for viral DNA synthesis, integration and protein processing. The structural proteins MA (matrix protein, p17), CA (capsid protein, p24) and NC (nucleocapsid protein, p9) are gag gene products. Some of the adjust genes, such as tat, rev and nef produce the regulatory factors required for HIV replication. The functions of other adjust genes are not clearly understood at this time (See reviews by Cullen, 1992; Hahn, 1994). HIV isolates show extensive genetic variability, resulting from the relatively low fidelity of RT in conjunction with the extremely high turnover of virions in vivo.
Figure 1.1 Replicative cycle of HIV-1, showing the sites of action of antiviral agents.

RT, reverse transcriptase; cDNA, complementary DNA; mRNA, messenger RNA; Tat, a protein that regulates viral transcription and affects the rate of replication; RNaseH, ribonuclease H; gp120, envelope glycoprotein. (Adapted from Hayden, 1995)
1.3 TARGETS FOR CHEMOTHERAPY OF AIDS

Therapy for AIDS is based on the premise that the replication of HIV is involved in the pathogenesis of the disease (See reviews by Fauci, 1996; Levy et al., 1993). Theoretically, therefore every step in the HIV replicative cycle is a possible target for the treatment of HIV infection. The possible targets for chemotherapy of AIDS based on the life cycle of HIV replication are shown in Table 1.1 and Figure 1.1.

1.3.1 Adsorption and fusion

HIV env encodes a glycoprotein precursor called gp160 that is cleaved by cellular enzymes into gp120 and gp41, which are surface and transmembrane proteins (Hahn, 1994). The first step of HIV infection is binding of the gp120 to the CD4 receptor located on the surface of the target cells. This interaction causes the conformational changes in gp120, allowing it to bind to another HIV co-receptor (Cohen, 1996a; Wain-Hobson, 1996). The HIV co-receptors are the β-chemokine receptors including CCR5 and CXCR-4 which are present on the surface of different types of cells. It has been proposed that after the chemokine receptor has fused with the CD4-gp-120 complex, the amino terminus of gp41 is uncovered, allowing penetration of the host cell membrane and fusion of the viral and cell membranes. Then the capsid complex moves into the cell (Alkhatib et al., 1996; Feng et al., 1996; Trkola et al., 1996; Wu et al., 1996; Zhang et al., 1996).
<table>
<thead>
<tr>
<th>HIV replication Steps</th>
<th>HIV inhibitors</th>
</tr>
</thead>
</table>
| Adsorption and fusion | CD4 derivatives  
Polyanionic compounds  
Lectins, polypeptides, albumin, betulinic acid derivatives  
Bicyclams  
β-chemokines |
| Reverse transcription | Dideoxynucleoside analogs: AZT, ddi, ddC, d4T and 3TC  
Other classes of RT inhibitors:  
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs):  
Nevirapine and Delavirdine  
Phosphonoformate (PFA)  
Acyclic Nucleoside Phosphonates (ANPs), e.g. PMEA |
| Integration | Hexapeptides  
DNA binding agents: MAP30 and GAP31  
Antisense oligonucleotides |
| Gene expression | *Tat* inhibitors: e.g. Ro 5-3335 and Ro 24-7429  
*Rev* inhibitors: e.g. Win 46569  
Antisense oligonucleotides  
Ribozymes may destroy HIV mRNA |
| Protein processing | Protease inhibitors: Saquinavir, Indinavir, Ritonavir and Nelfinavir |
| Protein modification | Glycosylation inhibitors: e.g. castanospermine  
Myristoylation inhibitors: e.g. 13-oxatetradecenoic acid |
| Viral package | Antisense against packing sequence |
| Budding | Interferons |
Adsorption and fusion inhibitors

(i) CD4 Derivatives

Two kinds of agents have been developed to block the adsorption of virus to the cells. Soluble CD4 (sCD4) molecules have been shown to bind selectively to the gp120 with the same avidity as the CD4 receptor of the native cell and prevent the viral particles from attaching to CD4 lymphocyte (Hussey et al., 1988). CD4 show good antiviral activity in vitro (Deen et al., 1988; Fisher et al., 1988; Hussey et al., 1988; Smith et al., 1987; Traunecker et al., 1988). However, clinical findings with the administration of sCD4 were disappointing due to short half life and poor plasma levels (Kahn et al., 1990; Schooley et al., 1990; Smith et al., 1987).

(ii) Polyanionic compounds

Polyanionic compounds, such as, dextran sulfate, heparin, suramin, aurinetricarboxylic acid and PSiW12O40 (JM1493) are good inhibitors of HIV (De Clercq, 1995a; 1995b). These compounds exert antiviral effect by binding to the envelope glycoprotein gp120, especially the V3 loop of gp120, thus blocking the adsorption of the virus to target cells. These substances can inhibit both viral replication and virus-induced syncytium formation in vitro. Considerable variation of HIV strains susceptibility to this class of compounds has been observed and it is probably due to the extensive variability of the V3 loop of gp120. Furthermore, different characteristics of these compounds, such as, molecular weight, chemical nature and electron density may also be responsible for the big differences in antiviral potency of different agents within this class of compounds. Dextran sulfate showed little antiviral effect after oral or I.V.
administration in human trials. The lack of efficacy appears to be due to the poor pharmacokinetic profiles of these compounds, such as low oral bioavailability and metabolic degradation. In addition, toxicity such as anticoagulant activity of sulfated polysaccharides also limits their clinical use.

(iii) Lectins, bicyclams and β-chemokines

Mannose-specific lectins (e.g. from Listera Ovata, Hippeastrum hybrid), polypeptides which represent domains of gp41 (e.g. DP-107), negatively charged albumin (e.g. succinylated-neoglycoproteins) and triterpene (e.g. betulinic acid) derivatives are the compounds targeted at the virus-cell fusion step. They inhibit HIV induced cytopathogenicity and block syncytium formation. However, these compounds do not inhibit virus-cell binding. At this time, the clinical use of these compounds is hard to predict because information is not available on toxicological and pharmacokinetic profiles (De Clercq, 1995a and 1995b).

Bicyclams class of compounds is composed of two cyclam (1,4,8,11-tetraazacyclotetradecane) moieties linked by various bridges. Two representatives of this class, JM2763 and JM3100 are highly potent and selective inhibitors of replication of HIV-1 and HIV-2 in cell cultures (De Clercq et al., 1992a; 1994). The bicyclams are classified as fusion/uncoating inhibitors since studies have shown that these compounds protected viral RNA from degradation by RNase A shortly after adsorption. This phenomenon indicates that in the presence of bicyclams, viral RNA was not dissociated (i.e. uncoated) from the surrounding viral capsid proteins or envelope glycoproteins (De Clercq et al., 1992a; 1994). Recent studies by recombination experiments between the
wild-type NL4-3 clone and the drug resistant mutants indicate that bicyclams interacts with gp120, thus leading to the inhibition of unfolding of gp120 and it's shedding from the gp41 fusion domain (De Vreese et al., 1996). Therefore, inhibition of fusion and/or uncoating was proposed as a possible mode of action of these compounds. Preclinical studies are needed to determine therapeutic potential of this class of compounds.

Another class of fusion inhibitors is β-chemokines. β-chemokine receptors are the co-receptors of HIV which are involved in the viral adsorption-fusion process (Alkhatib et al., 1996; Feng et al., 1996; Trkola et al., 1996; Wu et al., 1996; Zhang et al., 1996). These co-receptors play an important role in determining the infectivity of HIV-1 because some people who have both deletions of the genes of CCR5 (a β-chemokine receptor) are not infected by HIV-1 despite repeated exposure to the virus (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). β-Chemokines are the ligands of β-chemokine receptors. The binding of the β-chemokines with β-chemokine receptors may block the interaction of gp-120 with these receptors, thus, decreasing the infectivity of HIV-1. It has been shown that people with a higher level of certain kinds of β-chemokines, such as macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulation-upon-activation normal T expressed and secreted) exhibit slower progression of the disease compared to people with a lower level of these chemokines. β-Chemokines were found to have anti-HIV effects in vitro, however, the chemotactic and leukocyte-activating properties may induce toxicity in vivo (Arenzana-Seisdedos et al., 1996; Bleu et al., 1996; Cocchi et al., 1995; Dragic et al., 1996; Oberlin et al., 1996). Aminoxyxypentane (AOP)-RANTES is a potent inhibitor of HIV-1 replication in vitro, and is devoid of chemotactic and leukocytes-activating properties (Simmon et al.,
1997). These findings indicate the clinical potential of β-chemokines or their derivatives as anti-HIV drugs. Compared to the current approved HIV inhibitors, they have the advantage of being easy to produce in large scale and are relatively inexpensive.

1.3.2 Reverse transcription

With the fusion of viral and cellular membranes, the virus is internalized. After the virus is internalized, it is uncoated and viral RNA and enzymes are released into the cytoplasm. The double-stranded viral DNA is synthesized from single strand viral RNA by reverse transcriptase (RT) of HIV. Because of the unique association of RT with retroviruses, this enzyme has been one of the most attractive targets for the development of therapeutic agents for the treatment of HIV infection. RT inhibitors approved for the treatment of AIDS are discussed at the end of this chapter.

1.3.3 Integration

The next step in HIV replication is to integrate the double-strand of viral DNA into the host genome. This process is mediated by the viral integrase, an enzyme encoded at the 3'-end of the pol gene (Reviewed by Katz and Skalka, 1994). The integration reaction consists of three steps. First, 3'-processing: two nucleotides are removed from each 3'-end to produce new 3'-hydroxyl ends [(CA)H] for precise integration. Next, joining: after being transferred from the cytoplasm to the nucleus, the viral DNA is joined to host target DNA via staggered cleavage of the host DNA and ligation of (CA)H-3' viral DNA ends to the 5'-phosphate ends of the target DNA. The
final step is gap repair. Repair of the remaining gaps is probably accomplished by host enzymes. The integrated viral DNA serves as the template for the transcription of viral genes. Integration is thus essential for viral gene expression and the production of progeny viruses (Engelman et al., 1991).

Integration inhibitors

Although HIV integrase is a critical target for selective antiviral therapy, not much work has been done on the development of inhibitors of integrase. Two proteins, MAP30 and GAP31, which come from the plants Momorolica charantia and Gelonium multiflorum were reported to inhibit the infection of HIV-1 in T lymphocytes and monocytes (Lee-Huang et al., 1995). Both proteins exhibit dose-dependent inhibition of HIV-1 integrase. Inhibition was observed for all three specific reactions catalyzed by the integrase, viz., 3'-processing (specific cleavage of the dinucleotide GT from the viral substrate), strand transfer (integration) and “disintegration” (the reversal of strand transfer). Curumin (diferuloylmethane, a yellow pigment in turmeric) and a hexapeptide (HCKFWW) have also been shown to inhibit integrase activity (IC$_{50}$: 40 μM and 2 μM respectively) (Mazumder et al., 1995; De Clercq, 1995a). The recently established oligonucleotide-based microtiter plate assay and the elucidation of the three-dimensional structure of HIV-1 integrase should boost the antiviral screen of chemical libraries as well as the structure based design of the integration inhibitors (Dyda et al., 1994).

1.3.4 Regulatory proteins-HIV Tat and Rev proteins
After integration into the cellular genome, the viral DNA is transcribed to mRNA and then translated to proteins by cellular machinery. This viral gene expression requires both cellular and viral transcriptional factors. HIV-Tat, which is required for viral replication in vitro, is a potent trans-activator of viral transcription. Tat interacts with the trans-activation responsive (TAR) region at the 5'-end of viral mRNA (located downstream of the initiation site) for transcription. Tat is responsible for stimulating effective RNA elongation which gives rise to full-length HIV-1 transcripts (Karn and Graeble, 1992).

Another HIV regulatory protein is Rev. To produce its activity, Rev binds to the Rev-responsive region within the coding sequence of the env gene. Rev exerts its regulatory activity by increasing the transport/stability of the mRNAs encoding the structural proteins of HIV. Gag and env encoded protein synthesis is severely diminished and only small multiple spliced RNA accumulates in the cytoplasm in the absence of Rev (reviewed by Cullen, 1992; Felber et al., 1993).

(i) Tat inhibitors

Tat is an attractive target for the development of antiviral drugs because inhibition of Tat would arrest the virus at an early stage. Two of the benzodiazepene derivatives, 7-chloro-5-(2-pyryl)-3H-1,4-benzodiazepin-2(H)-one (Ro 5-3335) and its congenor Ro 24-7429 (in which the NH-CO functionality has been replaced by the N=C(NH-CH₃) are potent Tat antagonists. They are highly active in vitro against HIV-1, HIV-2 and AZT-resistant clinical isolates. (Hsu et al., 1991; 1992). These benzodiazepene analogs are unique inhibitors of HIV because they are active against
HIV in chronic infection, since they target the post integration step of replication. In addition, Tat antagonists do not lead to the development of resistance, even after a 2-year exposure in cell culture (Hsu et al., 1993).

Ro 5-3335 and Ro 24-7429 drugs predominately inhibit Tat dependent initiation and also exert a measurable effect on RNA elongation (Cupelli and Hsu, 1995). The drug-treated cells remained viable, showed significantly reduced levels of the full length and spliced HIV-1 mRNA and maintained integrated HIV-1 DNA (Shahabuddin et al., 1992). However, the preclinical trial with Ro 24-7429 failed to demonstrate its anti-HIV effect in vivo. Recent studies indicate that Tat-defective HIV-1 is able to replicate in MT-2 cells and cytokine-stimulated T cells, and the Tat inhibitor Ro 24-7429 lost its anti-HIV effect in these systems (Luznik et al., 1995). This may partly explain why the preliminary clinical trials using Ro 24-7429 showed no efficacy (Haubrich et al., 1995).

(ii) Rev inhibitors

Recently, a series of 8-alkyl-2-(4-pyridyl)-pyrido(2,3-d)pyrimidin-5 (8H)-ones have been reported to inhibit Rev activity. Win 49569 inhibits HIV replication at a concentration (ED_{50}: 2-3 µM) 10x less than that toxic to cells (25 µM) in cell culture (Ciccarelli et al., 1994). More potent and less toxic Rev inhibitors may be found by modifying the structure of this series of compounds.

1.3.5 Protease of human immunodeficiency viruses

HIV protease is a viral encoded, homodimeric aspartyl protease that is characterized by C2 symmetry (Lapatto et al., 1989; Navia et al., 1989). HIV protease
processes the *gag-pol* gene precursor molecule through post-translational cleavage to release the *gag* proteins P17, P24, P6 and P7. Protease also releases the viral enzymes present in the *pol* reading frame including RT, RNase H, integrase and protease itself.

The viral protease plays an important role in late stages of viral replication. Degradation of large polypeptide precursors into smaller, functional protein fragments by protease is essential for the packaging and infectivity of budding virions. Mutations of the protease gene leads to the production of morphologically immature and non-infectious viral particles (Kohl *et al.*, 1988, Peng *et al.*, 1989). The polyprotein cleavage sites of HIV protease include an unusual Phe/Tyr-Pro dipeptide which is not recognized by eukaryotic proteases of aspartic class, such as, pepsin, gastrisin, cathepsin D and E and renin (Bragman 1996; Debouck, 1992). Because of this unique property of HIV protease, specific inhibitors of this enzyme have been developed.

Most protease inhibitors are mimetic of known peptidic substrate sequences, where the amide bond has been replaced with a non-hydrolyzable isostere containing a hydroxyl group which mimics the transition state (Huff, 1991). This hydroxyl group has been shown through X-ray analyses of numerous inhibitor-enzyme complexes to interact with the two catalytic aspartate residues of the enzyme and contributes significantly to inhibitor potency. Most inhibitors bind in an extended conformation through the multiple hydrogen bonds to the enzyme and contain aromatic or aliphatic groups which interact with the lipophilic core of the active site (Huff, 1991).

At present, four protease inhibitors, namely, *saquinavir* (Ro 31-8959, Invirase), *ritonavir* (ABT-538, Norvir), *indinavir* (L-735, 524, MK-639, Crixivan) and *nelfinavir* (AG1343, Viracept) have been approved by the US Food and Drug Administration
(FDA) for clinical use in combination with RT inhibitors (Kaldor et al., 1997; Pakyz and Israel, 1997). HIV protease inhibitors approved for use and those in clinical trials or in preclinical development are listed in Table 1.2, the structures of HIV protease and approved drugs are shown in Figure 1.2. Most of these compounds are potent and specific inhibitors of HIV replication. For most viral isolates the ED$_{95}$ (concentration required to obtain 95% inhibition) is in the range of 25-200 nM (Craig et al., 1991; Kempf et al., 1991; 1995). Protease inhibitors are active against acutely infected cells as well as the chronically infected cells, such as mononuclear cells, because they inhibit viral replication after viral DNA integration (Lambert et al., 1992). HIV-1 infected cells treated with protease inhibitors produce morphologically immature virions. These viral particles lack a central core and are characterized by the presence of envelope knobs and the double layered ribonucleoprotein shell directly attached to the inner leaflet of the bilipid bilayer (Schatzl et al., 1991). They also contain unprocessed $gag$ precursors and are non-infectious (McQuade et al., 1990). A similar pattern is observed with viruses containing deleterious mutations in the protease gene (Peng et al., 1989).

In vivo efficacy of HIV inhibitors depends on the good pharmacokinetic profile, because the pharmacokinetics parameters determine the effective drug level in target organs. All of the protease inhibitors approved for use have good oral bioavailability, steady-state concentration and tissue distribution. Clinical benefit has been achieved by using either the monotherapy (protease inhibitors only) or by combination of protease inhibitors with other drugs approved for the treatment of HIV infection.
<table>
<thead>
<tr>
<th>Drug name</th>
<th>Combination chemotherapy</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>ddI, ddC, saquinavir, nevirapine</td>
<td>Bone marrow toxicity Myopathy</td>
</tr>
<tr>
<td>ddI</td>
<td>nevirapine</td>
<td>Pancreatitis Peripheral neuropathy</td>
</tr>
<tr>
<td>ddC</td>
<td>AZT, saquinavir</td>
<td>Peripheral neuropathy Pancreatitis</td>
</tr>
<tr>
<td>d4T</td>
<td>ddI, 3TC, nelfinavir</td>
<td>Peripheral neuropathy Pancreatitis</td>
</tr>
<tr>
<td>3TC</td>
<td>AZT, d4T, AZT plus 3TC; is well studied with all protease inhibitors</td>
<td>Neutropenia</td>
</tr>
<tr>
<td>Nevirapine</td>
<td>AZT, AZT plus ddI</td>
<td>Rash, usually transient</td>
</tr>
<tr>
<td>Delavirdine</td>
<td>AZT, ddI, AZT plus ddI</td>
<td>Rash, usually transient</td>
</tr>
<tr>
<td><strong>Protease inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saquinavir</td>
<td>AZT, ddC, AZT plus ddC, ritonavir</td>
<td>Nausea, diarrhea</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>AZT plus 3TC, AZT plus ddC, saquinavir</td>
<td>Nausea, diarrhea</td>
</tr>
<tr>
<td>Indinavir</td>
<td>AZT, AZT plus 3TC, AZT plus ddI</td>
<td>Painful kidney stones</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>d4T, AZT plus 3TC</td>
<td>Diarrhea, usually transient</td>
</tr>
</tbody>
</table>

1. One or two drugs are used in combination.
Figure 1.2 Structures of protease inhibitors and model of x-ray structure of inhibitor-protease complex.
Protease inhibitors: Saquinavir, Indinavir and Ritonavir and Nelfinavir

Saquinavir is a potent inhibitor (ED$_{50}$ 1-2 nM) of HIV replication (Galpin et al., 1994). In AIDS patients, 100 mg oral dose three times daily produced a maximum median CD4 lymphocyte increase of 32 cells/mm$^3$ in one study and 54 cells/mm$^3$ in another study (Pillay et al., 1995). These effects were seen at 4-8 weeks and diminished after 16 weeks. Reduction of serum p24 antigen, plasma HIV RNA and plasma viraemia was also seen in these patients. A recent study using higher doses in clinical trial (3600 mg/d and 7200 mg/d) showed better antiviral effect and clinical benefit in 40 patients (Schapiro et al.. 1996). The trials of combination of lower dose of saquinavir (1800 mg/d) and other RT inhibitors (AZT and/or ddC) which lasted for 24 weeks and 74 weeks, respectively, have shown that combination therapy provides greater and more sustained immunological and virological responses than treatment with saquinavir alone (Collier et al., 1996a; Deeks et al., 1997).

More impressive results have been reported from the clinical trials using indinavir. 13 of the 26 patients taking indinavir alone (monotherapy) had no detectable virus in their blood after four months of treatment and 24 out of 26 (92%) patients combining indinavir with two other drugs (AZT and 3TC) achieved that result.

The other protease inhibitor, ritonavir, has also shown promising results. In a large international study (1090 patients) with severely compromised immune systems were given either ritonavir or a placebo along with whatever antiviral they were already taking. Researchers in seven countries monitored the patients health and survival rates for seven months. The morality rate was 4.8% and 8.4% for ritonavir and placebo group, respectively.
Nelfinavir (Agouron Pharmaceuticals, Inc.) has been recently approved for the treatment of AIDS. This drug inhibits the replication of HIV-1 and HIV-2 isolates [ED$_{50}$ range 9 to 60 nM (Patick et al., 1996)]. The clinical trials in AIDS patients demonstrated that nelfinavir has potent antiviral effect. Phase II study in 33 patients with moderately advanced disease showed that nelfinavir in combination with d4T produced sustained reduction of HIV RNA levels (1.3-1.9 log decline) and CD4 cell increase of 0.09 to 0.11$^9$/L for five months. In another clinical trial, the patients receiving the combination regimen of AZT, 3TC and nelfinavir had a mean 3.6 log reduction of HIV RNA levels (Deeks et al., 1997).

However, drug resistant variants of HIV can be selected in vitro and have been isolated from patients. The decreased antiviral effect of protease inhibitors within several months or half year was observed in clinical trials, especially at the low doses of monotherapy. Cross-resistance among the protease inhibitors exists. Combination therapy with RT inhibitors appears to decrease the chance of resistant strain development (Deeks et al., 1997).

In general, the protease inhibitors are well tolerated at recommended doses for treatment. The most common adverse effect is nausea and other GI disturbances. Kidney stones occurred in 4% of patients treated with indinavir (Cohen, 1996a). Liver toxicity manifested as elevated levels of serum triglyceride and γ-glutamyl transferase for ritonavir and aminotransferase for ritonavir and saquinavir have been reported (Danner et al., 1995; Markowitz et al., 1995; Schapiro et al., 1996). Ritonavir may interfere with liver enzyme cytochrome p450 (Cohen, 1996b). This can have serious consequences for patients on multiple drug therapy.
In summary, although the antiviral effect of protease inhibitors is promising, long-term effects of protease inhibitors in patients still need to be determined.

1.3.6 Myristoylation and glycosylation:

Myristoylation and glycosylation of the viral protein is necessary for virion maturation and budding. At present, myristic acid derivatives such as 13-oxatetradecanoic acid and 12-azidoctadecanoic acid showed best antiviral effect among this class (De Clercq, 1995a). However, since myristoylation and glycosylation processes are also essential for cell machinery, these agents are relatively toxic to the cells. Thus, in relation to the RT and protease inhibitors of HIV, these compounds lack selectivity.

1.4 REVERSE TRANSCRIPTASE INHIBITORS

A crucial step in the replication cycle of retrovirus, which makes it distinct from the replication cycle of other viruses, is the reverse transcription of viral RNA to the double-strand DNA (dsDNA) catalyzed by reverse transcriptase (RT). HIV RT is an attractive target for treatment of AIDS not only because it is a unique enzyme essential for HIV replication, but also it is not required for normal host cell metabolism.

Encoded by pol gene, HIV-1 RT is a heterodimer composed of p66 and p51 subunits (Figure 1.3). The p51 polypeptide is a proteolytic product of p66. HIV RT appears to have one polymerase active site, one RNase H (ribonuclease H) active site and one tRNA binding site (Kohlstaedt et al, 1992; Tantillo, et al, 1994). The reaction catalyzed by RT is a complex enzymatic reaction. For optimum activity, this enzyme
Figure 1.3 Model of reverse transcriptase (RT), showing the locations of the nucleoside analog-binding site and non-nucleoside inhibitor-binding pocket in the context of the HIV-1 RT p66/p51 heterodimer. The white circle (•) and asterisk (*) denote the locations of the nucleoside and non-nucleoside inhibitor-binding sites, respectively. (Adapted from Tantillo et al., 1994).
requires four substrates (dATP, dGTP, dCTP, and dTTP), a primer, a template, and a number of co-factors. HIV RT uses a lysine transfer tRNA as a primer to make a minus strand DNA copy of the viral RNA as an RNA-DNA hybrid. The RNA of the RNA-DNA hybrid is then degraded by RNase H activity of RT. The RT then catalyzes the production of a positive strand DNA, and eventually, a double-strand (ds) DNA is formed (Hahn, 1994). The linear dsDNA is then integrated into the cellular chromosome by viral integrase.

RT inhibitors can be broadly classified into two major categories: (1) Dideoxynucleoside analogs (ddNs); (2) Other classes of RT inhibitors, include (i) Non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as nevirapine, delavirdine and TIBO, etc.; (ii) Phosphonoformate (PFA) and (iii) Acyclic nucleoside phosphonate analogs (ANPs), such as, PMEA (adefovir) and PMPA, etc.

1.4.1 Dideoxynucleoside analogs (ddNs):

1.4.1.1 General comments and mechanism of action

At present, five of the marketed drugs for the treatment of AIDS are dideoxynucleoside analogs (AZT, ddI, ddC, d4T and 3TC). The use of these drugs singly (monotherapy) or in combination (combination therapy) has been shown to slow the progress of the disease and decrease the mortality of AIDS patients.

All regular pyrimidine and purine 2',3'-dideoxynucleosides (ddNs) with the exception of dideoxyuridine (ddUrd), namely, dideoxythymidine (ddThd), dideoxycytidine (ddC), dideoxyadenosine (ddAdo), dideoxyinosine (ddI) and dideoxyguanosine (ddGuo) inhibit the replication of HIV in vitro (Mitsuya and Broder,
However, the antiretroviral activity of ddNs is not restricted to HIV. These compounds also have potent activity against a broad spectrum of human and animal retroviruses, including human T-cell lymphotropic virus type-1, animal lentiviruses, and murine retroviruses (Dahlberg et al., 1987; Matsushita et al., 1987; Waqar et al., 1984).

$2',3'$-dideoxynucleoside analogs (ddNs) are different from the endogenous deoxyribonucleosides because they lack the $3'$-OH group (Figure 1.4). ddNs are phosphorylated by cellular enzymes to corresponding $5'$-triphosphates (ddNTPs) intracellularly (Chart 1.1) (Balzarini et al., 1989a; 1994a; Cooney et al., 1986; 1987; Furman et al., 1986; Hart et al., 1992; Ho and Hitchcock, 1989; Starnes and Cheng, 1987). ddNTPs then bind to the dNTP binding site of p66 subunit of RT (Figure 1.3). At the RT level, ddNTPs inhibit HIV replication by two mechanisms: competitive inhibition and chain termination. ddNTPs compete with the naturally occurring $2'$-deoxynucleosides (dNTP) for binding to the RT (competitive inhibition). As a result, incorporation of natural dNTP into viral DNA is slowed and this leads to decreased synthesis of viral DNA. Because the structures of ddNs closely resemble natural deoxyribonucleosides, ddNTPs can be added to the newly synthesized DNA during reverse transcription. However, elongation of the DNA chain is blocked (chain terminator) because the $3'$,5'$-phosphodiester linkage of DNA can not be made due to the lack of a $3'$-OH functional group (Mitsuya et al., 1987).

However, ddNs show variations in their effectiveness as inhibitors of HIV replication and different toxicities to the host cells. Three major factors possibly contribute to the differences observed for ddNs. (i) The relative ability of ddNs to
Dideoxynucleoside analogs (ddNs)

AZT

d4T

ddC

3TC

ddI

Non nucleoside reverse transcriptase inhibitors (NNRTIs)

Nevirapine (ST-AG-587)

BHAP U-90152 (Delavirdine)

Figure 1.4 Structures of reverse transcriptase (RT) inhibitors. Dideoxynucleoside analogs: AZT, 3'-azido-2',3'-dideoxythymidine; d4T, 2',3'-didehydro-2',3'-dideoxythymidine; ddC, 2',3'-dideoxycytidine; 3TC, (--)3'-thia-2',3'-dideoxycytidine; ddI, 2',3'-dideoxyinosine. NNRTIs: Nevirapine, (dipyridodiazepinone); Delavirdine, [bis(heteroaryl)piperazine; BHAP].
Chart 1.1 Phosphorylation pathways of dideoxynucleosides.
generate their corresponding 5′-triphosphates (ddNTPs) is a basis for the substantial differences in activity and toxicity profiles of these compounds (Balzarini et al., 1988; Hao et al. 1988). The capacity to generate ddNTPs is also different in resting cells and replicative cells. This contributes to the different toxicities of ddNs. (ii) The effectiveness and selectivity of these ddNTPs to act as inhibitors of HIV RT. The pharmacological effectiveness of ddNs depends upon their relatively selective interference with viral DNA replication in the absence of significant toxicity to cellular DNA replication in the patient. Viral RT is approximately 200 times more sensitive to these compounds than cellular DNA polymerase α, and this is possibly one reason for their selective anti-HIV activity. However, ddNs also inhibit cellular DNA polymerases (β, γ), and as a result serious side effects are observed with the use of these drugs (Balzarini, 1994a; Lewis and Dalakas, 1995; Yarchoan et al., 1991). (iii) Pharmacokinetic profiles such as oral absorption, bioavailability, plasma concentration, half-life and metabolism of these compounds are also very important for the anti-HIV activity of ddNs in vivo. Because these factors determine the concentration of drugs in targeted cells/organs, these pharmacokinetic parameters largely determine the clinical efficacy of these drugs.

Salient properties of ddNs approved for the treatment of AIDS are discussed below. Structures are shown in Figure 1.4 and metabolite pathways are summarized in Chart 1.1. Pharmacokinetic properties are listed in Table 1.3.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Plasma concentration (µM)</th>
<th>Bioavailability (%)</th>
<th>Vss (L/kg)</th>
<th>CSF/plasma (%)</th>
<th>Metabolites</th>
<th>Clearance (L/hr/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>2-10</td>
<td>1.5-10</td>
<td>65</td>
<td>1.6-3.9</td>
<td>15-135</td>
<td>AZT-TP</td>
<td>1-1.8</td>
</tr>
<tr>
<td>ddI</td>
<td>3-6</td>
<td>6-9</td>
<td>38</td>
<td>1</td>
<td>20</td>
<td>ddA-TP</td>
<td>0.8-1</td>
</tr>
<tr>
<td>ddC</td>
<td>0.01-0.5</td>
<td>0.04-2</td>
<td>88</td>
<td>0.5</td>
<td>9-37</td>
<td>ddC-TP</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>d4T</td>
<td>0.67-4.0</td>
<td>5-19</td>
<td>82</td>
<td>0.5</td>
<td>10-70</td>
<td>d4T-TP</td>
<td>0.5-0.6</td>
</tr>
<tr>
<td>3TC</td>
<td>0.25-8.0</td>
<td>1-25</td>
<td>82</td>
<td>1-1.6</td>
<td>NA²</td>
<td>3TC-TP</td>
<td>22-26</td>
</tr>
<tr>
<td>PFA</td>
<td>16000</td>
<td>&lt;33</td>
<td>12-22</td>
<td>0.5-0.74</td>
<td>10-68</td>
<td>PFA</td>
<td>0.10-0.11</td>
</tr>
</tbody>
</table>

1. The data were taken from references: Dudley, 1995; Minor and Báltz, 1991.
2. Vss: Volume of distribution.
3. NA: Not available.
1.4.1.2 3'-Azido-2',3'-dideoxythymidine (AZT, Zidovudine, Retrovir)

AZT is a thymidine analogue in which the 3'-hydroxyl group of the 2'-deoxyribose moiety is replaced by an azido(-N₃) group. It was synthesized by Horowitz et al. in the early 1960s and found to be an inhibitor of murine retrovirus replication in vitro (Horowitz et al., 1964; Krieg et al., 1978; Ostertag et al., 1974). The anti-HIV activity of AZT was discovered through the large-scale screen of compounds (Mitsuya et al., 1985). AZT was approved for the treatment of AIDS in March 1987 (Yarchoan et al., 1991). AZT monotherapy is still the first choice of treatment for HIV infected patients in most countries.

AZT is a potent inhibitor of HIV-1 replication (ED₅₀: 0.002-2.4 μM) in susceptible target T cell cultures (Balzarini et al., 1988; Coates et al., 1992; De Clercq 1992b; Mitsuya et al., 1985). The dose that causes 95% viral inhibition (ED₅₅) is less than 1 μM for most clinical isolates (Larder et al., 1989a). The intracellular half-life of AZT-TP is about 3 hours. One study showed that four hours after removal of AZT from the culture medium, the level of AZT-TP was still about 1 μM, which is much greater than the Kᵢ of HIV-1 RT (Balzarini, 1994a). The CC₅₀ [cytotoxic concentration required to reduce the viability of the (mock-infected) host cells by 50%] is in the range of 3.5-400 μM (Coates et al., 1992; Pauwel et al., 1987).

AZT permeates the cell membrane by passive diffusion and is a substrate for the cit nucleoside transpoter in human erythrocyte and lymphocyte (Zimmerman et al., 1987). AZT is phosphorylated to its 5'-triphosphate (AZT-TP) by cellular enzymes of the thymidine-phosphorylation pathway (Chart 1) (Furman et al., 1986; Zimmerman et
Cytosolic thymidine (dThd) kinase (TK) is responsible for the phosphorylation of AZT to its monophosphate (AZT-MP) (Balzarini et al., 1989; Furman et al., 1986). AZT is a good substrate for the cellular TK and is phosphorylated as efficiently as thymidine. AZT-MP is subsequently converted to AZT-diphosphate (AZT-DP) by cellular thymidylate kinase (dTMP kinase) (Furman et al., 1986). The dTMP kinase phosphorylates AZT only inefficiently since AZT-MP is a substrate inhibitor of dTMP kinase and thus seriously limits its own conversion to the 5′-DP derivative (De Clercq 1992b). Cellular pyrimidine nucleoside diphosphate kinase (NDP kinase) may be responsible for the further conversion of AZT-DP to AZT-TP. The rate of phosphorylation is substantially lower in resting monocytes/macrophages than that in T-cells where the nucleoside kinase activity is known to be low (Richman et al., 1987a; Perno et al., 1988).

AZT-TP competes with dTTP for binding to HIV-1 RT (Furman et al., 1986). With poly (rA)-oligo (dT) as the template/primer, the IC₅₀ (50% inhibitory concentration) for HIV-1 RT ranged from 0.002 to 0.1 μM (De Clercq, 1992a).

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AZT is rapidly absorbed after oral administration with the oral bioavailability about 65%, plasma half-life (t₁/₂ ≈ 1 hour) and peak plasma concentrations achieved are in the range of 1.5 to 10 μM after oral dose of 2 to 10 mg/kg (Blum et al., 1988; Klecker et al., 1987; 1988; Yarchoan et al., 1986a). The protein binding of AZT is 20 to 38% (Neuzil, 1994). AZT is well distributed in the body and penetrates readily into the cerebrospinal fluid (CSF). The CSF concentrations of the drug are 15-135% of those
found in plasma (Blum et al., 1988; Gitterman et al., 1990; Klecker et al., 1987; Yarchoan et al., 1988). AZT is metabolized extensively (50% - 80%) by hepatic glucuronidation to form 3’-azido-2’,3’-dideoxy-5’-glucuronylthymidine, an apparently inert metabolite. The metabolite and rest of the drug is excreted in the urine (Good et al., 1986; Klecker et al., 1987). AZT is also metabolized to 3’-amino-3’-deoxythymidine (AMT) and 3’-amino-3’-deoxythymidine glucuronide by both human and rat liver microsomes in vitro (Cretton et al., 1991a; 1991b). These metabolites are also detected in plasma of rhesus monkeys (Morse et al., 1993). The former contributes to the toxicity of AZT. The total body clearance (CL) is about 1.0 to 1.8 L/h/kg, indicating a pattern of high clearance (Blum et al., 1988, Klecker et al., 1987). The renal clearance (C\textsubscript{r}) are reported to be 188 ml/min (11.3 L/h) for AZT and 293 ml/min (17.6 L/h) for the glucuronide (Blum et al., 1988).

The use of AZT even at low doses (regimen), has been shown to induce severe bone marrow toxicity, skeletal myopathy and reversible hepatotoxicity in patients. Some patients receiving AZT experience nausea, vomiting, abdominal discomfort, headaches, confusion, malaise and fatigue (Arnaudo et al., 1991; Dalakas et al., 1990; Dournon et al., 1988; Fischl et al., 1990; Melamed et al., 1987; Neuzil, 1994; Peters et al., 1993).

Further discussion of bone marrow and skeletal myopathy toxicities due to AZT are given in chapter 3 (section 3.1).

The efficacy for AZT in the treatment of ARC and AIDS has been extensively studied around the world (Danner, 1996). The results of these clinical trials are briefly summarized below:
1. Benefits of AZT therapy in advanced HIV-infected patients include prolonged survival, delayed disease progression, reduction in opportunistic infection, stabilization of weight, improved performance, stabilization of HIV-associated dementia, reduction in prenatal transmission, reduction in viral burden and increase in CD4 counts. However, the benefits of AZT monotherapy are limited. The antiviral effects decrease with the prolonged use of AZT. The clinical failure often parallels the appearance of mutations in the viral RT gene with reduced sensitivity of AZT.

2. All the clinical trials demonstrated that the early intervention of the disease by AZT results temporarily in clinical benefits. It is hard to conclude that the use of AZT in the treatment of early stage of HIV infection will indeed show clinical benefits or not. The ineffectiveness of AZT in asymptomatic patients may be due to the development of AZT resistant strains (Danner 1996).

\textit{1.4.1.3 Dideoxyinosine (ddI, Didanosine, Videx)}

Dideoxyinosine (ddI) was the second antiretroviral agent approved by the FDA for the treatment of HIV infection. ddI is currently approved for patients who have either not responded to AZT therapy or who are intolerant to its adverse effects (Shelton \textit{et al.}, 1992)

ddI inhibits the HIV-1 replication in lymphocytes test system (ED\textsubscript{50}: 0.08-12 \textmu M) (Chu \textit{et al.}, 1988). It is also active against AZT-resistant strains of HIV-1 (Morse \textit{et al.}, 1993). ddI is less toxic to lymphocytes compared to AZT (CC\textsubscript{50} 100 -1000 \textmu M) (Coates \textit{et al.}, 1992; Chu \textit{et al.}, 1988).
ddI is a purine analog of deoxyinosine. It penetrates cells by either passive process or facilitated diffusion (McGowan et al., 1990; Dudley, 1995). ddI exerts its antiviral activity by its ability to generate the active anabolite, ddATP. The initial phosphorylation of ddI to ddIMP is catalyzed by cytosolic 5′-nucleotidase (Johnson and Fridland, 1989). ddIMP is then converted to dideoxyadenosine monophosphate (ddAMP) by adenylosuccinate synthetase and adenylosuccinate lyase. Subsequently, ddAMP is converted to ddA di- and tri-phosphates (Chart 1.1) (Johnson et al., 1988). The intracellular half-life of ddATP varies from 12 to 24 hours depending on the presence of its natural nucleoside.

ddATP has a high affinity for the HIV-1 and HIV-2 RT, but not for DNA polymerases (Sommadossi, 1993). ddATP inhibits HIV-1 RT with $K_i$ values ranging from 0.083 to 0.22 μM when using poly (U)-oligo (dA) or poly (A-T) as template/primer (Hao et al., 1988).

ddI is acid labile and is readily hydrolyzed to dideoxyribose and hypoxanthine. Therefore, ddI requires an anti-acid buffer combined with the drug when it is given orally. The buffered tablets are used clinically. ddI is rapidly absorbed with bioavailability about 20-40% when given orally (Drusano et al., 1992; Hartman et al., 1990; Knupp et al., 1991; Pai et al., 1992; Singlas et al., 1992). The presence of food can significantly reduce the absorption of ddI (Shyu et al., 1991).

The peak plasma concentrations are about 6 to 9 μM with oral dose of 3-6 mg/kg of ddI (Hartman et al., 1990; Singlas et al., 1992). The volume of distribution is about 1 L/kg. ddI can permeate into the CSF with a CSF/plasma ratio of 20% (Hartman et al., 1990). The serum elimination half-life of ddI after oral administration ranges from 0.6-
1.5 hour (Hartman et al., 1990; Singlas et al., 1992). However, the active metabolite ddATP remains in cell for a long time $t_{1/2} > 12$ hours (Ahluwalia et al., 1993). ddI is metabolized by either a non-enzymatic hydrolytic cleavage or by purine nucleoside phosphorylase to $2',3'$-dideoxyribose and hypoxanthine. Hypoxathine may be used in the generation of endogenous nucleotides or further catabolized to and excreted as uric acid (Johnson, et al, 1988; Shelton et al., 1992). Up to 60% of ddI is excreted unchanged in the urine. The rest is metabolized to ddATP, hypoxanthine and uric acid (Hartman et al., 1990; Sommadossi, 1993).

The major life threatening toxicity of ddI is pancreatitis. Pancreatitis can occur at any point with the treatment, and occurs with standard dose of ddI after prolonged treatment in 5% of patients (Pike and Nicaise, 1993). The manifestations of ddI-related pancreatitis include abdominal pain, elevated serum amylase and/or lipase and elevated triglyceride concentrations. The risk of developing ddI related pancreatitis is higher (30%) in patients with a prior history of pancreatitis (Shelton et al., 1992). The mechanism of ddI pancreatitis is not understood and is probably related to the effect of ddI on mitochondrial damage (Lewis and Dalakas, 1995).

Another ddI-related toxicity is peripheral neuropathy, which occurred in about 12-34% of ddI-treated patients (Shelton et al., 1992). The onset and progression of neurologic toxicity are related to the daily and cumulative doses of ddI (Rozencweig et al., 1990). The mean time of onset of ddI neuropathy is 20 weeks (Simpson and Tagliati, 1995). ddI neuropathy is clinically similar to that of ddC, but is usually less severe. Patients complain of tingling, burning or aching sensation that typically begins at the soles of the feet and may progress upward to the legs (Shelton et al., 1992; Simpson and
Reduced distal vibratory sensation and depressed ankle reflexes may also occur. Nerve conduction studies often failed to demonstrate significant changes (Rozencweig et al., 1990; Yarchoan et al., 1990). The symptoms may continue to worsen during the first week after discontinuation and are usually resolved in 3-5 weeks in most patients (Kieburtz et al., 1992). The neuropathy may be due to ddI’s action on mitochondrial DNA of neural cells (Simpson and Tagliati, 1995).

Other side effects of ddI include diarrhea, nausea and vomiting, headache, insomnia, rash, hepatitis and seizures. Elevations in serum uric acid and hepatic aminotransferase may also be observed in ddI treated patients (Moyle et al., 1993).

The low toxicity and favorable safety profile of ddI led to the clinical trial of ddI (Cooley et al., 1990; Yarchoan et al., 1989). The patients treated with ddI experienced decrease of p24 concentration in serum and increase of CD4 count and the clinical benefits seen were similar to AZT. These effects observed in some patients over one year of treatment (Yarchoan et al., 1990). Switching to ddI after AZT treatment can delay disease progression, increase survival rate and decrease the frequency to opportunistic infection compared to patients with continued AZT treatment (Spruance et al., 1994). ddI treatment can also improve the HIV dementia in certain patients (Yarchoan et al., 1990).

1.4.1.4 Dideoxycytidine (ddC, Zalcitabine, Hivid)

The third nucleoside analog approved by FDA for the treatment of HIV infection was dideoxycytidine (ddC). ddC monotherapy is indicated for adults with advanced HIV disease who either are intolerant to AZT or who have experienced disease progression
while receiving AZT (Skowron, 1996). In addition, ddC is preferred for patients who are intolerant of ddI or with risk factors for pancreatitis. Combination of AZT/ddC may be considered for initial therapy of symptomatic patients with no prior antiretroviral treatment and advanced HIV disease (Skowron, 1996).

ddC is a very potent inhibitor of the HIV-1 replication in lymphocyte cell line (ED₅₀: range 0.001-0.9 μM) and CC₅₀ range from 5 to > 356 μM (Coates et al., 1992; Pauwels et al., 1988). ddC shows synergistic activity in combination with AZT against both AZT sensitive and AZT-resistant clinical isolates of HIV-1 (Eron et al., 1992).

ddC appears to enter cells by both facilitated and non-facilitated mechanisms (Plagemann et al. 1989, Ullman et al. 1988). Intracellularly, ddC is phosphorylated to ddC-MP by cellular enzyme, deoxycytidine kinase. ddC is a weak substrate for cytosolic dCyd kinase with Kₘ value 180 μM compared to 3 μM for deoxycytidine (Balzarini et al., 1987a; Johnson et al., 1987; Starnes et al., 1987). The Vₘₐₓ for ddC phosphorylation is also significantly lower than the Vₘₐₓ for dCyd. ddC-MP is further phosphorylated to its di- and triphosphate forms, probably catalyzed by CMP/dCMP kinase and nucleoside diphosphate (NDP) kinase, respectively (Chart 1). Phosphorylation of ddC is inhibited by deoxycytidine and enhanced by dThd (Balzarini et al., 1987a; Broder, 1990; Conney et al., 1986). Besides the phosphate forms of dCyd, ddCDP-choline and ddCDP-ethanolamine adducts are also formed to a significant extent (Hao et al., 1993). ddCTP competes with dCTP to inhibit HIV-1 RT with Kᵢ value of 0.26 μM (Hao et al., 1988).

ddC is rapidly absorbed after oral administration with bioavailability of 88%. The peak oral plasma concentration ranged from 0.04-2 μM depending on the dosages of ddC. Values for the Vₘₐₓ is 0.5 L/kg, which is less than AZT and ddI. ddC can penetrate
into the CSF, the CSF/plasma ratio is about 9-37% (Gustavson et al., 1990; Klecker et al., 1988; Yarchoan et al., 1988b). Till now, no hepatic metabolites of ddC have been identified in humans. About 8-9% of ddC was deaminated to dideoxyuridine in monkeys. The elimination half-life of ddC is 1-2 hours which is similar to that for other dDNs (Gustavson et al., 1990; Klecker et al., 1988). The intracellular half-life of ddCTP is about 2.6 hours (Starnes and Cheng, 1987). About 75% of ddC is excreted unchanged in urine (Klecker et al., 1988).

The major toxic manifestation of ddC is **neuropathy**. ddC neuropathy is dose related and occurred in 25-66% patients in phase I/II studies. The manifestations of neuropathy are pain, numbness of lower extremities, occasionally weakness, loss of ankle deep tendon reflexes studies (Berger et al., 1993; Yarchoan et al., 1988b). Human receiving ddC show clinical and electrophysiologic evidence of distal symmetric axonal neuropathy (Dubinsky et al., 1989; Berger et al., 1993). The time of onset ranged from 8-23.5 weeks depending on the dosages of ddC. The recovery of neuropathy occurred 3-5 months after ddC withdrawal. Some patients experience a period of intensification of symptoms when ddC treatment is discontinued (Blum et al., 1993; Berger et al., 1993). Like ddI, pancreatitis also has been seen in patients (2%-5%) treated with ddC (Abrams et al., 1994). Other toxicities of ddC include ulcerative stomatitis esophagitis (17% cases) (Fischl et al., 1993). Mucocutaneous eruptions, cardiomyopathy/congestive heart failure and anaphylactoid reactions were also observed in patients with ddC therapy [(McNeely et al., 1989; package insert, HIVID (Zalcitabine), 1992].

Three clinical trials compared efficacy of ddC to AZT. 636 AIDS or ARC patients with less than three months of prior AZT treatment were given either ddC or
AZT. The trial was stopped one year later because continued therapy with AZT was superior in respects of survival, opportunistic infection, CD4 counts and weight gain (Skowron, 1996).

ddC was also compared to AZT in the three arm trial ACTG 155 (Fischl et al., 1995). The patients with at least six months AZT prior therapy were randomized to receive AZT, ddC or both AZT and ddC. There was no significant difference with ddC treatment compared to AZT monotherapy, and ddC compared to AZT/ddC combination therapy with respect to disease progression and survival. However, the greater toxic responses were observed in combination therapy group, especially in patients with CD4 cells <50 per ml. ddC treatment was also compared to ddI monotherapy in patients who have failed or were intolerant to AZT. The sixteen months observation showed there were no significant differences between the two treatment in respect to disease progression and death (Abrams et al., 1994).

The regimens of ddC alternating with AZT were designed to minimize the toxicity of individual drugs. Some alternating regimens suppressed p24 antigenemia and increased CD4 cell counts for up to one year during therapy. Hematological toxicity was markedly reduced with alternating therapy; rates of neuropathy were similar or higher than that of AZT monotherapy. Studies of concurrent ddC/AZT combination therapy showed that except in participants with fewer than 150 CD4 cells/ml, generally the combination therapy had more pronounced and prolonged rises in CD4 counts and drop in plasma viral RNA concentration than AZT monotherapy (Meng et al., 1992).

1.4.1.5 Didehydrodideoxythymidine (d4T, Stavudine, Zerit)
2′,3′-Didehydro-2′,3′-dideoxythymidine (d4T) is the fourth nucleoside analog approved by FDA for the treatment of advanced HIV-1 infection in adults who demonstrated clinical progression or immunologic deterioration while receiving AZT and are intolerant to ddI or ddC (Riddler et al., 1995).

In most cell systems, the anti-HIV potency of d4T is comparable to AZT (ED$_{50}$: 0.009 - 4.1 μM). Cellular toxicity of d4T for lymphoblast cell lines is in the range of 1.2-119 μM (Riddler et al., 1995).

d4T enters cells by non-facilitated diffusion (August et al., 1991). Phosphorylation to the d4T-MP by thymidine kinase and d4T-DP by dTMP kinase is much less efficient than AZT and is the rate limiting anabolic step in the formation of active metabolite d4T-TP (Balzarini et al., 1989a; Ho and Hitchcock, 1989). Interestingly, d4T-MP does not inhibit dTMP kinase, so that d4T-MP does not accumulate inside the cell. Furthermore, d4T does not cause the increase of dTMP pools of the cells. The ratio of d4T mono-to di to triphosphate is relatively constant, ranging from 1:1:1: to 1:1:3 (Balzarini et al., 1989a). d4T-TP inhibits HIV-RT with IC$_{50}$ value of 0.03 μM when using poly(a).oligo(dT) as template/primer (Matthes et al., 1987).

d4T is stable at gastric pH, is rapidly absorbed after oral administration (bioavailability about 82%) and gives plasma concentrations in the range of 5-19 μM with oral dosages from 0.67 to 4 mg/kg. The drug is well distributed throughout the body (Vss value ≈ 0.5 L/Kg), penetrates into CSF (CSF levels are 10-70% of those in plasma), and a half-life of 1-1.6 hours (Dudley et al., 1992; Horton et al., 1995). The studies in humans indicate that approximately 40% of the dose is excreted unchanged in urine and the remaining drug is broken down at the glycosidic linkage to form thymine.
(Cretton et al., 1993; Schinazi et al., 1990). The metabolic fate of d4T in humans is not very clear at present.

Peripheral neuropathy is the principal dose limiting toxicity of d4T and occurs in 15-21% of the patients (Browne et al., 1993; Murray et al., 1995). The symptoms of neuropathy included bilateral numbness or tingling progressing to pain in the feet and lower legs were similar to the neuropathy associated with ddI and ddC (Skowron, 1995). The symptoms resolve within 1 week to several months after interruption of the treatment (Petersen et al., 1995).

Recent studies suggest that neuropathy induced by d4T may be mediated by mechanisms different from those of ddI and ddC (Sommadossi, 1995). In an in vitro system, a correlation was observed between inhibition of neurite outgrowth and decreased mtDNA synthesis for ddI and ddC. Although d4T was as toxic as the other two ddNAs in altering neurite outgrowth, it did not inhibit mtDNA synthesis. Lack of the inhibition of mtDNA synthesis by d4T may be due to the fact that d4T is a poor substrate for mitochondrial thymidine kinase or due to the poor affinity of d4T-TP toward a hypothetical transport carrier at the mitochondrial membrane (Chen and Cheng, 1992; Munch-Pertersen et al., 1991).

Pancreatitis was reported in 1% of d4T treated patients (Stavudine, 1994). The other side effects include elevated levels of hepatic SGOT and aspartate aminotransferase (Skowron, 1995).

To date, most clinical trials have been done in patients previously treated with AZT. The phase III trial studied the effect of d4T in patients with CD4 counts of 50-500 cells/mm$^3$ who had received at least 6 months of prior AZT therapy. The patients
switched to d4T therapy (40 mg b.i.d) had a more favorable surrogate marker's changes for considerably longer period than the subjects continued on AZT treatment. The patients switched to d4T therapy also had less hematological toxicity (Riddler et al., 1995). The use of d4T for initial therapy has not been assessed in the clinical trials performed to date. The exploratory analysis of the naive subjects enrolled in the phase I and II studies of d4T and the retrospective comparison with the subjects with AZT as initial therapy showed that the CD4 cell increases in the d4T group (94 cell/mm$^3$) exceeded that in the AZT group (53 cell/mm$^3$) and appear to be maintained above baseline level for up to 48 weeks compared to 30 weeks on AZT. This results suggest that d4T should be directly compared to AZT as initial monotherapy for HIV-infected adults (Friedland et al., 1996).

1.4.1.6 (-)2′-Deoxy-3′-thiacytidine (3TC, Lamivudine, Epivir)

3TC [(-) enantiomer] is an analog of ddC approved for the treatment of HIV infection in combination with AZT.

3TC differs from ddC by the substitution of a sulphur atom at the 3′-position of the ribose ring. This drug is a potent inhibitor of HIV-1 and HIV-2 replication (ED$_{50}$: 0.0025 to 0.67 μM) in cell cultures. 3TC is considerably less toxic for T-lymphocyte cell lines (CC$_{50}$: 0.5 to 5 mM) compared to AZT and ddC (Coates et al., 1992). It is also active against AZT-resistant HIV variants (Larder et al., 1995). 3TC crosses the cell membrane through non-facilitated passive diffusion and is anabolized to its 5′-mono-, 5′-di- and 5′-tri-phosphate derivatives by intracellular kinases (Chang et al., 1992a;
1992b; Coates et al., 1992). The intracellular half-life of 3TC-TP in peripheral blood lymphocytes was found to be 10-15 hours (Cammack et al., 1992).

Similar to other ddNs, 3TC is a competitive inhibitor of HIV-1 RT. It also acts as a chain terminator. In vitro, 3TC-TP competes with dCTP for HIV-1 RT (Kₘ: 10.6 to 12.4 μM) depending on the template and primer used. The IC₅₀ of 3TC-TP for HIV-1 RT is 23.4 μM, while the values for the cellular DNA polymerase α, β and γ are 175, 24.8 and 43.8 μM, respectively. The lack of selectivity in inhibition of DNA pol β and γ by 3TC suggests that competitive inhibition of RT activity does not appear to account for the potent and selective antiviral effect. In contrast, the chain terminating effect by 3TC may play an important role in inhibiting the viral replication (Gray et al., 1995).

3TC is well absorbed with the average oral bioavailability about 82%. The peak oral plasma concentration is about 1 to 25 μM with oral dose of 0.25 to 8.0 mg/kg. 3TC has a volume of distribution of 1.0-1.6 L/kg. The serum elimination half-life of 3TC is 2.5 hours. About 68-71% of the drug is excreted unchanged in urine (Horton et al., 1994; Van Leeuwen et al., 1992). The pharmacokinetics of 3TC was not altered after three days of concomitant administration of AZT (Horton et al., 1994).

In general, 3TC is well tolerated when used alone (monotherapy) and in combination with AZT. In a phase I/II study, about 2.1% patients developed neutropenia at highest dose 20 mg/kg/d (Pluda et al., 1995). There was a trend toward greater reduction in neutrophil count and hemoglobin in the high-dose combination of 3TC (300 mg twice daily) with AZT (Katlama et al., 1996; Staszewski et al., 1996). The other adverse effects observed in clinical trials were headaches, insomnia, nausea, diarrhea and abdominal pain (Pluda et al., 1995).
The phase I/II study in patients with advanced HIV infection showed that 3TC monotherapy only has a transient anti-HIV activity as measured by CD4 counts and viral load in blood (Pluda et al., 1995). Another clinical trial in asymptomatic or mild symptomatic patients demonstrated that the peak viral suppression was achieved at study week 4 or week 8, the antiviral effect of 3TC decreased afterwards (Ingraud et al., 1995). This phenomenon could be partially explained by the rapid development of high-level resistance of HIV to 3TC in vitro and in vivo (Boucher et al., 1993; Gao et al., 1993; Schuurman et al., 1995; Tisdale et al., 1993;).

The clinical trials of the combination of 3TC with AZT were conducted based on the observation that 3TC is active against AZT-resistant isolates and is synergistic with AZT in vitro (Larder et al., 1995). The results showed that the antiviral effects, namely, decrease in the viral load and increase of the CD4 lymphocyte counts are superior to and sustained longer than that of AZT monotherapy and AZT-ddC combination therapy (Bartlett et al., 1996; Katlama et al., 1996; Staszewski et al., 1996). 3TC also slowed the development of AZT-resistant strains in combination therapy (Katlama et al., 1996; Larder et al., 1995).

1.4.2 Other classes of RT inhibitors

1.4.2.1 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

The NNRTIs represent a structurally diverse group of compounds which inhibit HIV-1 replication with very high specificity and potency. There are at least 25 groups of compounds categorized as NNRTIs (De Clercq, 1996a). To date, two of NNRTIs, namely, nevirapine (Viramune) and delavirdine (Rescriptor, U-90152) have been
approved for use in the United States. A third NNRTI, efavirenz (Sustiva, DMP-266, L-743,726,) is currently available in the United States through an expanded access program (Holtzer et al., 1998). Structures of these drugs are shown in Figure 1.4.

NNRTIs are potent and highly specific inhibitors of HIV-1 replication (ED\textsubscript{50} 0.006 to 4.7 \(\mu\)M). These compounds have low cytotoxicity (CC\textsubscript{50} 100 to 1,000 \(\mu\)M). NNRTIs specifically interact with a molecular site of HIV-1 RT. These compounds do not interact with other DNA polymerases (\(\alpha, \beta, \gamma, \delta\)) and therefore are selectively virotoxic (De Clercq, 1996a).

A number of classes of NNRTIs such as the derivatives of dipyridodiazepinones (e.g. nevirapine), bis(heteroaryl)piperazine (BHAP, e.g. delavirdine), tetrahydroimidazo-benzodiazepinone (TIBO), hydroxyethoxymethylphenylthiohyamine (HEPT), show synergistic anti-HIV activity in cell cultures when combined with ddNs such as AZT and/or ddI and/or ddC (De Clercq, 1996a). However, most of the NNRTIs did not show synergistic effect with AZT-TP at enzymatic level (Yuasa et al., 1993). The synergistic effects of NNRTIs and ddNs can be partially explained on the basis that these drugs may lead to the mutually non-complementary or mutually antagonistic resistance mutations of the virus (De Clercq, 1996b).

**Mechanism of action**

Crystallographic analysis of thiazolobenzimidazole (TBZ) and dipyridodiazepinones showed a “butterfly”-like structure (Figure 1.5) (Mui et al., 1992; Chimirri et al., 1995). Thus, while these groups seemingly look unrelated based on simple, two-dimensional structures, many do share a common three-dimensional
Figure 1.5 Butterfly-like structure of thiazolobenzimidazoles (TBZ). (Adapted from De Clercq, 1996a).
structure. A closer analysis of their chemical structure indicated that most of them have a common "butterfly-like" conformation, that is, the hydrophilic center (body) which is characterized by (thio)carboxamide, (thio)acetamide or (thio)urea etc., surrounded by two hydrophobic outskirts (wings).

NNRTIs interact directly with HIV-1 RT and do not need to be activated intracellularly. The binding of NNRTI with HIV-1 RT allosterically inactivate the RT activity (Ding et al., 1995a; 1995b). NNRTIs inhibit the HIV-1 RT activity by mainly inhibiting the RNA-dependent DNA polymerization step, not the ribonuclease H or DNA-dependent DNA polymerization (Althaus et al., 1993; Debyser et al., 1991; Merluzzi et al., 1990; Tramontano and Cheng, 1992). Furthermore, they also show a template/primer preferences. NNRTIs tend to inhibit the chain elongation with a template/primer of poly(C)-oligo(dG), rather than with a template/primer of poly(A)-oligo(dT), poly(U)-oligo(dA) or poly(I)-oligo(dC) (Baba et al., 1991a; Balzarini et al., 1992; Debyser et al., 1991; Goldman et al., 1991; Pauwels et al., 1990; Tramontano and Cheng 1992). NNRTIs are noncompetitive inhibitors with respect to both substrate (dGTP) and template/primer [poly(C)-oligo(dG)]. However, they could also be competitive inhibitors. For example, HEPT derivatives compete with dTTP if using poly(A)-oligo(dT) as template/primer, and TIBO R82150 is a competitive inhibitor with respect to dATP if directed by poly(U)-oligo(dA) (Baba et al., 1991a; 1991b; Balzarini et al., 1992). This dual behaviors suggests that the HIV-1 RT binding site for the NNRTIs must be functionally and possibly also spatially related to the natural substrate binding site. This hypothesis was strongly supported by crystal structure
analysis of RT with nevirapine which shows that nevirapine binding site on RT is only about 10Å apart from the polymerase active site (Ding et al., 1995a; 1995b).

The clinical trials of nevirapine, delavirdine and other NNRTIs, such as TIBO R82913, TIBO R91767, pyridinone L697, 661, BHAP U90152 and α-APA R89439 demonstrated that NNRTIs produce marked but transient antiviral effects. The rapid rebound in viral markers and decrease in CD4 lymphocyte counts could be observed within the several weeks of NNRTIs monotherapy (De Clercq, 1996b; Kilby and Saag 1996). The loss of the antiviral activity of NNRTIs are closely related to the rapid emergence of drug-resistant virus strains (De Clercq, 1996b). The development of resistant virus strains has been generally perceived as a serious problem, thus compromising the clinical usefulness of NNRTIs.

The drug-resistance is due to the mutations of HIV-1 RT. The most frequent and general amino acids mutated in RT when it become cross-resistance to NNRTIs are: L100, K103, V106, V108, Y181, Y188 and G190. For example, the most generalized cross-resistance is the mutation Y181C, which results in resistant to TIBO, HEPT, nevirapine, pyridinone, BHAP, TSAO, α-APA and quinoxalines (Baba et al., 1991b; Balzarini et al., 1993a; 1993b; 1993c; 1993d; Byrnes et al., 1993; Kleim et al; 1993; Mellors et al., 1992; Nunberg et al., 1991; Nguyen et al., 1994; Ríchman et al., 1991; Sardana et al., 1992). Interestingly, resistance to one NNRTI may or may not show cross-resistance to other NNRTIs. However, even for the Y181C mutant, the PETT compounds as well as some other compounds were still proven to be effective (Ahgren et al., 1995; Balzarini et al., 1994b; Brennan et al., 1995; Buckheit et al., 1995; Williams et al., 1993). A very straightforward strategy, “knocking out” the virus (high
concentrations of drugs that can completely suppress virus replication) has been suggested to prevent the virus from becoming resistant for NNRTIs (De Clercq 1996b). The “knock-out” procedures of NNRTIs should be considered and applied in clinical trials, especially in combination with the other anti-HIV drugs.

The clinical trials of NNRTIs in combination with nucleoside analogs and protease inhibitors has shown dramatic and sustained anti-HIV effects. The use of two or three drug regimens: delavirdine + AZT + ddi; nevirapine + AZT + ddi and efavirenz + indinavir significantly increased CD4 cell counts in AIDS patients. Most impressively, about 60-89% of the patients had undetectable viral load after 48-56 weeks of the treatments. (Holtzer et al., 1998).

NNRTIs are well tolerated in patients. The most common adverse effect of nevirapine, delavirdine and efavirenz was rash. To date, NNRTIs have been recommended for second-line therapy for AIDS patients.

The salient characteristics of ddN analogs and NNRTIs are summarized in Table 1.4

1.4.2.2 Phosphonoformate (Foscarnet sodium, PFA)

Phosphonoformate (PFA), a pyrophosphate analog, is an effective inhibitor of several DNA and RNA viruses (See reviews, Chrisp and Clissold, 1991; Oberg, 1989; Wood and Geddes, 1987). The selective activity of PFA is due to its specific inhibitory effects on the viral-associated DNA polymerases (Cheng et al., 1981; Datta and Hood, 1981; Eriksson et al., 1982; Helgstrand et al., 1978; Oberg, 1986). PFA has been shown to be an effective inhibitor of HIV-1 replication in vitro in a dose-dependent manner.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ddN analogues</th>
<th>NNRTIs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Potency</td>
<td>at nM conc.</td>
<td>at nM conc.</td>
<td></td>
</tr>
<tr>
<td>SI</td>
<td>$\leq 10^4$</td>
<td>up to $10^5$ (and higher)</td>
<td></td>
</tr>
<tr>
<td>Target enzyme</td>
<td>RT (all retroviruses)</td>
<td>RT (HIV-1 only)</td>
<td></td>
</tr>
<tr>
<td>Target site</td>
<td>Catalytic</td>
<td>Allosteric</td>
<td></td>
</tr>
<tr>
<td>Mode of action (at target site)</td>
<td>Chain termination</td>
<td>Inactivation</td>
<td></td>
</tr>
<tr>
<td>Intracellular metabolism</td>
<td>to 5'-triphosphate form (ddNTP)</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Interaction with natural substrate</td>
<td>Competitive</td>
<td>Non-competitive</td>
<td></td>
</tr>
<tr>
<td>Ability to “clear” cells of virus</td>
<td>No</td>
<td>At “knock-out” concentrations</td>
<td></td>
</tr>
<tr>
<td>Drugs resistance development</td>
<td>Probable (not very rapid)</td>
<td>Highly (very rapid)</td>
<td></td>
</tr>
</tbody>
</table>

1. Adapted from reference De Clercq, 1996a with modification.
2. Compared to didexoxynucleoside analogs, one of the common events observed in NNRTIs is the rapid development of drug-resistant virus strains both in vitro and in vivo.
(ED$_{50}$ 1.4 to 25 μM) (Eriksson and Schinazi, 1989; Kong et al., 1991; Koshida et al., 1989). Exposure of HIV-1 infected cells to 132 and 680 μM of PFA resulted in 98% and 100% inhibition of HIV-1 replication (Sandstrom et al., 1985). PFA shows synergistic activity in combination with AZT, 3’-fluoro-thymidine and d4T against HIV replication in cell culture without concomitant increase in toxicity to the host cell (Cox et al., 1991; Eriksson and Shinazi, 1989; Kong et al., 1991). PFA is less toxic to the cells (CC$_{50}$ about 1000 μM) compared to the most of the ddNs (Stenberg et al., 1983; 1985; Stenberg and Larsson, 1978).

HIV-1 RT is uniquely sensitive to PFA (Vrang and Oberg, 1986). The IC$_{50}$ of HIV-1 RT has been reported to be in the range of 0.1 μM to 0.5 μM and IC$_{100}$ at 5 μM (Sandstrom et al., 1985; Sarin et al., 1985; Vrang and Oberg, 1986; Vrang et al., 1988).

PFA is considered a product analogue in the context of RT inhibition because of its structural resemblance to pyrophosphate, one molecule of which is formed each time the enzyme catalyses addition of a nucleotide to a nascent DNA chain (Figure 1.6) (Vrang and Oberg, 1986). PFA actively inhibits HIV-1 RT in its parent form and does not require phosphorylation for optimal antiviral activity. PFA is a noncompetitive inhibitor of HIV RT with respect to substrate and template (Vrang and Oberg, 1986). The exact binding site of PFA to HIV RT is not very clear. PFA probably interacts with HIV-1 RT at a site where pyrophosphate is split off during polymerization of nucleoside triphosphates (Figure 1.6) (Sandstrom et al., 1985; Vrang and Oberg, 1986). The noncompetitive inhibitor pattern of PFA indicated that PFA does not interact at the binding site for dNTP on the enzyme (Vrang and Oberg, 1986). Combination of PFA and AZT-TP has additive effects at HIV-1 RT level (Starnes and Cheng, 1989). This
Figure 1.6 Structure of phosphonoformate and its mode of action. A. Chemical structure of trisodium phosphonoformate (foscarinet, PFA). B. Schematic representation of DNA chain elongation catalysed by DNA polymerase showing target site of phosphonoformate. Abbreviations and symbols: A=adenine; C=cytosine; G=guanine; T=thymine; =phosphodiester bond; P=phosphate; PPI=pyrophosphate. (Adapted from Chrisp et al., 1991).
mutually exclusive inhibition plus the site directed mutagenesis studies of HIV-1 RT suggest that the binding site of PFA and AZT-TP on HIV-1 RT may be overlapping (Larder et al. 1987).

PFA is approved for use in the treatment of cytomegalovirus (CMV) retinitis, one of the most common and devastating afflictions in patients with advanced HIV infection. The clinical trials suggest that treatment with PFA has an in vivo antiretroviral effect, which include the decrease of HIV RNA level, reduced p24 antigen level, and increase of CD4 counts in blood (Gaub et al., 1987; Farthing et al., 1987; Jacobson et al., 1988, Kaiser et al. 1995). In two clinical trials, the extent of decreases of p24 and HIV RNA level in blood in AIDS patients was comparable to that observed with other antiretroviral drugs (Chaisson et al., 1988, Jabobson et al. 1988, Kaiser et al. 1995; Yerly et al., 1995a; 1995b). A multicentre trial involving 234 patients with AIDS and CMV retinitis demonstrated convincingly that PFA was better tolerated in AIDS patients receiving other myelosuppressive medication such as AZT (Hirsh, 1992).

PFA has low oral bioavailability (12-22%) so that drug has to be administrated by i.v. route, and often requires the use of an infusion pump (Sjovall et al., 1988). The patients on PFA therapy usually need the close monitoring for electrolyte abnormalities. The toxicities of PFA consists primarily of reversible renal dysfunction to some degree as well as disturbances of mineral and electrolyte balance such as hypokalemia, hypercalcaemia, hypocalcemia, hypomagnesemia and hyperphosphatemia, etc. (Hirsch, 1992). Seizures, anemia, hallucinations, fever and rash may also occur in patients receiving PFA treatment (Hirsch 1992; Minor and Baltz 1991). These disadvantages of PFA limit the clinical use of PFA for long-term AIDS therapy.
1.4.2.3 Acyclic nucleoside phosphonates (ANPs)

The acyclic nucleoside phosphonates (ANPs) is another class of RT inhibitors. Representatives ANPs with good activity against HIV include: [9-(2-(phosphonylmethoxyethyl)adenine] (PMEA), [(R)-9-(2-(phosphonylmethoxypropyl)adenine] (PMPA), [(S)-9-(3-fluoro-2-(phosphonylmethoxypropyl)adenine] (FPMPA), [9-(2-(phosphonylmethoxyethyl)-2,6-diaminopurine] (PMEDAP), [(S)-9-(3-fluoro-2-phosphonylmethoxypropyl)-2,6-diaminopurine] (FPMPDAP) and [(R)-9-(2-(phosphonylmethoxypropyl)-2,6-diaminopurine] (PMPDAP) (Figure 1.7). These compounds are structurally like the dideoxynucleoside-5′-phosphate (ddNMP) analogues because the first phosphate group has been built in as a phosphonate. The ANPs are phosphorylated to their corresponding disphosphorylated derivatives in the cells by either PRPP synthetase or AMP kinase (Balzarini et al., 1991a). The ANP diphosphates then inhibit HIV replication by acting as the competitive inhibitors of RT with respect to dATP, or as DNA chain terminators (Balzarini et al., 1991a; Cronn et al., 1992).

PMEA and its congeners are effective against a wide range of retroviruses including HIV in cell culture with ED₅₀ values in the range of 0.02-45 μM (Balzarini et al., 1991b; Pauwels et al., 1988). Most interestingly, however, their in vivo antiviral activity seems more potent than may be predicted from their in vitro antiviral potency. For example, the in vivo anti-retroviral effects of PMEA are superior to that of AZT in animal models (Balzarini et al., 1989b; 1990a). This may be related to the long half-life of the active metabolite, PMEApp intracellularly (t½: 16-18 hours) which in turn leads to long-lasting antiviral action of PMEA (Balzarini et al., 1990b; 1991a; Naesens et al., 1991).
Figure 1.7 Structures of acyclic nucleoside phosphonates: 9-(2-(phosphonyl-methoxy-ethyl))-adenine (PMEA) and -2,6-diaminopurine (PMEDAP); (S)-9-(3-fluoro-2-phosphonylmethoxypropyl)-adenine (FPMPA) and -2,6-diaminopurine (FPMPDAP); and (R)-9-(2-phosphonylmethoxypropyl)-adenine (PMPA) and -2,6-diaminopurine (PMPDAP).
ANPs have slow cellular uptake (probably by an endocytosis-like process) and poor oral bioavailability (Cundy et al., 1994; Srinivas et al., 1993). In order to overcome these problems, the pro-drug bis(pivaloyloxymethyl) [bis(pom)] derivative of PMEA has been synthesized (Starrett et al., 1992). The pro-drug has better cellular uptake and oral bioavailability compared to that of the parent compound (Cundy et al., 1994; Srinivas et al., 1993). Preclinical studies on PMEA and its derivatives are now in progress.

1.5 DRUG RESISTANCE

The replication of all single-strand RNA viral genomes is highly prone to error and not subject to DNA proofreading mechanisms (Holland et al., 1992). The high replication rate of the virus and a mutation rate of $3 \times 10^4$, leads to the emergence of resistant mutants under selective pressure like drug treatment (Mansky et al., 1995). HIV has been shown to develop resistance to most of the drugs currently approved for the treatment of AIDS (Condra et al., 1995; Deeks et al., 1997; Gao et al., 1992; Goulden et al., 1996; Larder et al., 1989a; 1989b; Patick et al., 1996; Richman et al., 1992). Although the clinical relevance of virus-drug resistance and its role in disease progression remains to be determined, it is generally felt that the emergence of drug-resistance is related to the loss of antiviral activity.

The drug resistance strains on exposure to HIV inhibitors in situ as well as from patients undergoing therapy have been isolated. However, the emergence of drug resistant strains of HIV is dramatically different among the different classes of HIV inhibitors (De Clercq, 1996a). For example, NNRTIs are notorious for the rapid
development of resistant strains and this limits the clinical use of NNRTIs. Drug resistant strains were isolated from patients treated with nevirapine for 1 week and delavirdine for 8 weeks. The development of resistance strains occurs relatively slowly with ddN's, usually after 3-6 months of treatment (Demeter et al., 1997; Larder et al., 1989a; Richman et al., 1994)

Studies using RT and protease (PR) inhibitors have shown that the development of resistant strains to these compounds are due to the mutations of RT and PR molecules, respectively. The drug-resistant subpopulations of HIV to ddNs, NNRTIs and PR inhibitors have been identified in previously untreated patients (Lech et al., 1996; Najera et al., 1994; 1995). These mutants are selected under the drug pressure. For drugs such as 3TC, nevirapine and other NNRTIs, a single nucleotide change can confer 100- to 1000-fold reductions in drug susceptibility (Gao et al., 1993; Nunberg et al., 1991; Richman et al., 1991; Tisdale et al., 1993); whereas such high levels of resistance to other drugs, such as AZT and PR inhibitors, require the cumulative acquisition of as many as five or more mutations. The selection of variants with such mutations requires ongoing high levels of replication in the face of drug pressure for 6 months to several years (Condra et al., 1995; Larder et al., 1989a; Larder and Kemp, 1989b). Most of the mutations that confer resistance to drugs occur at or near the binding site of RT or PR inhibitors, thus directly affecting the binding of these compounds to their target enzymes (Pillay et al., 1995). Drug-resistant mutations have been also located close to the template binding site of RT and this mutation indirectly decreases the binding of RT inhibitors (Tachedjian, 1995; 1996). Mutations of HIV virus observed for approved drugs are listed on table 1.5.
Table 1.5 (A). Patterns of HIV drug resistance for reverse transcriptase (RT) inhibitors currently used for the treatment of AIDS\(^1\)

<table>
<thead>
<tr>
<th>Mutation (RT)</th>
<th>AZT</th>
<th>ddi</th>
<th>ddc</th>
<th>d4T</th>
<th>3TC</th>
<th>nevirapine</th>
<th>deavirdine</th>
</tr>
</thead>
<tbody>
<tr>
<td>41 Met→Leu</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 Ile→Thr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>65 Lys→Arg</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67 Asp→Asn</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>69 Thr→Asp</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 Lys→Arg</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>74 Leu→Val</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75 Val→Thr</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>98 Ala-Gly</td>
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<td>219 lys-Gln/Glu</td>
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<td>236 pro-Leu</td>
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1. The data were taken from references: De Clercq, 1995a and Holtzer et al., 1998.

Table 1.5 (B). Patterns of HIV drug resistance for protease (PR) inhibitors currently used for the treatment of AIDS\(^2\)

<table>
<thead>
<tr>
<th>Mutation (PR)</th>
<th>Saquinavir</th>
<th>Ritonavir</th>
<th>Indinavir</th>
<th>Nelfinavir</th>
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<tr>
<td>8 Arg→Gln/Lys</td>
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<tr>
<td>10 Leu→Phe</td>
<td>+</td>
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<td>45 Lys→Ile</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 Met→Ile/Leu/Phe</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>47 Ile→Leu</td>
<td>+</td>
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<td>48 Gly→Val</td>
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<td>90 Leu→Met</td>
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</tbody>
</table>

2. The data were taken from references: De Clercq, 1995a and Kakuda et al., 1998.
Cross-resistance does not happen between ddNs and NNRTIs, and within each group, the compounds do not necessarily show cross-resistance (De Clercq, 1995). Furthermore, the viral strains resistant to one drug may show increased susceptibility to other drugs, for example, PFA resistant strains are hypersensitive to AZT, TIBO and nevirapine (Mellors et al., 1995, Tachedjian et al., 1995). Therefore, one of the important strategies to overcome the drug resistant problem is to develop new HIV inhibitors, so that patients have the option to switch to other anti-HIV drugs when resistance occurs to one drug. Another method to overcome resistance problem is to use combination chemotherapy.

1.6 COMBINATION CHEMOTHERAPY

The benefit of anti-HIV effects wanes with prolonged monotherapy, due to the incomplete suppression of HIV replication and emergence of the resistant virus. Furthermore, the dose-related toxicities of anti-HIV agents limit the use of one compound over prolonged period of time. These limitations of monotherapy can be overcome by using two or three drugs simultaneously (combination chemotherapy) or alternating use of different drugs. Although the sequential administration of agents has been associated with clinical benefits, for example, in the patients who switched from AZT to ddI or to d4T, or to ddC, this benefit does not persist for very long with the continued treatment using one nucleoside antiviral agent (Abrams et al., 1994; Kahn et al., 1992; Spruance et al., 1997; 1994). The concurrent use of different drug combination regimens therefore represent a more promising approach in the treatment of HIV infection. In theory, combination of different anti-HIV drugs for treating HIV infection
has three virtues: (1) **Additive or synergistic antiviral activity.** The maximum suppression of HIV should be achieved when HIV inhibitors targeted at different viral proteins (e.g. RT and PR inhibitors) or different sites within the same protein are used in combination. (2) **Reduced toxicity.** Most of the toxicities of anti-HIV compounds are dose related. When these drugs are used in combination, a substantially lower concentration of each drug will be required to effect the same degree of virus inhibition. The reduced dosage of the individual compound result in lower toxicity for the host. Thus, the concurrent use of two inhibitors with different toxicity profiles, for example AZT/ddC or AZT/ddI are useful for the prolonged treatment of AIDS patients (Hammer *et al.*, 1996; Meng *et al.*, 1992; Saravolatz *et al.*, 1996). (3) **Reduce risk of virus-drug resistance development.** This is the most valuable benefit observed with use of combination chemotherapy. The emergence of resistant mutants is reduced because of rapid decline of virus load in the body and the susceptibility of naturally occurring resistant virus particles to different drugs.

*In vitro* assays can be used to assess the relative antiviral potency of various combinations and their potential for cross-resistance. Synergistic anti-HIV activity has been demonstrated with a large number of combinations, including (1) two drug combinations: AZT+ddC, AZT+ddI, AZT+PFA, AZT+protease inhibitor saquinavir etc. (Antonelli *et al.*, 1994; Craig *et al.*, 1993; Koshida *et al.*, 1989); (2) three drug combinations: AZT+ddI+3TC, AZT+ddThd+PFA, AZT+ddI+nevirapine, AZT+rsCD4+IFN-α etc. (Chow *et al.*, 1993; St. Clair *et al.*, 1995); and (3) even four drug combinations: AZT+NNRTIs (HEPT+E-EPU+nevirapine or TIBO) etc. (Baba *et al.*, 1991c; Brennan *et al.*, 1995). *In vitro* evaluation of combination demonstrates, as a
rule, the multi-drug regimens are more effective with increasing the number of drugs in the combination (Mazzulli et al., 1994; St. Clair et al., 1995). Although such assays cannot predict clinical outcome, they can help provide a rational basis for moving a particular combination forward into clinical trials.

Human studies using a variety of different antiviral combinations have been completed or are underway (Table 1.2). AZT has been included in most of the combination regimen studies. Similar to in vitro studies, clinical trials have demonstrated that two drug anti-HIV regimens are generally superior to monotherapy, and triple-drug regimens are superior to two drug regimens. Among the triple-drug regimens, two RT inhibitors combined with one of the protease inhibitors produce more potent antiviral effects compared to the other regimens. For example, the combination of AZT, 3TC and indinavir was shown to reduce virus levels in blood from between 20,000 and 1,000,000 RNA copies per ml of plasma to below the levels of detection as measured by PCR and by culture. This reduction lasted for periods up to 1 year in at least 90% of treated patients (Richman, 1996). This suppression of the viral levels is sufficient to delay the rate of disease progression. However, this dramatic reduction of the viral population does not mean that the virus has been eradicated from the body, and it seems that the HIV infected persons need prolonged combination therapy.

AIDS is a chronic disease and AIDS patients require long-term or more likely life-time chemotherapy. In summary, seven HIV RT inhibitors (AZT, ddC, ddI, d4T, 3TC, nevirapine and delavirdine) and four HIV protease inhibitors (saquinavir, indinavir, ritonavir and nelfinavir) are currently approved for use singly or in combination to treat HIV infection. The use of these drugs has been shown to suppress the viral replication
and thus permit some restoration of immunity in AIDS patients. The drug induced toxicities and loss of antiviral effects due to the emergence of drug-resistant virus strains are the major problems of these drugs on long-term use in therapeutics.

1.7 GOALS AND OBJECTIVES

The design of biologically active compounds with selectivity against HIV and improved Pharmacological and Toxicological profile is the goal of this programme. The objective is to develop Pharmacophores with desirable attributes for long-term therapy of infection in humans. This research programme was initiated in 1990 with initial funding from the MRC.

When these studies were initiated in our laboratory, there were only two drugs (AZT and ddI) approved for the treatment of HIV infection. Both these drugs were shown to produce multi-organ toxicosis on long-term therapy in AIDS patients. The toxicities were attributed to the incorporation of these antimetabolites into the cellular and mitochondrial DNA of cells. It was rationalized that compounds which would not require 'activation' by host enzymes for anti-HIV activity should produce minimum host toxicity.

Hypothesis:

SYNTHESIZE NUCLEOSIDE ANALOGS WHICH WOULD NOT REQUIRE METABOLIC TRANSFORMATION FOR ANTI-HIV ACTIVITY.
Rationale and Advantages:

(i) These *Pharmacophores* would not be phosphorylated intracellularly to 5'-triphosphates. Therefore, these molecules would not be incorporated into cellular or mitochondrial DNA because they cannot serve as substrates for DNA polymerases. Furthermore, these compounds will not disturb dNTP pools (precursors needed for DNA synthesis) in the cell.

Thus problems of delayed toxicity due to defective cellular or mitochondrial DNA replication would be minimized or obviated.

(ii) These compounds should also inhibit HIV replicates resistant to 2',3'-dideoxynucleosides (ddNs). This is based on the premise that mechanism of action of these unique nucleoside analogs would be different than ddNs. If this hypothesis is correct, then these Pharmacophores should show favorable drug interactions, additive or synergistic, when used in combination with other anti-HIV drugs (e.g. ddNs and protease inhibitors).

Thus these Pharmacophores should prove useful for the treatment of infection due to HIV-resistant replicates of ddNs when used singly (monotherapy) or in combination with other anti-HIV therapeutics (combination chemotherapy).

In order to test this hypothesis, a programme for the synthesis of carboxyphosphonyl (CP) analogs of ddNs and deoxyribonucleosides was undertaken by my colleagues Drs Sashi, V.P. Kumar and Allan L. Stuart. Purified compounds were given to me for biological studies. Antiviral activity of CP analogs of ddNs and deoxyribonucleosides against HIV was determined by different assay methods. Cytotoxicity against lymphoblast cell lines, CBMC and PBMC was evaluated.
Pharmacology and toxicity studies on compounds with most therapeutic potential were undertaken. Results of these studies are discussed in this thesis. In addition to these investigations, antiviral activity of hydroxymethyldeoxyuridine and its derivatives against HIV was determined by a quantitative focal immunoassay. Rationale for these investigations and results of these studies are discussed in chapter 2.
2.0 STUDIES ON HIV INHIBITORS

INTRODUCTION

Several assay methods have been developed to determine antiviral activity of inhibitors of HIV replication. The antiviral activity and cytotoxicity of HIV inhibitors has been shown to vary considerably depending on the cell line, virus strains and the assay method used (Whalley et al., 1991). The merits and limitations of four assay methods currently used by researchers to evaluate activity of HIV inhibitors in situ are described below:

2.1 ANTIVIRAL ASSAYS

2.1.1 Cytopathic inhibition assay

HIV replication results in lysis and cell death. Agents capable of inhibiting the replication of HIV have been shown to protect the cells from the damage due to HIV (inhibition of HIV-induced cell death). The viability of the cells can be determined by MTT assay or trypan blue-exclusion assay.

(i) MTT assay: This assay method was originally developed for the screening of 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 the yellow colored tetrazolium salt (MTT) by mitochondrial dehydrogenases of metabolically active cells to a dark blue formazan product, which can be measured spectrophotometrically. The
cytopathic inhibition assay has been widely used by many investigators for the evaluation of HIV 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 HIV. 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 growth-inhibited (sick-slow growth), can give false results because the enzyme machinery of the cell is still viable.

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

2.1.2 Suspension-phase infectivity assay (SPIA)

When HIV replicates, it produces a number of viral enzymes and proteins. The levels of these viral products in the supernatant fluid reflect the extent of viral replication. This assay is based on the detection of products of viral replication: namely, reverse transcriptase (RT) and p24 antigen in the supernatant of cell culture. The suspension-phase infectivity assay is simple and easily performed. The primary advantage of SPIA is that a better picture of viral dynamics is achieved.

(i) Reverse transcriptase (RT) assay: The cell cultures can be maintained for a long period by using a suitable cell line (e.g. monocyte macrophages). RT activity of infected cultures can be assayed over a long period (up to several weeks) after exposure to HIV inhibitors. Labeled nucleotide oligo template and primer is used to detect activity of the enzyme in the supernatant of cells infected with HIV. RT assay is very sensitive and reliable (especially for RT inhibitors), and is commonly used for the screening of compounds by
drug companies and AIDS research centers. The only disadvantage of this assay method is that it is labor intensive and requires the use of radioisotopes.

(ii) gag p24 antigen assay (ELISA): This assay determines level of p24 core antigen in the supernatant of cells infected with HIV by enzyme-linked immunosorbent assay (ELISA). Levels of p24 antigen can be measured over a period of time. The assay is simple, sensitive and reliable for the identification of HIV infection. However, kits used for detecting p24 antigen are very expensive. Thus this assay is valuable to confirm the antiviral activity of compounds.

2.1.3 Focal immunoassay (FIA)

The confluent monolayers of HT4-6C cells, (HeLa CD4: HeLa cells transfected with CD4 receptor) are used to detect the infectious particles (plaques) of HIV in a quantitative manner. The immuno-color staining of the fixed cells for HIV antigen is used for enumeration of plaques. This assay for determination of activity of HIV inhibitors was developed by Dr. Chesebro et al., (1988).

Initially, the antiviral activity of compounds synthesized under AIDS research programme was determined by focal immunoassay in Saskatoon. Compounds were given to me with code numbers. The active compounds were then sent to Professor De Clercq (Director, Rega Institute for Medical Research, Leuven, Belgium), Dr. Lazdins, (Ciba-Geigy Research Laboratories, Basle, Switzerland) and Professor Wainberg, (Director, AIDS Research Centre, McGill University, Montreal, Canada) under code names. Assays with different endpoints were used to determine the activity of compounds against HIV by these investigators. The activity against HIV was determined using MTT assay (Professor De Clercq) and RT assay (Professor Wainberg and Dr. Lazdins). This approach enabled us to
obtain antiviral screen data against various HIV strains by antiviral assays using different end points.

2.2 MATERIALS AND EXPERIMENTAL PROCEDURES

2.2.1 Tissue culture supplies, cell lines and virus strains

Sterile plastics-tissue culture flasks, microtitre trays 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), DMEM high-glucose (4.5g/L), and other tissue culture supplies: fetal bovine serum (FBS), L-glutamine, sodium pyruvate, penicillin-streptomycin, fungizone were obtained from Gibco, Burlington, Canada.

Cell lines

The cell lines used were: HT4-6C (HeLa CD4), HUT 78, H9, MT-2, MT-4, human cord blood mononuclear cells (CBMC) and human peripheral blood monocyte macrophages (PBMC). HT4-6C cell line is the HeLa cells (from human cervical epithelial carcinoma) expressing CD4 receptor on the cell surface. This cell line was kindly made available from Dr. B. Chesebro, Rocky Mountain Laboratories, Hamilton, Montana, U.S.A. HUT 78 and MT-2 are T cell lines and were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). MT-4 cell line is HTLV-1 transformed human T cells isolated from a patient with adult T-cell leukemia and were obtained from National Institutes of Health (NIH, Rockville, MD, USA). H9 cell line is a single cell clone which was selected for high yield permissive growth with HIV-1. CBMC and PBMC were obtained from HIV-seronegative donors and were used by Dr. Wainberg and Dr. Lazdins for RT assays, respectively. Additional information on cell lines is given in Table 1 of Appendix.
Cell cultures

HT4-6C cells were cultured using RPMI 1640 culture medium supplemented with 8% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 50 Unit/ml penicillin, 50 mg/ml streptomycin and 1% fungizone in 75 cm$^2$ tissue culture flasks. The cells were incubated at 37°C in a humidified 5% CO$_2$ atmosphere. The monolayers were trypsinized, and one fifth of the cells were transferred to another flask twice weekly for propagation of cells.

Umbilical CBMC were obtained from HIV-seronegative donors and isolated by Ficoll-Hypaque gradient centrifugation. Cultures were stimulated with phytohemagglutinin (PHA). After 3 days, pools of cells from three CBMC donors were established and used as feeder cultures. Cultures were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine/ml, 200 Units of penicillin/ml, and 200 mg of streptomycin/ml (Salomon et al. 1994).

Human blood monocyte derived macrophages were obtained by the method described by Lazdins et al. (1990). Briefly, peripheral blood monocytes were isolated from normal HIV sero-negative donors by a combination of lymphocytapheresis and counter current centrifugation elutriation. After isolation the monocytes were cultured in petri dishes using DMEM high-glucose (4.5g/L), supplemented with 10% (v/v) human type AB serum, 50 unit/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. The cells acquired the phenotype of macrophages after culture in vitro for 2 weeks. The adherent cells were detached by placing the monolayers in cold PBS (no Ca$^{2+}$/Mg$^{2+}$) and incubating at 5°C for 30 min. After washing, the cells were resuspended in the above described medium and plated in 96-well plate at 6 x 10$^4$ cells/well in 0.3 ml of medium.
The MT-2 cells and MT-4 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heated-inactivated FBS and 20 mg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. Every 3-4 days, cells were spun down and seeded at 3 x 10^5 cells/ml in new cell culture flasks.

HUT 78 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heated-inactivated FBS, 2 mM L-glutamine, 50 unit/ml penicillin, 50 mg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. One fifth of the cells were transferred to another flask twice weekly for propagation of cells.

H9 cells were grown in DMEM medium supplemented with 8% (v/v) heated-inactivated FBS, 2 mM L-glutamine, 50 unit/ml penicillin, 50 mg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. One fifth of the cells were transferred to another flask twice weekly for propagation of cells.

Cells were routinely tested for mycoplasma contamination and only cells that were negative in this test were used for the studies described in the thesis.

**HIV strains**

The virus strains used were: HIV-1 (IIIB, MO, ADA and RF; AZT-resistant mutants ADP/141, 1075 and 1082) and HIV-2 strains (EHO and ROD). HIV-1 IIIB (also called HTLV-IIIB/H9) is the wild-type strain of HIV-1. It was isolated from several patients with AIDS or related disease and was used to establish a permanent productive infection in H9 cells. This strain is widely used for the testing of HIV inhibitors in cell cultures. Other strains of HIV-1 (RF) and HIV-2 (EHO and ROD) were isolated from AIDS patients and are part of the panel of virus isolates used in Professor De Clercq's laboratory to evaluate HIV inhibitors. HIV-1 MO was isolated from an AIDS patient and was obtained from the Center for Disease Control, Ottawa, Ontario, Canada. HIV-1 ADA strain is a monocytotropic
isolate and was used in Dr. Janis K. Lazdins laboratory. The virus isolate was provided by Dr. H. Gendelman, Walter Reed Army Institute of Research, Washington, D.C.

AZT resistant HIV-1 strains 1075 (mutation at 215F), 1082 (mutation at 215F) and ADP/141 [(also designated RTMC) (mutations at 67N, 70R, 215F and 219Q)] were used. Additional information on virus isolates is given in Table 2 in the Appendix.

HIV-1 MO virus was grown in HUT 78 cells until about 90% HIV cytopathic effect. The virus titre was determined using HT4-6C cells. HIV-1 IIIB, 1075 and 1082 were propagated in Dr. Wainberg's laboratory. HIV-1 IIIB, RF, ADP/141 and HIV-2 EHO and ROD were propagated in Dr. De Clercq's laboratory and HIV-1 ADA were propagated in Dr. Lazdins' laboratory.

2.2.2 Drugs and chemicals

Control drugs, azidothymidine (AZT), dideoxycytidine (ddC), and dideoxyinosine (ddI) were purchased from Raylo Chemicals, Edmonton, Alberta, Canada. Dideoxy-2',3'-didehydrothymidine (d4T) was synthesized using published procedures (Mansuri et al. 1989). Phosphonoformic acid (PFA) was purchased from Sigma Chemical Company, Ontario, Canada.

Compounds used in these investigations were synthesized by Dr. Sashi Kumar in our laboratory. 5'-Hydroxymethyldeoxyuridine (1, HMdUrd), 2',3'-dideoxy-5-hydroxymethyluridine (2, HMdUrd), 3'-azido-2',3'-dideoxy-5-hydroxymethyluridine (3, AZHMdUrd), 3'-fluoro-2',3'-dideoxy-5-hydroxymethyluridine (4, FHMdUrd), and 2',3'-dideoxy-2',3'-didehydro-5-hydroxymethyluridine (5, HMd4Urd) were synthesized. The structures of HMdUrd and its derivatives (compound 2-5) are shown in Scheme 2.1.

Carboxyphosphonyl (CP) derivatives of dideoxynucleosides (ddNs), namely, 5'-carboxyphosphonylazidothymidine (6, CP-AZT), 5'-carboxyphosphonyl-2',3'-dide-
1. HMdUrd
2. HMddUrd
3. AZHMddUrd
4. FHMddUrd
5. HMd4Urd

1. 5-Hydroxymethyl-2'-deoxyuridine (HMdUrd)
2. 2',3'-Dideoxy-5-hydroxymethyluridine (HMddUrd)
3. 3'-Azido-2',3'-dideoxy-5-hydroxymethyluridine (AZHMddUrd)
4. 3'-Fluoro-2',3'-dideoxy-5-hydroxymethyluridine (FHMddUrd)
5. 2',3'-Dideoxy-2',3'-didehydro-5-hydroxymethyluridine (HMd4Urd)

Scheme 2.1 5-Hydroxymethyl-2'-deoxyuridine and its derivatives
oxycytidine (7, CP-ddC), 5'-carboxyphosphonyl-2',3'-dideoxyinosine (8, CP-ddI) and 5'-
carboxyphosphonyl-d4T (9, CP-d4T) were prepared. The structures of 5'-CP derivatives of
ddNs are shown in Scheme 2.2. Carboxyphosphonyl (CP) derivatives of naturally occurring
nucleosides, namely, 5'-carboxyphosphonyldeoxythymidine (10, CP-dThd), 5'-
carboxyphosphonyldeoxyuridine (11, CP-dUrd), 5'-carboxyphosphonyl-deoxyinosine
(12, CP-dlno), 5'-carboxyphosphonylinosine (13, CP-Ino) and 5-substituted
deoxyribonucleoside, 5'-carboxyphosphonyl-5-methoxymethyldeoxyuridine (14, CP-
MMDUrd) and 5'-carboxyphosphonyl-5-ethyldeoxyuridine (15, CP-EtdUrd) were
synthesized. The structures of nucleoside analogs are shown in Scheme 2.3. All
carboxyphosphonyl analogs were purified by DEAE-Sephadex chromatography and HPLC.
The purity of the compounds was at least 95% and generally higher 98 ~ 99% based on
HPLC profiles. The structures were confirmed by Nuclear Magnetic Resonance (NMR)
spectral analysis. HMdUrd (1) and its derivatives (2-5) are crystalline compounds.
Microanalysis of these samples was also performed to confirm structure. Salient properties
of (molecular weight and spectral characteristics) compounds used in these investigations
are summarized in Table 2.1.

For antiviral assays, stock solution of compounds was made in phosphate buffered
saline (0.1M), pH 7.2 and used immediately or stored frozen at -20°C. The concentration of
the stock solution for each compound was determined spectrophotometrically. Appropriate
concentrations of the test compound were made with culture medium immediately prior to
antiviral assay.

Ficoll-hypaque and Poly(rA).oligo(dT)12-18 were purchased from Pharmacia,
Uppsala, Sweden. Human type AB serum, trypsin, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl
tetrazolium bromide (MTT), isopropanol, Tris hydrochloride (Tris-HCl), Triton X-100,
ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(b-aminoethyl ether) N,N,N',N'-
Phosphonoformic Acid (PFA) = \[ \text{HO-} \text{P} \text{O}_2 \text{OH} \]

AZT; R=H
ddC; R=H
ddI; R=H
d4T; R=H

6. CP-AZT; R=CP
7. CP-ddC; R=CP
8. CP-ddI; R=CP
9. CP-d4T; R=CP

6. 5'-Carboxyphosphonyl-3'-azidothymidine (5'-CP-AZT)
7. 5'-Carboxyphosphonyl-2',3'-dideoxycytidine (5'-CP-ddC)
8. 5'-Carboxyphosphonyl-2',3'-dideoxynosine (5'-CP-ddI)
9. 5'-Carboxyphosphonyl-2',3'-dideoxy-2',3'-didehydro-thymidine (5'-CP-d4T)

Scheme 2.2 5'-Carboxyphosphonyl derivatives of 2',3'-dideoxynucleosides
Scheme 2.3 5'-Carboxyphosphonyl derivatives of deoxyribonucleosides
Table 2.1 Absorption characteristics and molecular weight of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon_{\text{max}}$ (pH 7.0)</th>
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<tbody>
<tr>
<td>1. HMdUrd</td>
<td>258.2</td>
<td>264</td>
<td>9,600</td>
</tr>
<tr>
<td>2. HMddUrd</td>
<td>242.2</td>
<td>264</td>
<td>9,600</td>
</tr>
<tr>
<td>3. AZHMddUrd</td>
<td>283.2</td>
<td>264</td>
<td>9,600</td>
</tr>
<tr>
<td>4. FHMddUrd</td>
<td>260.2</td>
<td>264</td>
<td>9,600</td>
</tr>
<tr>
<td>5. HMd4Urd</td>
<td>240.2</td>
<td>264</td>
<td>9,600</td>
</tr>
<tr>
<td>6. CP-AZT</td>
<td>375.2</td>
<td>267</td>
<td>9,700</td>
</tr>
<tr>
<td>7. CP-ddC</td>
<td>319.2</td>
<td>271</td>
<td>9,000</td>
</tr>
<tr>
<td>8. CP-ddI</td>
<td>344.2</td>
<td>249</td>
<td>12,200</td>
</tr>
<tr>
<td>9. CP-d4T</td>
<td>332.2</td>
<td>267</td>
<td>9,700</td>
</tr>
<tr>
<td>10. CP-dThd</td>
<td>350.2</td>
<td>267</td>
<td>9,700</td>
</tr>
<tr>
<td>11. CP-dUrd</td>
<td>336.2</td>
<td>262</td>
<td>10,200</td>
</tr>
<tr>
<td>12. CP-dIno</td>
<td>360.2</td>
<td>249</td>
<td>12,200</td>
</tr>
<tr>
<td>13. CP-Ino</td>
<td>376.2</td>
<td>249</td>
<td>12,200</td>
</tr>
<tr>
<td>14. CP-MMdUrd</td>
<td>380.2</td>
<td>265</td>
<td>9,700</td>
</tr>
<tr>
<td>15. CP-EtdUrd</td>
<td>364.2</td>
<td>267</td>
<td>9,800</td>
</tr>
</tbody>
</table>

| AZT           | 267.2            | 267                          | 9,700                               |
| ddC           | 211.2            | 271                          | 9,000                               |
| ddI           | 236.2            | 249                          | 12,200                              |
| d4T           | 224.2            | 267                          | 9,700                               |
| PFA           | 126.0            | -                            | -                                   |
| dThd          | 242.2            | 267                          | 9,700                               |
tetraacetic acid (EGTA), peroxidase-conjugated goat anti-human antibodies, 3-amino-9-ethylcarbazole (AEC), ethylene glycol, dithiothreitol (DTT), trichloroacetic acid (TCA), reduced glutathione (GSH), crystal violet, MgCl₂, KCl, NaCl, hydrogen peroxide, sodium pyrophosphate (Na-PPi) were purchased from Sigma Chemical Company, Ontario, Canada. Human antibodies against HIV were obtained from patient serum from the Royal University Hospital, Saskatoon, Saskatchewan, Canada. Radiolabelled thymidine triphosphate [³H]dTTP was purchased from NEN, Boston, MA, USA. DE51 cellulose discs and GF/A discs were from Whatman Credman Sciences, Fairfield, New Jersey, USA. Scintillation fluid EcoLite(+)™ was from ICN Pharmaceuticals, Inc. Montreal, Quebec, Canada.

2.2.3 Acute cytotoxicity

Cytotoxicity is an important parameter for evaluation of the selectivity of compounds. Methods used to determine cytotoxicity of compounds as part of the antiviral screen by different investigators are described. (i) The toxicity of compounds on HT4-6C cells was assessed as follows: Confluent monolayer HT4-6C cells (2.5 x 10⁴ cells/well) were exposed to series of dilutions of the test compound. Cell controls (culture medium only) were run simultaneously. After 72 hours incubation, cells were washed with PBS and stained with 1% crystal violet in 70% ethanol. The inhibition of cell growth was grossly estimated by the density of monolayers structure compared to that of cell controls. The concentration required to inhibit cell growth by 50% (CC₅₀) was calculated from the dose-response curves. (ii) The toxicity of compounds was determined using MTT assay in Professor De Clercq’s laboratory and in our laboratory when MT-2 and MT-4 cells were used for antiviral assays. The method is described in detail in section 2.2.4.3 (iii) The toxicity of compounds against CBMC was examined microscopically (Dr. Wainberg’s laboratory) and PBMC using DAPI method (Dr. Lazdins laboratory) (section 2.2.4.2). (iv)
The acute toxicity of compounds against CEM cells was determined by trypan blue-exclusion method in our laboratory. Briefly, $2 \times 10^5$ CEM cells/ml (3 ml/well) were cultured in RPMI1640 containing 5% FBS in 12 well microtitre plates for 4 days in humidified 5% CO$_2$ atmosphere. The growth medium was changed on day 2. Cells were counted on day 2 and 4. The alive and dead cell were checked by trypan blue-exclusion method. Further details of this methodology are given in chapter 3 section 3.3.1.

2.2.4 Drug inhibition assays

2.2.4.1 Focal immunoassay (FIA)

Antiviral activity was determined by a quantitative focal immunoassay according to the recommendation of Chesebro and colleagues (Chesebro et al., 1988).

For FIA assays, $2.5 \times 10^4$ cells/well in 1 ml medium were seeded into a 24 well microtitre tray and incubated at 37°C for 24 hours. The confluent monolayers were washed with PBS (2 x 24 ml). Graded concentrations of the drug or test compound (2 x desired concentration) in 0.5 ml medium without FBS were added and immediately thereafter infected with 50-100 plaque forming units of HIV-1 MO strain in 0.2 ml medium. Plates were incubated for one hour, 0.3 ml of growth medium (25%FBS) was added and microtitre trays were kept at 37°C for 3 days in a humidified CO$_2$ (5%) atmosphere. The monolayers were fixed and immunochemically stained using the procedure of Chesebro et al. (1988) with minor modifications. Briefly, the medium was removed, monolayers fixed for 5 min with methanol, solvent was aspirated and cells were rinsed three times with TNE (0.01 M Tris HCl, pH 7.5; 0.15 M NaCl; 0.002 M EDTA) supplemented with 1% FBS (TNE-1%FBS buffer). To each well, 0.2 ml of 1:100 dilution of human AIDS patient serum with antibody against HIV was added and the plates were left for 30 min at 22°C. At the end of incubation
period, wells were washed three times with TNE-1% FBS to remove serum; 0.3 ml of peroxidase-conjugated goat anti-human antibodies (1:500 dilution in TNE-1% FBS buffer) was added to each well and plates were left at 22°C for 45 min. The cells were washed again with TNE-1%FBS buffer and treated with 0.5 ml of freshly prepared peroxidase substrate (Nexo, 1977). After 15-20 min at 22°C, plates were rinsed once with TNE and with water (2x 24 ml), and left for drying at 22°C for 24 hours. The foci of infected cells were counted using an inverted microscope at a magnification of 40X (Figure 2.1, magnification 100x). In each experiment, toxicity controls (containing test compound and media only), cell controls (containing media only) and virus controls (containing virus and media) were run simultaneously. The anti-HIV effect was expressed as a percentage of plaque reduction (No. of plaque of tested compound/No. of plaque of cell control). From dose response curves, the concentration required to reduce the number of plaques by 50% ($ED_{50}$) was determined.

2.2.4.2 Reverse transcriptase (RT) assay:

(a) Assay procedures using cord blood mononuclear cells (CBMC)

The method used for evaluation of anti-HIV activity was according to recommendations of Hoffman et al., (1985) and Lee et al., (1987). Briefly, umbilical CBMC was obtained from HIV-seronegative donors as described earlier (2.2.1). Cells were infected with HIV-1 positive supernatant for 2 hours. The HIV-1 inoculum selected for CBMC ranged from 1000 to 2000 of 50% tissue culture infective dose (TCID$_{50}$). After infection, cells were washed with medium and $4 \times 10^5$ cells in 0.2 ml medium per well were dispersed in 96-well microtiter tray and exposed immediately to series of concentrations of tested compounds. AZT and ddI were used as positive controls and dThd was included as a
Figure 2.1 Photomicrograph of HT4-6C (HeLa CD4) cells
negative control. The culture medium was changed on day 4, such that half of the medium was replaced with fresh medium containing the original drug concentration in each well. HIV-1 replication was assayed by measuring the reverse transcriptase activity in cell free supernatant fluid harvested on day 7. The supernatant was divided into aliquots and RT activity was tested or stored at -70°C till assay. The RT activity present was expressed as cpm/ml supernatant. Each value was derived from duplicate determinations in each experiment and represents the mean value. The 50% effective dose (ED$_{50}$) of test compounds was calculated on the basis of reverse transcriptase activity in the culture fluid vs. concentration of drug.

(b) Assay procedures using monocyte-macrophages (PBMC)

Human blood monocyte derived macrophages (PBMC) from HIV-seronegative donors were isolated as described previously (section 2.2.1). Four hours after confluent macrophage monolayers were established, the supernatant was removed and the cells were infected with 50 µl of HIV-1/ADA supernatant (7.5 x 10$^6$ cpm RT activity/ml, TCID$_{50}$ 1 x 10$^4$ /ml). After six hours infection, virus containing supernatant was removed and fresh medium containing compounds was added. Medium was removed on days 6, 10, 14 and 17. At these time points, fresh medium containing test compounds was further added. The experiment was terminated on day 21 post infection. At the indicated time points, reverse transcriptase activity of the supernatant fluid was evaluated. Each value was derived from triplicate determinations. The variation among triplicates was less than 10%. In each experiment, toxicity controls were run simultaneously. Non infected cell monolayers were exposed to compounds as for infected macrophages. On day 21 after start of addition test compounds, the cell number in the monolayers was determined using 4'-6'-diamino-phenylindole-2HCl (DAPI), a DNA-specific fluorescent dye for nuclear quantification, to
determine the cytotoxicity of the compounds (Lazdins et al. 1991). AZT was used as a positive control.

(c) Measurement of RT activity

RT activity of cell supernatants was determined by the incorporation of $[^{3}H]dTTP$ into the complementary DNA chain of Poly(rA).oligo(dT) using the procedures of Lee et al. (1987). The standard assay mixture contained 50 mM Tris-HCl (pH 7.9), 5 mM MgCl$_2$, 150 mM KCl, 0.5 mM EDTA. 0.05% of Triton X-100, 2% ethylene glycol, 5 mM DTT, 0.3 mM GSH, 50 mg/ml of Poly (rA).oligo(dT)$_{12-18}$, tritiated $[^{3}H] dTTP$ 1 mCi (specific activity 50-80 Ci/mmol) and 50 µl clarified HIV culture fluid. Total reaction volume was 100 µl. The assay mixture was incubated at 30°C for 22 hours. The reaction was terminated by adding 1 ml of 10% cold TCA in 20 mM sodium pyrophosphate, and samples were left in ice bath for 2 hours. The samples were filtered using GF/A disks, washed with cold 10% TCA followed by absolute ethanol and dried in air for 24 hours. Radioactivity was counted using 5 ml of EcoLite(+)™ scintillation fluid.

2.2.4.3 Cytopathic inhibition assay (MTT assay)$^1$

The studies described in this thesis using cytotoxicity assay were carried out in Professor De Clercq's laboratory (Pauwels et al. 1987). For anti-HIV assay, flat bottom, 96 well plastic microtiter trays were filled with 100 µl of complete medium. Subsequently, serial dilutions of 25 µl (9 x final test concentration) of compounds were added to two series of triplicate wells so as to allow simultaneous evaluation of their effects on HIV- and mock-infected cells. Untreated control HIV- and mock-infected cell samples were included for

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$^1$ Preliminary studies on CP analogs of ddNs were carried out by Dr. Ayisi in this laboratory.
each compound. 50 µl of HIV at 100 TCID₅₀ or medium was added to either infected or mock-infected part of a microtitre tray. Exponentially growing MT-4 cells were centrifuged for 5 min at 140x g and the supernatants were discarded. 50 µl of exponentially growing MT-4 cells at concentrations of 6 x 10⁵ cells/ml culture medium were then added to the two series of replicate wells. The outer row wells were filled with 100 µl of medium. The cells cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. Five days after infection the viability of mock and HIV infected cells was examined spectrophotometrically by the MTT method as follows:

To each well of the microtiter trays, 20 µl of a solution of MTT (7.5 mg/ml) in PBS was added. The trays were further incubated at 37°C for one hour. The medium (150 µl) was then gently removed from each well without disturbing the MT-4 cell clusters containing the formazan crystals. Solubilization of the crystals was achieved by adding 100 µl 10% (v/v) Triton X-100 in acidified isopropanol (2 ml concentrated HCl per 500 ml solvent). Complete dissolution of the formazan crystals could be obtained after the trays had been placed on a plate shaker for 10 min. Finally, the absorbencies were read in an eight-channel computer-controlled photometer (Multiskan MCC. ICN Flow) at two wavelengths (540 nm and 690 nm). The absorbance measured at 690 nm was automatically subtracted from the absorbance at 540 nm, so as to eliminate the effects of non-specific absorption. Blanking was carried out directly on the microtitre trays with the first column wells which contained all reagents except for the MT-4 cells. All data represent the average values for a minimum of three wells. The 50% cytotoxic dose was defined as the concentration of compound that reduced the absorbency (OD₅₄₀) of the mock-infected control sample by 50%. The percent protection achieved by the compounds in HIV infected cells was calculated by the following formula:

\[
\frac{(OD_T)_\text{HIV}-(OD_C)_\text{HIV}}{(OD_C)_{\text{mock}}-(OD_C)_{\text{HIV}}}
\]
whereby \((\text{OD}_T)_{\text{HIV}}\) is the optical density measured with a given concentration of the compound in HIV infected cells; \((\text{OD}_C)_{\text{HIV}}\) is the optical density measured for the control untreated HIV infected cells; \((\text{OD}_C)_{\text{mock}}\) is the optical density measured for the control untreated mock-infected cells. The dose achieving 50% protection according to the above formula was defined as the 50% effective dose (ED\(_{50}\)).

2.2.5 Thymidine kinase (TK) activity

Fresh peripheral blood lymphocytes were stimulated with PHA (5 \(\mu\)g/ml) for 48 hrs. After pipetting, the cells were suspended in 50 mM Tris-HCl (pH 8.0) containing 2.5 mM DTT, sonicated (2 fifteen seconds bursts at 20 watts each) and the suspension was centrifuged at 100, 000 x g for 60 min. The supernatant was divided into aliquots and stored at \(-70^\circ\text{C}\).

The standard assay mixture contained 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM MgCl\(_2\), 10 mM KF, 2.5 mM DTT, 1 mg/ml bovine serum albumin, 5 \(\mu\)M dThd, tritated \(^{3}\text{H}\) thymidine 1 \(\mu\)Ci (Specific activity: 84 Ci/mmol), varying concentrations of the test compounds and 25 \(\mu\)l enzyme extract. Total reaction volume was 100 \(\mu\)l. The assay mixtures were incubated for 30 min at 37°C and the reactions were terminated by chilling the samples to 0°C in an ice bath. The samples (50 \(\mu\)l) were applied onto DE51 cellulose discs, dried and washed with 1 mM ammonium formate followed by absolute ethanol and dried. Radioactivity was counted using a toluene based scintillation fluid (6 g PPO and 0.075 g POPOP per liter of scintillation grade toluene). The dissociation constant of the enzyme-inhibitor complex (K\(_i\)) was used to express the affinity of the compounds for TK. K\(_i\) was calculated using equation: 

\[ K_i = \frac{\text{IC}_{50}}{[1+(S/K_m)]} \]

where \(\text{IC}_{50} = 50\% \) inhibitor
concentration, \( S \) = substrate concentration, \( K_m \) = Michaelis constant of the substrate [0.5 \( \mu M \) for TK1 (Cheng and Prusoff, 1973; Munch-Petersen et al., 1991)].

2.3 RESULTS: BIOLOGICAL ACTIVITY

2.3.1 Studies on hydroxymethyldeoxyuridine and its derivatives

2.3.1.1 Antiviral activity and cytotoxicity of HMdUrd and its derivatives

The antiviral activity of HMdUrd (1) and compounds HMddUrd (2), AZHMddUrd (3), FHMddUrd (4) and HMd4Urd (5) against HIV was determined by FIA using HT4-6C cells (Table 2.2 and Figure 2.2). AZT, d4T and FdThd were included as positive controls. Compounds 2 to 5 had an \( ED_{50} \) of \( > 200 \mu M \) compared to HMdUrd (\( ED_{50} \) : 8 to 10 \( \mu M \)). The average concentrations required to inhibit HIV by 50% for AZT, d4T and FdThd were 0.1 \( \mu M \), 0.04 \( \mu M \) and 0.24 \( \mu M \), respectively. The average concentrations required to inhibit cell proliferation of HT4-6C cells by 50% (\( CC_{50} \)) for AZT, d4T, FdThd and HMdUrd were 7-8 \( \mu M \), 95 \( \mu M \), 80 \( \mu M \) and 35 \( \mu M \), respectively. The \( CC_{50} \) values for HMdUrd (2) was greater than 800 \( \mu M \) and for compounds 3-5 were greater than 300 \( \mu M \) (highest concentration tested).

HMdUrd showed similar antiviral potency (\( ED_{50} \approx 10 \mu M \)) in PBMC macrophages infected with HIV-1 strain ADA (Figure 2.3). Interestingly, HMdUrd at low concentration (0.1 \( \mu M \)) stimulated the viral growth. The \( CC_{50} \) value for HMdUrd for uninfected macrophages was greater than 90 \( \mu M \) (highest concentration tested).

2.3.1.2 Affinity for human cellular thymidine kinase

The affinity of HMdUrd (1), AZHMddUrd (3) and HMd4Urd (5) for human cellular thymidine kinase (HTK) was determined by competition assay. AZT was included as positive control. The results are shown in Table 2.3 and Figure 2.4. AZT had high affinity
Table 2.2 Antiviral activity of HMdUrd and its derivatives against human immunodeficiency virus in HT4-6C (HeLa CD4) cells by focal immunoassay.

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>ED$_{50}$ (µM)</th>
<th>CC$_{50}$ (µM)</th>
<th>SI$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>0.1±0.01</td>
<td>7.6±2.1</td>
<td>76</td>
</tr>
<tr>
<td>d4T</td>
<td>0.04±0.01</td>
<td>95±8</td>
<td>2375</td>
</tr>
<tr>
<td>F-dThd</td>
<td>0.24±0.22</td>
<td>80±10</td>
<td>333.3</td>
</tr>
<tr>
<td>HMdUrd (1)</td>
<td>9±1.8</td>
<td>35±8.5</td>
<td>3.9</td>
</tr>
<tr>
<td>HMddUrd (2)</td>
<td>&gt; 800</td>
<td>&gt; 800</td>
<td>-</td>
</tr>
<tr>
<td>AZHMddUrd (3)</td>
<td>&gt; 200</td>
<td>&gt; 400</td>
<td>-</td>
</tr>
<tr>
<td>FHMddUrd (4)</td>
<td>&gt; 400</td>
<td>&gt; 400</td>
<td>-</td>
</tr>
<tr>
<td>HMd4Urd (5)</td>
<td>&gt;200</td>
<td>&gt;300</td>
<td>-</td>
</tr>
</tbody>
</table>

1. HIV-1 MO strain was used. Virus input 50-100 PFU.
2. ED$_{50}$: Inhibitory concentration (mean ± SD) required to reduce viral plaques by 50%.
3. CC$_{50}$: Cytotoxic concentration (mean ± SD) required to reduce cell growth by 50%.
4. SI: Ratio of CC$_{50}$/ED$_{50}$.
Figure 2.2 Anti-HIV activity of HMdUrd and its derivatives by FIA assay. HIV-1 MO strain was used, virus input: 50 - 100 PFU. HMdUrd (■■), HMddUrd (●●), AZHMddUrd (◆◆), FHMddUrd (□□) and HMd4Urd (▽▽).
Figure 2.3 Anti-HIV activity of HMsUrd in PBMC system by RT assay. Virus strain: HIV-1 ADA. Virus input: 500 TCID<sub>50</sub>. HMsUrd: 0.1 µM (■), 1 µM (□) and 10 µM (□).
### Table 2.3 Affinities of HMdUrd and its derivatives for cellular thymidine kinase

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>$K_i$ ($\mu$M) $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>1.0</td>
</tr>
<tr>
<td>HMdUrd (1)</td>
<td>28.3</td>
</tr>
<tr>
<td>AZHMddUrd (3)</td>
<td>61.4</td>
</tr>
<tr>
<td>HMd4Urd (5)</td>
<td>$&gt;363.6$</td>
</tr>
</tbody>
</table>

1. $K_i$: The dissociation constant of the enzyme-inhibitor complex.
Figure 2.4 Interaction of AZT, HMdUrd, AZHMddUrd and d4HMddUrd with phosphorylation of \(^3\text{H}\)-dThd by cellular thymidine kinase. AZT (■-■), HMdUrd (●-●), AZHMddUrd (□-□) and d4HMddUrd (△-△).
for the kinase with $K_i$ value of 1.0 $\mu$M. When assayed under similar conditions, HMdUrd had significantly less affinity compared to AZT, with $K_i$ values of 28.3$\mu$M. The $K_i$ values for AZHMddUrd (3) and HMd4Urd (5) were 61.4 $\mu$M and greater than 363.6 $\mu$M, respectively.

2.3.2 Studies on dideoxynucleosides (ddNs) and their corresponding 5’-carboxyphosphonyl derivatives

2.3.2.1 Antiviral activity and cytotoxicity of AZT, ddC, ddI, d4T, CP-AZT, CP-ddC, CP-ddI and CP-d4T

(a) Focal immunoassay (FIA)

The antiviral activity of CP-AZT (6), CP-ddC (7), CP-ddI (8) and CP-d4T (9) was determined against HIV-1 using HT4-6C cells (Table 2.4 and Figure 2.5). AZT, ddC, ddI, d4T and PFA were included as positive controls. The average concentrations required to inhibit HIV by 50% ($ED_{50}$) for AZT, ddC, ddI and d4T were 0.1 $\mu$M, 0.4 $\mu$M, 4.6 $\mu$M and 0.04 $\mu$M, respectively. Under similar assay condition, the average concentrations required to inhibit viral replication by 50% for compounds 6, 7, 8 and 9 were 4.4 $\mu$M, 130 $\mu$M, 150 $\mu$M and 17 $\mu$M, respectively. All CP derivatives of ddNs were less active than the parent compound. However, the most dramatic loss of activity was seen for d4T and ddC analogues (300 ~ 400 fold). Interestingly, the potency of CP-AZT was similar to ddI. On the basis of potency, these compounds can be ranked: d4T > AZT > ddC > CP-AZT ≈ ddI > CP-d4T > PFA > CP-ddC > CP-ddI (Table 2.4). The CP-ddNs are not very toxic to the uninfected host cells. The average concentrations required to decrease HT4-6C cell growth by 50% ($CC_{50}$) ranged from 500 $\mu$M to greater than 2000 $\mu$M for compounds 6 to 9 (Table 2.4). Under similar assay conditions, the average $CC_{50}$ values for AZT, ddC, ddI, d4T and PFA was 7.6 $\mu$M, >474 $\mu$M, 937 $\mu$M, 95 $\mu$M and 900 $\mu$M. The selectivity index ($ED_{50}/ CC_{50}$) for
Table 2.4 Relative antiviral activity of dideoxynucleosides and their corresponding 5'-carboxyphosphonyl analogs against human immunodeficiency virus in HT4-6C (HeLa CD4) cells by focal immunoassay

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>Antiviral Activity ( \text{ED}_{50} ) (µM)</th>
<th>( \text{ED}_{90} ) (µM)</th>
<th>Cytotoxicity</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{ED}_{50} ) (µM)</td>
<td>( \text{ED}_{90} ) (µM)</td>
<td>Cell Morphology (MCC) (µM)</td>
<td>Cell Growth (CC(_{50})) (µM)</td>
</tr>
<tr>
<td>AZT</td>
<td>0.1±0.01</td>
<td>1.8±0.3</td>
<td>0.7</td>
<td>7.6±2.1</td>
</tr>
<tr>
<td>CP-AZT (6)</td>
<td>4.4±1.1</td>
<td>28±1</td>
<td>62</td>
<td>515±148</td>
</tr>
<tr>
<td>ddC</td>
<td>0.4±0.1</td>
<td>2.4±0.3</td>
<td>64</td>
<td>&gt;474(^6)</td>
</tr>
<tr>
<td>CP-ddC (7)</td>
<td>130±17</td>
<td>&gt;1000(^6)</td>
<td>250</td>
<td>&gt;2000(^6)</td>
</tr>
<tr>
<td>ddI</td>
<td>4.6±2.3</td>
<td>17±1.4</td>
<td>375</td>
<td>937±57</td>
</tr>
<tr>
<td>CP-ddI (8)</td>
<td>150±70</td>
<td>&gt;1000(^6)</td>
<td>500</td>
<td>945±78</td>
</tr>
<tr>
<td>d4T</td>
<td>0.04±0.01</td>
<td>0.5±0.1</td>
<td>23</td>
<td>95±8</td>
</tr>
<tr>
<td>CP-d4T (9)</td>
<td>17±3</td>
<td>58±2.8</td>
<td>300</td>
<td>&gt;1000(^6)</td>
</tr>
<tr>
<td>PFA</td>
<td>47±12</td>
<td>140±28</td>
<td>125</td>
<td>900±14</td>
</tr>
</tbody>
</table>

1. HIV-1 MO strain was used. Virus input 50-100 PFU.
2. \( \text{ED}_{50} \) and \( \text{ED}_{90} \): Inhibitory concentration (mean ± SD) required to reduce viral plaques by 50% and 90%, respectively.
3. MCC: Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
4. CC\(_{50}\): Cytotoxic concentration required (mean ± SD) to reduce cell growth by 50%.
5. SI: Ratio of CC\(_{50}/\text{ED}_{50}\).
6. Highest concentration tested.
Figure 2.5 Relative antiviral activity of AZT, ddC, ddI and d4T, control drugs; and 5'-carboxyphosphonyl analogs: CP-AZT, CP-ddC, CP-ddI and CP-d4T against HIV-1 in HT4-6C (HeLa CD4) cells by focal immunoassay. The assay for each compound was carried out in duplicate and each point represents an average of at least four independent determinations. Values shown represent the average percentages of viral control ± SD. HIV-1 MO strain was used. Virus input 50 - 100 PFU. Panel A, CP-AZT (■ ■), AZT (● ●); panel B, CP-ddC (■ ■), ddC (● ●); panel C CP-ddI (■ ■), ddI (● ●) and panel D, CP-d4T (■ ■), d4T (● ●). In each experiment, virus controls (virus and media), cell controls (media only) and toxicity controls (test compound and media) were run simultaneously.
CP-AZT was greater than AZT. In marked contrast the selectively index for other CP-ddNs was significantly lower than ddC, ddI and d4T.

(b) Reverse transcriptase assay using peripheral blood derived monocyte-macrophages (PBMC)

The antiviral activity of CP-AZT, CP-ddC and CP-ddI was evaluated against HIV-1 ADA strain using human peripheral blood derived monocyte-macrophage by RT assay in Dr. Lazdins laboratory, Ciba-Geigy Research Laboratories, Basle, Switzerland. The minimum concentration required for the maximum antiviral effect has been used to compare the relative potency of these compounds because each compound was tested at only three concentrations. The concentrations of CP-ddC and CP-AZT required to inhibit HIV replication (≥ 98%) were 0.8 μM and 6.7 μM, respectively. In contrast, the maximum inhibition achieved at the highest concentration of CP-ddI 7.3 μM was around 80%.

The long-term effects of these compounds on the virus inhibition were also investigated by exposing PBMC for 21 days to CP-AZT, CP-ddC and CP-ddI. The RT activity of the supernatant fluid was determined on day 6, 10, 14, 17 and 21 post-infection. The salient findings of these studies were: (1) The pattern of inhibition of the RT activity (measurement of HIV replication) was similar for CP-ddN analogs. (2) CP-AZT and CP-ddC were better inhibitors of HIV replication compared to CP-ddI. Exposure of HIV-infected PBMC to CP-ddC (7.8 μM), CP-AZT (6.7 μM) and CP-ddI (7.3 μM) resulted in 86%, 80% and 40% loss of RT activity on day 6 post infection. The inhibitory effect was maintained for the entire duration of the experiment (21 days). RT activity was barely detectable in samples exposed to CP-AZT and CP-ddC after 14 days post infection (inhibition ≥ 98%). (3) CP-ddC was the most potent inhibitor of viral replication. RT activity
was essentially undetectable after exposure to 7.8 μM of the drug. In contrast, CP-AZT was considerably less active inhibitor than CP-ddC and CP-ddI at lower concentrations (up to 0.73 μM) was inactive. At lower concentrations, CP-AZT (0.06 μM) and CP-ddI (0.07 μM) were found to stimulate the viral growth. Results are shown in Figure 2.6.

In summary, CP-ddC, CP-AZT and CP-ddI were found to be potent inhibitors of HIV-1 ADA in PBMC assay system at concentrations in the range of 7 to 8 μM. Of particular significance were the findings that the antiviral effect persisted for a long period (21 days of infection).

(c) Cytopathic inhibition assay

These studies were carried out in Professor De Clercq's laboratory, Rega Institute for Medical Research, Leuven, Belgium. The antiviral activity of CP-AZT (6), CP-ddC (7) and CP-ddI (8) was determined against HIV-1, HIV-2 and AZT-resistant virus ADP/141 by assessing inhibition of cytopathic effect in MT-4 cells. Drugs (AZT, ddC and ddI) and thymidine (dThd) were included as positive and negative controls, respectively. AZT and ddc and ddI were found to be potent inhibitors of both HIV-1 and HIV-2 virus. The antiviral potency of ddC and ddI was approximately 142 and 640 fold lower than AZT. These ED₅₀ values correspond to previously reported ED₅₀ values for AZT, ddC and ddI using MT-4 cells (Table 3 of Appendix).

The average concentrations required for 50% inhibition of the wild strains of HIV by CP-AZT (6), CP-ddC (7) and CP-ddI (8) were 0.07 μM, 40 μM and 130 μM, respectively. Thus, the introduction of carboxyphosphonyl group at 5'-position of the sugar molecule of ddN's resulted in a substantial decrease in potency ranging from 18 to 70 fold. AZT-resistant mutant, strain ADP/141 was refractory to CP-AZT, however remained sensitive to CP-ddC.
Figure 2.6 Relative antiviral activity of 5'-carboxyphosphonyl analogs: CP-AZT, CP-ddC and CP-ddI against HIV-1 in PMBC by RT assay. HIV-1 ADA strain was used. Virus input 500 TCID\textsubscript{50}. Panel A, CP-AZT: 0.067 µM (■), 0.67 µM (□), 6.7 µM; panel B. CP-ddC: 0.078 µM (■), 0.78 µM (□), 7.8 µM (□) and panel C CP-ddI: 0.073 µM (■), 0.73 µM (□) and 7.3 µM (□). In each experiment, viral controls (virus and media), cell controls (media only) and toxicity controls (test compound and media) were run simultaneously.
and CP-ddI. Interestingly, the concentrations of CP-ddC required for 50% and 90% inhibition of ADP/141 were approximately 3 fold lower than that of HIV-IIIB. Even though, the potency of CP-AZT was lower (approximately 20 fold) compared to AZT against sensitive strains. CP-AZT was a better inhibitor of virus replication compared to ddC and ddI, drugs currently approved for the treatment of HIV infection. The results were summarized in Table 2.5.

2.3.3 Antiviral activity of 5'-carboxyphosphonyl derivatives of naturally occurring deoxyribonucleosides (5'-CP-dNs)

Carboxyphosphonyl derivatives of deoxynthymidine (dThd), deoxyuridine (dUrd) and deoxyinosine (dIno), 5-methoxymethyldeoxyuridine (MMdUrd) and 5-ethyldeoxyuridine (EtdUrd) were synthesized to determine the effect of the introduction of the carboxyphosphonyl (CP) group on HIV replication. For comparative purposes CP-Inosine (CP-Ino) was also prepared and its anti-HIV activity was investigated. The rationale for the synthesis of these compounds was discussed earlier (chapter 1, section 1.7) and structure of active compounds are shown in scheme 2.3.

Initially, the activity of CP analogs was determined by a plaque reduction assay (FIA) by me. The most active compounds were then submitted to Professor Wainberg (McGill University, Montreal, Canada) and Professor De Clercq (Rega Institute for Medical Research, Leuven, Belgium) for antiviral screen. Results of these studies are discussed below.
Table 2.5 Relative antiviral activity of AZT, ddC, ddI, CP-AZT, CP-ddC and CP-ddI against HIV-1 and HIV-2 in MT-4 cells by cytopathic inhibition assay.¹

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>Antiviral Activity (ED₅₀ µM)²</th>
<th>HIV-1</th>
<th>HIV-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IIIIB</td>
<td>RF</td>
<td>ADP/141⁺</td>
</tr>
<tr>
<td>AZT</td>
<td>0.004</td>
<td>0.002</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(0.07)³</td>
<td>(0.006)</td>
<td>(0.06)</td>
</tr>
<tr>
<td>CP-AZT (6)</td>
<td>0.072</td>
<td>0.08</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>(0.13)</td>
<td>(0.29)</td>
<td>(&gt;133)</td>
</tr>
<tr>
<td>ddC</td>
<td>0.57</td>
<td>0.43</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>(1.00)</td>
<td>(0.95)</td>
<td>(0.76)</td>
</tr>
<tr>
<td>CP-ddC (7)</td>
<td>39.80</td>
<td>22.9</td>
<td>13.16</td>
</tr>
<tr>
<td></td>
<td>(79.30)</td>
<td>(53.3)</td>
<td>(26.95)</td>
</tr>
<tr>
<td>ddI</td>
<td>2.56</td>
<td>2.85</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(NA)⁵</td>
<td>(NA)</td>
<td>(NA)</td>
</tr>
<tr>
<td>CP-ddI (8)</td>
<td>129.6</td>
<td>363/&gt;436</td>
<td>142.4</td>
</tr>
<tr>
<td></td>
<td>(270)</td>
<td>(&gt;726)</td>
<td>(259)</td>
</tr>
</tbody>
</table>

1. Virus input: 100 TCID₅₀ units.
2. ED₅₀: Inhibitory concentration required for the 50% protection of MT-4 cells from viral cytopathic effect.
3. ED₉₀(bracket): Inhibitory concentration required for the 90% protection of MT-4 cells from viral cytopathic effect.
4. AZT-resistant strain HIV-1 ADP/141: RT site mutations at 67N, 70R, 215F and 219Q.
5. NA: Not available.
Relative antiviral activity of CP-dThd, CP-dUrd, CP-dIno, CP-Ino, and control drugs AZT, ddI and PFA

(a) Focal immunoassay

CP-dIno, CP-dUrd and CP-dThd were good inhibitors of HIV and their 50% inhibitory levels (ED$_{50}$) ranged from 22 to 37 μM. The antiviral potency of PFA was slightly lower than these compounds (ED$_{50}$ 47 μM). AZT and ddI were potent inhibitors with ED$_{50}$ values of 0.1 μM and 4.6 μM, respectively. The ED$_{50}$ values of AZT, ddI and PFA correspond closely to reported values (Iversen et al., 1996, Larder et al., 1990). CP-Ino (13) was a marginal inhibitor of HIV replication. 5-substituted carboxyphosphonyl analogs, CP-MMdUrd (14) and CP-EtdUrd (15) were toxic to HT4-6C cells and it was difficult to determine their antiviral activity. Therefore, further studies on compounds 13-15 were abandoned. Results are summarized in Table 2.6 and Figure 2.7.

(b) Reverse transcriptase assay using cord blood mononuclear cells (CBMC)

The activity of CP-dThd (10), CP-dUrd (11) and CP-dIno (12) was also determined against HIV-1 strain IIIB and AZT-resistant mutants isolated from patients and designated HIV-1 1075 and HIV-1 1082 in freshly isolated human CBMC by RT assay. AZT and ddI were included as positive controls. dThd was used as a negative control. Compounds 10-12 were potent and selective inhibitors of HIV-1 replication. The concentration of each compound that inhibited 50% of RT activity (ED$_{50}$) is given in Table 2.7. The antiviral potency of CP analogs was approximately 4 to 10 fold higher than ddI against all HIV strains. The average concentrations of CP-dThd, CP-dUrd and CP-dIno required for 50% inhibitory levels against the wild strain HIV-1 IIIB was 0.15 μM to 0.23 μM, respectively. Interestingly, the concentrations required for 50% inhibition of RT activity of AZT-resistant
Table 2.6 Relative antiviral activity of CP-dThd, CP-dUrd, CP-dIno, AZT, ddI and PFA against human immunodeficiency virus in HT4-6C (HeLa CD4) cells by focal immunoassay\(^1\)

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>Antiviral Activity(^2)</th>
<th>Cytotoxicity</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED(_{50}) ((\mu)M)</td>
<td>ED(_{90}) ((\mu)M)</td>
<td>Cell Morphology (MCC)(^3) ((\mu)M)</td>
</tr>
<tr>
<td>AZT</td>
<td>0.1± 0.01</td>
<td>1.8± 0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>CP-dThd (10)</td>
<td>37±11</td>
<td>670 ±195</td>
<td>200</td>
</tr>
<tr>
<td>CP-dUrd (11)</td>
<td>22±9</td>
<td>160 ±51</td>
<td>1000</td>
</tr>
<tr>
<td>ddI</td>
<td>4.6±2.3</td>
<td>17 ±1.4</td>
<td>375</td>
</tr>
<tr>
<td>CP-dIno (12)</td>
<td>22±8</td>
<td>252±114</td>
<td>1000</td>
</tr>
<tr>
<td>PFA</td>
<td>47±12</td>
<td>140±28</td>
<td>125</td>
</tr>
</tbody>
</table>

1. HIV-1 MO strain was used. Virus input: 50-100 PFU.
2. ED\(_{50}\) and ED\(_{90}\): Inhibitory concentration (mean ± SD) required to reduce viral plaques by 50% and 90%, respectively.
3. MCC: Minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.
4. CC\(_{50}\): Cytotoxic concentration (mean ± SD) required to reduce cell growth by 50%.
5. SI: Ratio of CC\(_{50}\)/ED\(_{50}\).
Figure 2.7 Relative antiviral activity of dideoxynucleosides (ddNs): AZT and ddi; 5'-carboxyphosphonyl analogs of deoxynucleosides: CP-dThd, CP-dUrd and CP-dIno against HIV-1 in HT4-6C (HeLa CD4) cells by focal immunoassay. The assay for each compound was carried out in duplicate and each point represents an average of at least four independent determinations. Values shown represent the average percentages of viral control ± SD. HIV-1 MO strain was used. Virus input 50-100 PFU. Panel A, CP-ddI (■■■■), ddi (○○○○); panel B, CP-dThd (■■■■), AZT (○○○○); panel C, CP-dUrd (■■■■), AZT (○○○○). In each experiment, virus controls (virus and media), cell controls (media only) and toxicity controls (test compound and media) were run simultaneously.
Table 2.7 Relative Antiviral Activity of CP-dThd, CP-dUrd, CP-dIno, AZT and ddI against human immunodeficiency virus type 1 (HIV-1) in cord blood mononuclear cells (CBMC) by reverse transcriptase assay

<table>
<thead>
<tr>
<th>Compound(^2) (Code #)</th>
<th>Antiviral Activity (ED(_{50}) μM)(^3)</th>
<th>Cytotoxicity CC(_0)(^4) (μM)</th>
<th>SI(^5) (CC(<em>0)/ED(</em>{50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IIIB</td>
<td>1082</td>
<td>1075</td>
</tr>
<tr>
<td>AZT</td>
<td>0.0043 ± 0.0024</td>
<td>1.0</td>
<td>0.28</td>
</tr>
<tr>
<td>ddI</td>
<td>0.6</td>
<td>2.2</td>
<td>4.0</td>
</tr>
<tr>
<td>dThd</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CP-dThd (10)</td>
<td>0.15 ± 0.021</td>
<td>0.24</td>
<td>0.30</td>
</tr>
<tr>
<td>CP-dUrd (11)</td>
<td>0.23 ± 0.021</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>CP-dIno (12)</td>
<td>0.23 ± 0.056</td>
<td>0.15</td>
<td>0.55</td>
</tr>
</tbody>
</table>

2. Drugs AZT and ddI (positive controls); thymidine (negative control).
3. ED\(_{50}\): Inhibitory concentration (mean ± SD) required to reduce RT activity by 50%.
4. CC\(_0\) (Highest concentration used in the assay). Microscopically detectable alteration of cell morphology was not observed.
5. SI: Ratio of CC\(_0\)/ED\(_{50}\) (CC\(_{50}\) values were not available).
6. Included for comparison (Gu et al., 1995)
7. ND: Not determined (not available).
strains were essentially similar for CP-dUrd and CP-dThd and marginally higher for CP-dIno against HIV-1 1075. AZT was a potent inhibitor of HIV-1 IIIB with potency approximately 35 to 140 fold greater than CP analogs and ddI, respectively. However, AZT-resistant variants (strains 1075 and 1082) displayed approximately 65 and 232 fold *decreases* in susceptibility to AZT compared to the wild strain HIV-1 III B. In contrast, the amount of ddI required to inhibit AZT-resistant replicates was only 4 to 7 fold higher compared to HIV-1 IIIB. Deoxythymidine was *devoid* of activity up to 100 μM (highest concentration tested) against HIV-1 IIIB and AZT-resistant variants.

**(c) Cytopathic inhibition assay**

The antiviral activity of CP-dThd (10), CP-dUrd (11) and CP-dIno (12) was determined against HIV-1, HIV-2 and AZT-resistant virus ADP/L41 by assessing inhibition of cytopathic effects in MT-4 cells. AZT and ddI (positive control) data was included for comparison. Deoxythymidine (dThd) was included in these assays as a negative control. Results of this antiviral screen are summarized in Table 2.8. **Due to wide variation observed for the concentration required for 50% inhibitory levels (ED$_{50}$) for both HIV-1 and HIV-2 strains (some times up to 50 fold) in repeat experiments in this assay system, it is difficult (if not impossible) to calculate the correct ED$_{50}$ values.**

The salient findings of antiviral serum using cytotoxicity assay studies were:

1. CP-dThd, CP-dUrd and CP-dIno showed only moderate activity against HIV-1 and HIV-2 strains by cytopathic inhibition assay.

2. CP-dIno was the most active inhibitor of HIV replication. The average concentration required for 50% inhibition of the wild strains of HIV varied from 5 μM to 90 μM.
Table 2.8 Relative antiviral activity of AZT, ddI, CP-dThd, CP-dUrd, CP-dI no and thymidine (dThd) against HIV-1 and HIV-2 in MT-4 cells by cytopathic inhibition assay

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>Antiviral Activity (ED₅₀ μM)²</th>
<th>HIV-1</th>
<th>HIV-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IIIB</td>
<td>RF</td>
<td>ADP/141 ³</td>
</tr>
<tr>
<td>AZT⁴</td>
<td>0.0006 ± 0.0003</td>
<td>0.0014 ± 0.0006</td>
<td>0.012 ± 0.008</td>
</tr>
<tr>
<td>CP-dThd (10)</td>
<td>145 ± 96</td>
<td>&gt;268 &gt;357</td>
<td>92 ± 94</td>
</tr>
<tr>
<td>CP-dUrd (11)</td>
<td>92 ± 1.5</td>
<td>386 &gt;373</td>
<td>31 ± 15.2</td>
</tr>
<tr>
<td>CP-dI no (12)</td>
<td>21 ± 5.3</td>
<td>90 ± 39.0</td>
<td>11 ± 3.0</td>
</tr>
<tr>
<td>ddI</td>
<td>2.6 ± 1.2</td>
<td>2.9 ± 0.5</td>
<td>NA⁵</td>
</tr>
<tr>
<td>dThd</td>
<td>55 ± 4.1</td>
<td>99 &gt;160</td>
<td>58 &gt;66</td>
</tr>
</tbody>
</table>

1. Virus input: 100 CCID₅₀ units.
2. ED₅₀: Inhibitory concentration required for the 50% protection of MT-4 cells from viral cytopathic effect.
3. AZT-resistant strain HIV-1 ADP/141: genotype RT site mutations at 67N, 70R, 215F and 219Q.
4. The ED₅₀ values (mean ± SD) from the antiviral screen data of ddNs were provided by Professor De Clercq. Assay runs against each strain as follow: AZT, IIIB (51), RF (4), ADP/141 (8), ROD (32) and EHO (11); ddI, III B (24), RF (2), ROD (8) and EHO (4).
5. NA: Data not available.
3. Deoxothyridine was a good inhibitor of HIV replication. These findings were quite surprising because dThd was found to be devoid of activity against HIV sensitive and AZT-resistant replicates by RT assay (Table 2.7).

4. AZT-resistant strain ADP/141 (mutations at 67N, 70R, 215F and 219Q of RT gene) was inhibited by compounds 10-12 at lower concentrations (hyper-sensitive) compared to the wild strain HIV-1 III B. The average concentration required for 50% inhibition by CP-dIno and CP-dUrd were 11 μM and 31 μM, respectively. In contrast, AZT-resistant variant HIV ADP/141 was approximately 20 fold less sensitive to AZT (ED_{50} 0.012 μM) compared to III B (ED_{50} 0.0006 μM).

5. CP-dThd, CP-dUrd and CP-dIno were found to be particularly good inhibitors of HIV-2 strain EHO and their 50% inhibitory levels (ED_{50}) ranged from 5 to 21 μM. The antiviral potency of CP-dIno was similar to ddI.

2.3.4 Acute toxicity of ddNs and carboxyphosphonyl analogs against lymphoblast cell lines and HT4-6C (HeLa CD4 cells)

Cytotoxicity is an important parameter for evaluation of the selectivity of anti-HIV inhibitors. To ascertain that the antiviral effect of a compound was not due to toxic effects on cells, the effect of increasing concentrations of antiviral drugs and synthesized compound on monolayers and rapidly growing cells using lymphoblast cell lines was examined. The toxicities studies using HT4-6C, MT-2 and CEM cells were carried out at University of Saskatchewan.

The results of cytotoxicity studies on anti-HIV drugs (AZT, ddC, ddI, d4T and PFA), CP analogs of dideoxynucleosides (CP-AZT, CP-ddC, CP-ddI and CP-d4T) and deoxyribonucleosides (CP-dThd, CP-dIno and CP-dUrd) against different lymphoblast cell lines and HT4-6C cells are summarized in Tables 2.9 and 2.10. The acute toxicity of each
Table 2.9 Inhibitory effects of ddNs (AZT, ddC, ddI, d4T), carboxyphosphonyl analogs (CP-AZT, CP-ddC, CP-ddI, CP-d4T, CP-dThd, CP-dUrd, CP-dlno, CP-EtdUrd), thymidine (dThd), and foscarnet (phosphonoformic acid, PFA) on cell morphology (MCC)\(^1\) and cell growth (CC\(_{50}\))\(^2\) against HT4-6C and MT-4 cells

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>Cell Line</th>
<th>MT-4(^4) (MT-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HT4-6C(^3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCC (µM)</td>
<td>CC(_{50}) (µM)</td>
</tr>
<tr>
<td>AZT</td>
<td>0.73</td>
<td>7.6±2.1</td>
</tr>
<tr>
<td>ddC</td>
<td>64</td>
<td>&gt; 474(^5)</td>
</tr>
<tr>
<td>ddI</td>
<td>375</td>
<td>937±57</td>
</tr>
<tr>
<td>d4T</td>
<td>23</td>
<td>95±7.8</td>
</tr>
<tr>
<td>CP-AZT (6)</td>
<td>62</td>
<td>515±148</td>
</tr>
<tr>
<td>CP-ddC (7)</td>
<td>250</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>CP-ddI (8)</td>
<td>500</td>
<td>945±78</td>
</tr>
<tr>
<td>CP-d4T (9)</td>
<td>300</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>CP-dThd (10)</td>
<td>200</td>
<td>&gt; 2000</td>
</tr>
<tr>
<td>CP-dUrd (11)</td>
<td>1000</td>
<td>&gt; 2000</td>
</tr>
<tr>
<td>CP-EtdUrd (15)</td>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>dThd</td>
<td>46</td>
<td>&gt; 413</td>
</tr>
<tr>
<td>PFA</td>
<td>125</td>
<td>900±14</td>
</tr>
</tbody>
</table>

1. MCC: Minimum cytotoxic concentration required to cause a microscopically detectable alteration of cell morphology.
2. CC\(_{50}\): Concentration (mean ± SD) required to reduce cell growth by 50%.
3. Cytotoxicity against confluent monolayers of HT4-6C (HeLa CD4) cells was examined by cell morphology (MCC) and cell density (CC\(_{50}\)) four days after drug treatment.
4. Cytotoxicity against MT-4 and MT-2 cells was examined by the viability of uninfected cells using the spectrophotometric assay (MTT method) five days after drug treatment.
5. Highest concentration used.
1. MT-2 cells (Values in bracket).
2. Included for comparison (Baba et al., 1987).
3. NA: Data not available.
Table 2.10 Inhibitory effects of ddNs (AZT, ddC, ddl, d4T), carboxyphosphonyl analogs (CP-AZT, CP-ddC, CP-dThd, CP-dUrd, CP-dIno) and thymidine (dThd) on Cell morphology (MCC)¹ and cell growth (CC₅₀)² for CEM, CBMC and PBMC cells.

<table>
<thead>
<tr>
<th>Compound (Code #)</th>
<th>Cell Line²</th>
<th>CEM</th>
<th>CBMC (PBMC)⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCC (µM)</td>
<td>CC₅₀ (µM)</td>
<td>MCC (µM)</td>
</tr>
<tr>
<td>AZT</td>
<td>32</td>
<td>&gt; 32 ⁵</td>
<td>&gt; 7.5 (37.5)⁶</td>
</tr>
<tr>
<td>ddC</td>
<td>0.5</td>
<td>12</td>
<td>&gt; 25 ⁶</td>
</tr>
<tr>
<td>ddl</td>
<td>NA ⁷</td>
<td>945</td>
<td>&gt; 33 ⁶</td>
</tr>
<tr>
<td>d4T</td>
<td>NA</td>
<td>90</td>
<td>25 ⁶</td>
</tr>
<tr>
<td>CP-AZT (6)</td>
<td>&gt; 250</td>
<td>&gt; 1000</td>
<td>(&gt; 6.7)</td>
</tr>
<tr>
<td>CP-ddC (7)</td>
<td>4</td>
<td>62</td>
<td>(&gt; 7.8)</td>
</tr>
<tr>
<td>CP-dThd (10)</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 286 (&lt; 7.1)</td>
</tr>
<tr>
<td>CP-dUrd (11)</td>
<td>&gt; 800</td>
<td>&gt; 800</td>
<td>&gt; 298</td>
</tr>
<tr>
<td>CP-dIno (13)</td>
<td>&gt; 800</td>
<td>&gt; 800</td>
<td>&gt; 278</td>
</tr>
<tr>
<td>dThd</td>
<td>NA</td>
<td>NA</td>
<td>&gt; 413</td>
</tr>
</tbody>
</table>

1. MCC: Minimum cytotoxic concentration required to cause a microscopically detectable alteration of cell morphology.
2. CC₅₀: Concentration required to reduce cell growth by 50%.
3. Cytotoxicity against CEM cells was examined by morphology and by the viability of cells using the trypan blue exclusion method four days after drug treatment. Cytotoxicity against CBMC was examined by morphology seven days after drug treatment. Cytotoxicity against PBMC was examined by DAPI method twenty one days after drug treatment.
4. PBMC (values in bracket).
5. Highest concentration tested.
6. Included for comparison. AZT (Lazdins et al., 1990), ddC and d4T (Gu et al., 1994), ddl (Gu et al., 1995).
7. NA: Data not available.
compound was determined using two parameters: MCC, the minimum cytotoxic concentration required to cause a microscopically detectable alteration of cell morphology and CC<sub>50</sub>, the concentration required to reduce cell growth by 50%. The salient findings of the cytotoxicity studies were:

1. The cytotoxicity of ddNs was cell dependent. AZT was the most toxic compound. AZT was very toxic to HT4-6C cells (MCC: 0.73 μM), and relative non-toxic to CEM cells (CC<sub>50</sub>: >32 μM). In contrast, ddC was most toxic to CEM cells, with MCC and CC<sub>50</sub> values of 0.5 μM and 12 μM, respectively, however, ddC was less toxic to HT4-6C cells (CC<sub>50</sub> > 474 μM). d4T was less toxic than AZT against HT4-6C and CEM cells, but more toxic for MT-4 cells. ddI in general was the least toxic ddNs tested. The CC<sub>50</sub> values of ddI ranged from 937 μM to greater than 1058 μM for lymphoblast cells. PFA was not very toxic for the panel of cell lines used in these studies (CC<sub>50</sub> values of PFA ranged from 460 μM to 1500 μM). Deoxythymidine (dThd) was not toxic to CBMC (MCC > 413 μM). However, it was more toxic for HT4-6C and MT-4 cells.

2. In general, carboxyphosphonyl derivatives of dideoxynucleosides (CP-ddNs) were less cytotoxic. The MCC values for CP-AZT (6), CP-ddC (7), CP-ddI (8) and CP-d4T (9) for HT4-6C cells ranged from 62 μM to 300 μM, and the CC<sub>50</sub> values ranged from 515 μM to greater than 2000 μM. Compounds 6-8 were also not toxic to MT-4 cells with the CC<sub>50</sub> values ranging from >133 μM to >726 μM (highest concentration tested). However, CP-ddC (7) was toxic to CEM cells. The MCC and CC<sub>50</sub> values for CP-ddC were 4 μM and 62 μM, respectively. In contrast, CP-AZT (6) was less toxic to CEM cells, (CC<sub>50</sub>: >1000 μM). The cytotoxic profiles of compounds 6 and 7 to CEM cell are similar to their corresponding parent drugs, AZT and ddC.
In summary, CP-ddNs were usually less toxic to cells compared to their corresponding ddNs. The cytotoxicity of compounds 6, 7 and 9 was 4 to 85 fold lower compared to AZT, ddC and d4T for different cell lines. Surprisingly, however, CP-ddI (9) was almost as toxic as ddI for HT4-6C cells.

3. 5'-carboxyphosphonyl analogs of deoxyribonucleosides have low cytotoxicity. The CC$_{50}$ values for CP-dThd (10), CP-dUrd (11) and CP-dIno (12) were greater than 700 µM, 800 µM and 2 mM for MT-4, CEM and HT4-6C, respectively. The cytotoxicity of compounds 10-12 was also greater than 300 µM (highest concentration tested) for CBMC.

CP-EtdUrd is the exception. It was very toxic to HT4-6C cells with the CC$_{50}$ value of 3 µM.

2.4 DISCUSSION

5-Hydroxymethyl-2'-deoxyuridine (HMDUrd) is a natural nucleoside produced by bacteriophages. HMDUrd is structurally similar to natural deoxythymidine in which the C5 methyl group of dThd is replaced by hydroxymethyl group. The findings that this naturally occurring thymidine analog has activity against retrovirus in vitro and in vivo (Gupta et al., 1991) are interesting. The previous studies showed that HMDUrd was able to be incorporated in the DNA of mammalian cells (Boorstein et al., 1992; Kaufman, 1986; Vilpo and Vilpo, 1988), and HMDUrd-5'-triphosphate (HMDUTP) is a moderate inhibitor of HIV RT (Tao et al., 1989). Therefore, the antiviral activity of HMDUrd is probably due to inhibition of RT by HMDUTP and incorporation of HMDUrd into the viral DNA. The low activity of HMDUrd in cell cultures compared to AZT is probably due to two reasons: firstly, HMDUrd has a very low affinity to cellular human thymidine kinase (HTK) compared to AZT, this may lead to a slow rate of phosphorylation of HMDUrd to HMDUTP, and results in a lower triphosphate level of HMDUrd in the cells. This in turn may decrease the ability
of HMdUrd to inhibit HIV RT. Secondly, one of the important mechanisms of anti-HIV activity of AZT is that AZT can stop the viral DNA chain elongation due to the lack of a 3'-OH group in its molecule, in contrast, HMdUrd, can be incorporated into DNA and would not be a chain terminator because of the presence of a 3'-OH group in the sugar moiety. Therefore, it is not surprising that HMdUrd only has moderate anti-HIV activity in cell culture.

In order to improve the potency of HMdUrd against HIV, synthesis of HMddUrd, AZHMddUrd, FHMddUrd and HMd4Urd was undertaken. It was reasonable to assume that by converting HMdUrd to the dideoxy, 3'-azido, 3'-fluoro and didehydridideoxy derivatives would result in a more active molecule (Gupta et al., 1995). This was based on the premise that the only structural difference between the compounds stated above and the corresponding thymidine analogs fluorothymidine and dideoxythymidine (ddT and d4T) is the presence of the hydroxymethyl group instead of a methyl group at the C5 position of the pyrimidine ring. Unfortunately, the expectation of increased potency was not fulfilled. The derivatives of HMdUrd were essentially devoid of activity against HIV. The results of enzyme assay indicated that the modification of the sugar ring further decreased the ability of these compounds for binding to HTK. Thus, one possible reason for the lack of anti-HIV activity of these compounds is a very slow rate of phosphorylation by the cellular thymidine kinase so that physiologically significant concentrations of the monophosphates (and ultimately triphosphates) are never reached in infected cells.

In summary, HMdUrd has moderate antiretroviral activity \textit{in vitro} and \textit{in vivo}. The effect of improving the antiviral effects of HMdUrd by modifying the chemical structure of HMdUrd failed. Therefore, further studies on these compounds were terminated.
The antiviral activity of two classes of 5'-CP nucleoside analogs, CP-dNs and CP-ddNs was investigated using three assay methods with different endpoints: MTT (cytopathy), RT (viral production) and FIA (infectious viral particles). The anti-HIV activity (ED$_{50}$ and ED$_{90}$) of compounds has been shown to vary with the assay methodologies used, virus strain, virus input and the cell line used for virus multiplication. For ddNs this has been attributed to the rate of intracellular phosphorylation of these compounds to their corresponding triphosphates. The ED$_{50}$ values of marketed ddNs (AZT, ddC, ddI and d4T) obtained in our studies are within the range reported by other investigators (Table 3 of Appendix). The same rank order of potency: AZT>ddC>ddI was obtained using MTT and FIA assay. The inhibitory values are similar to published results.

Among three assay methods used, RT assay was the most sensitive method to detect the activity of this type of compounds. The ED$_{50}$ values of 5'-CP-dNs obtained by RT assay were significantly lower (100 to 250 fold) in comparison to ED$_{50}$ inhibitory concentration obtained by FIA assay. Although, a similar pattern was seen for AZT and ddI, however, the disparity in inhibitory concentrations was much lower (8 to 23 fold). Although FIA method is less sensitive than RT assay, it is the most accurate quantitative method and correctly reflects the inhibition of infectious particles in the cell. In the MTT assay, considerable variation was observed for the concentrations required to inhibit HIV in repeat experiments for CP-dNs. Thus this assay method does not appear to be suitable for anti-HIV activity evaluation of this class of compounds.

The results of antiviral screen show that CP-dThd, CP-dUrd and CP-dIno are selective inhibitors of HIV replication in vitro. The CP-dNs inhibit viral replication in CD4+ cell lines (MT-4 and HT4-6C) and primary human CBMC cultures. CP-dNs are not only active against HIV type 1 (HIV-1), but also effective against HIV type 2 (HIV-2). Most
importantly, three of the HIV-1 AZT resistant strains showed essentially same or greater susceptibilities to this class of the compounds.

The antiviral activity of CP-dNs is lower than AZT in cell cultures by all assay systems. In contrast, CP-dNs were found to be more potent inhibitors of HIV-1 and AZT-resistant variants compared to ddi by RT assay (Table 2.7). In HT4-6C cell cultures, the potency of CP-dNs was similar or greater than PFA by FIA (Table 2.6). CP-dThd, CP-dUrd and CP-dino were found to have essentially similar potency when evaluated by RT and FIA assay. However, CP-dino was more potent than CP-dUrd and CP-dThd against all HIV strains in cytopathic inhibition assay.

Two interesting observations are obvious from the antiviral screen data of CP-dNs. Firstly, different types and strains of HIV showed different sensitivity to those compounds. Secondly, same viral strains showed different susceptibility to different compounds, even though these compounds inhibit HIV replication by similar mechanisms of action in MT-4 cells. Further studies on a panel of HIV isolates should be carried out by RT and FIA assays to determine whether same pattern is exhibited by CP-dNs. Interestingly, similar observations have been made for AZT. For example, AZT is not active against HIV-1 GB8 strain/JM cell and HIV-2 LAV-2 (1502)/MT-2 test systems (Coates et al., 1992). On the other hand, the replications of HIV-1 GB8 in JM cells and HIV-2 LAV-2 (1502) in MT-2 cells can be efficiently inhibited by the other ddNs, such as ddC, ddi and 3TC (Coates et al., 1992). The inhibitory values against these strains for ddC and ddi are comparable to other strains (Table 3 of Appendix).

At present, it is difficult to give reasons for the large discrepancies observed for inhibitory values of CP-dNs against HIV by different assay methods. It may be related to the unique characteristics of different HIV strains, cell line used for the assay, cellular uptake
and affinity of the CP-dNs (competitive antagonist) for the target molecule, and intracellular levels of deoxyribonucleosides.

CP-AZT, CP-ddC and CP-ddI were excellent inhibitors of HIV-1 ADA/PBMC test system. Moreover, long term (as long as 21 days) antiretroviral effects were observed in HIV-1 ADA/PBMC test system. This was the best assay system for evaluation of anti-HIV activity of these compounds. The introduction of carboxyphosphonyl group at 5'-position of anti-HIV drugs resulted in reduction of anti-viral potency compared to parent drugs. This was anticipated because these compounds are not likely to be 'anabolized' (activated) by similar mechanisms as ddNs.

The cytotoxicity of CP-dNs against three different types of cells, MT-4 (T-lymphocyte cell line), HT4-6C (cervical carcinoma epithelial cell line) and CBMC (primary human monocyte) are very low. In my studies, CC_{50} for CP-dNs was not reached even at 2 mM. Thus, in spite of lower potency, CP-dNs have high safety index. Indeed, one of the most valuable properties of CP-dNs is very low toxicity. As will be discussed later, they are much less toxic to bone marrow progenitor cells compared to AZT and they also did not exhibit delayed cytotoxicity; toxic manifestations commonly observed with the use of ddNs.

The cytotoxicity profile of carboxyphosphonyl analogs of anti-HIV drugs varied with the compound and cell line. For example, CP-ddI and ddI were toxic to HT4-6C cells at similar concentrations (CC_{50} ≈ 940 μM). Whereas CP-AZT was considerably less toxic than AZT for HT4-6C cells and CEM cells. In contrast, CP-ddC was very toxic to CEM cells and considerably less toxic for MT-4 and HT4-6C cells.

In summary, carboxyphosphonyl analogs of deoxyribonucleosides were found to have anti-HIV activity and low cytotoxicity. Therefore, biological studies were primarily focussed on compounds of this series.
3.0 TOXICITY STUDIES

3.1 INTRODUCTION

Two classes of anti-HIV agents inhibit reverse transcriptase (RT), the enzyme unique to this virus. The dideoxynucleoside analogs (ddNs): AZT, ddC, ddI, d4T and 3TC act as competitive inhibitors/alternate substrates of HIV RT after conversion to their 5'-triphosphates by cellular enzymes (Barry et al., 1998; Furman et al., 1986; Gray et al., 1995; Hao et al., 1988; Matthes et al., 1987). Phosphorylation of ddNs requires active cellular metabolism. The non-nucleoside RT inhibitors (NNRTIs) interact non-competitively with an allosteric site of the enzyme and inactivate it. NNRTIs do not require modification by cellular factors to bind to their target (Ding et al., 1995a; 1995b). The binding sites of NNRTIs are to a motif that is unique to HIV RT which confers specificity and selectivity. The NNRTIs currently approved for clinical use are nevirapine (NVP) and delavirdine (DEL). These drugs are much less toxic compared to the ddNs currently approved for the treatment of ARC and AIDS.

Although many of the anti-HIV agents currently available reduce viral load substantially, their effects are sustained only for a limited time due to the development of resistant strains and drug-induced toxicities (Arnaudo et al., 1991; Berger et al., 1993; Condra et al., 1995; Deeks et al., 1997; Gao et al., 1992; Goulden et al., 1996; Larder et al., 1989a; 1989b; Murray et al., 1995; Patick et al., 1996; Pluda et al., 1995; Richman et al., 1992; Shelton et al., 1992). In order to increase clinical outcome of chemotherapy and decrease the chances of development of viral resistant strains, the patient has to be given
combination of drugs (combination chemotherapy) on a long-term basis. However, the prolonged use of antiviral nucleoside analogs (ANAs) induces delayed multi-organ toxicities. Manifestations of delayed toxicities include peripheral neuropathy and pancreas failure caused by ddC, ddI, d4T and myopathy induced by AZT. These unique multi-organ toxicities suggest defective mitochondrial DNA (mtDNA) due to inhibition of mtDNA polymerase gamma (mtDNA polymerase $\gamma$) by triphosphate forms of ddNs.

As mentioned previously, combination chemotherapy has been found to be the most valuable therapeutic regime for the treatment of AIDS. The possibility of using several anti-HIV drugs of the same and different classes in tandem was examined in three large scale clinical trials (Barry et al. 1998). The effectiveness of AZT alone (monotherapy) or AZT in combination with ddC, ddI or 3TC was compared in AIDS patients with low CD4 counts (<500 cells/ml). Based on these trials, recommendations to date for initial viral combination therapies are either two ddNs with a protease inhibitor or two ddNs with a NNRTI. AZT is always a preferred drug for use in combination because of its potency and more importantly its ability to improve the HIV dementia compared to other anti-HIV drugs.

However, AZT has been shown to cause haematopoietic suppression manifested by anemia, neutropenia, and overall bone marrow failure in patients (Richman et al. 1987b). Several investigators have identified AZT-induced marrow toxicity to be the result of drug-induced inhibition of haematopoietic progenitor stem cells (Dainiak et al., 1988; Faraj et al., 1994; Gallicchio et al., 1993a; Sommadossi et al., 1987a; 1987b). The mechanism(s) of AZT induced marrow toxicity is multifactorial, including inhibition of nuclear DNA synthesis and disturbances of deoxynucleoside triphosphate pools (Sommadossi, 1993).

In summary, the characteristics of chemical structure of ddNs (lack of 2'-OH group) determine the ultimate multi-organ toxicities manifested by ddNs. The delayed toxicities and bone marrow toxicity of ddNs are mainly influenced by the cellular availability and
abundance of the ddNs, the ability of cellular enzymes to phosphorylate ddNs and the ability of ddNTP (as competitive inhibitor/chain terminator of cellular DNA polymerases) to inhibit nuclear or mitochondrial (mt)DNA synthesis.

The rationale for the synthesis of carboxyphosphonyl analogs of nucleosides was discussed earlier (Section 1.7). It was postulated that this class of compound should have less toxicity potential, because these compounds would not serve as substrates for the cellular or nuclear kinases and thus not be converted to triphosphates. CP analogs of AZT and ddC [CP-AZT (6) and CP-ddC (7)] and deoxyribonucleosides [CP-dThd (10), CP-dUrd (11) and CP-dlno (12)] are moderate and selective inhibitors of HIV including AZT-resistant variants of HIV (Tables 2.4 ~2.8 and Figures 2.5 ~ 2.7). CP-dUrd, CP-dThd, CP-dlno and CP-AZT have low cytotoxicity (Tables 2.9 and 2.10). The dose of compounds 10-12 and CP-AZT required to reduce HT4-6C cells growth by 50% was > 2000 μM and 515 μM, respectively. The lack of cytotoxicity against rapidly dividing cells suggested that CP analogs most likely do not serve as substrates for cellular kinases in uninfected or HIV-infected cells. CP analogs likely remain intact inside the cell, and the antiviral activity of these compounds therefore would be due to intrinsic activity of these molecules. If this hypothesis is correct, then these pharmacophores should have low potential for causing delayed and bone marrow toxicity.

Several investigations on ddN compounds indicate that in vitro assays of delayed cytotoxicity using lymphoblastoid cell lines and clonogenic assay of bone marrow haematopoietic progenitor cells are adequate models for predicting the delayed and bone marrow toxicities of anti-HIV inhibitors (Faraj et al., 1994). Therefore, I chose these in vitro assays to evaluate the potential toxicities of CP analogs.

CP derivatives of dideoxynucleoside: CP-AZT and CP-ddC; CP derivatives of
deoxynucleosides: CP-dUrd, CP-dIno and CP-dThd were evaluated for acute cellular toxicity (growth inhibition within 2 ~ 4 days) and delayed cytotoxicity (14 days exposure) using human derived CEM cells. ddC and AZT were used as controls. In addition, the toxicities against CEM cells of two potent antiherpes agents: 5-methoxymethyl-2'-deoxyuridine (MMdUrd) and 5-methoxymethyl-2'-deoxycytidine (MMdCyd) were also studied. The triphosphates, MMdUTP and MMdCTP were synthesized and their substrate properties for mtDNA polymerase γ were investigated. Results of these investigations are discussed in this section.

CP-dUrd and CP-dIno were used to undertake bone marrow toxicity studies. The object of these studies was to evaluate the ability of these compounds on murine bone marrow haematopoietic progenitor CFU-GM and BFU-E. Foscarnet and AZT were used as positive controls. The effects of deoxyribonucleosides (dThd, dUrd and dIno) were also investigated using this assay system. Results of these investigations on murine CFU-GM are discussed below. The work on murine haematopoietic progenitor BFU-E was discontinued because of technical difficulties (see section 3.3.3.3).

Finally, I chose CP-dUrd as a representative of CP-dN analogs to investigate the in vivo toxic profile. A single dose of CP-dUrd was given to mice. The LD₅₀ value of the compound was determined and its effect on haematological and clinical chemistry parameters were examined. The results of acute toxicity study of CP-dUrd are also discussed in this chapter.

3.2 MATERIALS AND METHODS

Drugs, chemicals, reagents, cells and animals

The CP derivatives of nucleosides were synthesized as described previously (Section 2.2.2). Antiherpes compounds MMdCyd and MMdUrd and their corresponding
triphosphates MMdCTP and MMdUTP were also synthesized in our laboratory. The purity of antiherpes compounds was analyzed by HPLC and was greater than 95%. Drugs (AZT, ddC and PFA) and dThd were purchased from commercial source (Section 2.2.2). dIno and dUrd were obtained from Rose Scientific Ltd. Alberta, Canada. The sources of media, serum and tissue culture supplies have been described previously (Section 2.2.1). Granulocyte-macrophage colony stimulating factor [GM-CSF, mouse recombinant (E. Coli), specific activity > 1.0 x 10⁷ U/mg] was purchased from Boehringer Mannheim Biochemica. Laval, Quebec, Canada. Lympholyte M (density 1.0875 ± 0.003 g/ml) was obtained from Cedar Lane Laboratories Ltd, Hornby, Ontario, Canada. Agar (granulated solidifying agent) was purchased from DIFCO, Detroit, MI, USA. The stock solution of each compound was made using PBS and concentration was determined spectrophotometrically. The stock solution was used immediately or divided into aliquots and stored frozen till use.

Coulter counter (model Z1 Coulter Electronics Ltd, Burlington, Ontario, Canada) was used for cell counts. Hematology analyzer Coulter Counter (Model S-plus IV Coulter Electronic. Inc., Hialeah, FL, USA), HEMA-TEK II slide stainer (Miles Scientific, Naperville, IL, USA) and Chemistry Analyzer Spectrum Series II (Abbott Laboratories Limited, Ontario, Canada) were used for analysis of blood and clinical chemistry. Plasma separator tubes (Microtainer, Becton Dickinson) were obtained from Source Medical, Saskatoon, Saskatchewan, Canada.

CEM cells, a human T lymphoblast cell line was used for acute and delayed cytotoxicity studies. The stock culture of CEM cells was kindly provided by Dr. Robert Warrington, Department of Biochemistry, University of Saskatchewan. The cells were grown in RPMI 1640 medium containing 5% FBS for several generations till the doubling time was fairly constant. For CEM cells, the average doubling time was in the range of 22–26 hours. The viability of the cells was checked by the trypan blue-exclusion method.
C3H female mice (7-8 weeks old, ≈ 20-24 g) were used for bone marrow toxicity studies. The mice were obtained from Charles River Laboratories, Massachusetts, USA. For the toxicological studies, male white Swiss CD-1 mice weighing 17 to 20 g were obtained from the Animal Resources Center, University of Saskatchewan. Mice were housed in groups of four in clear autoclavable polycarbonate cages in the animal facility maintained at 21 ± 1°C and lighting was controlled to provide 12 hours light and 12 hours darkness per day. Commercially prepared mouse pellets (Prolab, PMI Feeds Inc., St. Louis, MO, USA) and tap water in plastic bottles fitted with stainless-steel sipper tubes were available ad-libitum at all times.

3.3 EXPERIMENTAL PROTOCOLS

3.3.1 Acute and delayed cytotoxicity assay

CEM cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum. To determine the effect of nucleoside analogs on cell growth, the cells (2 x 10^5 cells/ml) were incubated with various concentrations of the test compound and ddC (positive control) at 37°C in 5% CO₂ humidified atmosphere. Every 2 days, the cell number was determined by the Coulter counter. The cells were then centrifuged at 400 g for 5 minutes. The cells (2 x 10^5 cells/ml) were resuspended in the medium containing drug. The cycle was repeated and incubation was continued for 14 days. Doubling time was calculated using the formula: number of population doubling n = [log_{10} (N/N_0)] x 3.33; doubling time = (T_N - T_{N_0})/n, where N_0 is the cell number at the beginning of the period; N is the cell number at the end of the period; T_{N_0} is the time at the beginning of the period; T_N is the time when cells were counted.
3.3.2 Enzyme studies

DNA polymerase γ was purified from the crude mitochondrial fraction of myelogenous leukemia cells by the procedure described previously (Kukhanova et al., 1996). The purification procedure included the following chromatography steps: DE-52 cellulose, phosphocellulose, heparin, and finally single-stranded DNA cellulose. The ability to use poly (rA).oligo(dT) as template and primer, and high sensitivity to ddCTP indicated that the DNA polymerase activity was that of polymerase γ.

Incorporation of MMdUTP and MMdCTP into the 3'-end of primer annealed with DNA polymerase γ was carried out using a reaction mixture containing 20 mM Tris-HCl buffer (pH 8.0), 6 mM MgCl₂, 1 mM dithiothreitol, 80 mM KCl, 0.5 mM EDTA and 0.1 μM 5'-32P-labelled primer-template complex. Various concentrations of dNTP and other analogs were added, and reaction was started by the addition of 2 units of enzyme. The reaction mixture was incubated for 20 min at 37°C and products of primer extension were subjected to 15% polyacrylamide-8 M urea gel electrophoresis. One unit of enzyme activity is defined as the amount of enzyme needed to incorporate 1 nmol of dTMP/h into the acid-insoluble fraction at 37°C. Enzyme activity studies were carried out by Dr. Kukhanova in Professor Cheng’s laboratory at the Department of Pharmacology, Yale University, School of Medicine, New Haven, Connecticut, USA.

3.3.3 Bone marrow toxicity studies: Assay for granulocytic-monocytic progenitors

CFU-GM (colony forming units of granulocyte-macrophage)

3.3.3.1 Isolation of murine mononuclear cells (MNCs)

Mononuclear cells were isolated by using the modified procedure described previously (Gallicchio et al. 1989). Mice were sacrificed by cervical dislocation and femurs
were removed under sterile conditions. The marrow cells were flushed from the femur, under sterile conditions, with DMEM using a 21-gauge needle into a bottle. Single cell bone marrow suspension was prepared by repeated passage of the cell suspension through a 25-gauge needle. The cell pellet was isolated by slow speed centrifugation (400g) for 10 min and resuspended in cell culture medium. The cell number was determined using Coulter Counter. The cell density (approximately $1 \times 10^7$ cell/ml) was adjusted with DMEM and the cells were gently layered over an equal volume of lympholyte M. The interface of low density mononuclear cells was collected by centrifugation at 800g for 20 min at 22°C. The residual lympholyte M from mononuclear cell suspension was removed by suspending the cells in culture medium and slow speed centrifugation at 22°C. The process of resuspension and centrifugation cycle was repeated three times. The cell number was determined using Coulter Counter. Cell viability determined by trypan blue exclusion was greater than 95%.

3.3.3.2 Assay methods for murine hematopoietic progenitors CFU-GM

The assays for cloning CFU-GM were carried out using the modified bilayer soft-agar procedure (Sommadossi et al. 1987a and Warrington et al. 1985). Preliminary studies were carried out to determine the amount of the growth factor GM-CSF for these experiments. Based on the literature, 20 and 50 units of GM-CSF were used in initial experiments. To obtain approximately 100 colonies/dish, 50 units were found to be optimum in bone marrow assays. Three ml agar (0.5%) in cell culture media containing 5% FBS was poured in 60 mm plastic Petri dishes and allowed to solidify at 22°C (bottom layer). The top layer (2 ml) containing $5 \times 10^4$ cells ($2.5 \times 10^4$ mononuclear cells /ml), GM-CSF 50 units, agar (0.3%) in culture media supplemented with 20% FBS and drugs (HIV inhibitor) or deoxyribonucleosides at the appropriate concentrations was gently poured over the solidified bottom layer. The plates were incubated in a humidified 5% CO₂-air mixture
at 37°C for 14 days. In each experiment, cell controls (without drugs) were run concurrently. Colonies consisting of 50 or more cells were scored as CFU-GM using an inverted microscope (Nikon, TMS). Foscarnet (PFA) and AZT were used as positive controls. Deoxyribonucleosides (dUrd, dThd and dIno) were used as negative controls. The assays were carried out in triplicate and repeated at least twice. Dose response curves were plotted and the concentrations required to inhibit 50% (IC₅₀) and 90% (IC₉₀) colonies were calculated.

3.3.3.3 Assay for murine hematopoietic progenitors BFU-E (Burst-forming unit of erythroid)

The assays for BFU-E were also performed. Although several methods were tried, all attempts for the generation of erythrocyte colonies failed. The most likely reason for repeated failures of BFU-E assay was the poor quality of recombinant mouse erythropoietin supplied by Boehringer Mannheim Biochemica. Further attempts on BFU-E assays were terminated.

3.3.4 Acute toxicity study of CP-dUrd in mice

3.3.4.1 Treatment of animals

Groups of 12 randomly selected male mice weighing 17 to 20g were given a single intraperitoneal (i.p.) injection of CP-dUrd at dosages of 100 mg/kg and 1,000 mg/kg. CP-dUrd was dissolved in PBS (0.1M, pH 7.2). The drug concentration was adjusted so that the volume injected into any animal was not more than 0.2 ml. Control mice were treated in an identical manner (i.p.) with an equal volume of PBS. Mice were sacrificed at three different intervals following administration of drug and monitored for gross pathological, hematological and clinical chemistry parameters. Four mice from the 100 mg/kg group and
control group and two mice from 1000 mg/kg group were sacrificed on days 2, 5 and 7 post CP-dUrd administration. All mice were weighed at the beginning of the experiment and at the time of sacrifice.

3.3.4.2 Hematology and clinical chemistry

Mice were euthanized by CO$_2$ inhalation. The abdominal muscle and the rib cage was quickly incised to expose the beating heart. The blood sample was collected using a tuberculin syringe fitted with a 3/8 inch, 25 G needle. The dead space of the syringe was filled with undiluted heparin (1:1,000) to prevent clotting. The right ventricle was penetrated and blood (0.7 to 1.0 ml) was slowly withdrawn. Blood samples were immediately transferred into plasma separator tubes coated with heparin. Hematological profiles were obtained using a hematology analyzer Coulter Counter. Total white blood cell (WBC) counts, red blood cell (RBC) counts, hemoglobin concentrations (Hgb), hematocrits (HCT), mean corpuscular volumes (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentrations (MCHC) and red cell distribution width (RDW) were determined. Duplicate blood smears were made from fresh whole blood and stained on a HEMA-TEK II slide stainer. The HEMA-TEK II utilizes a modified Wright-Giemsa Romanowsky stain. The WBC differential and RBC morphology was checked by microscope. The plasma protein was measured using a refractometer. Each animal was then carefully examined for the presence of gross visible pathological lesions. The following tissues were removed from each mouse and placed in 10% phosphate-buffered formalin for fixation: heart, lung, liver, kidney, spleen, gut (stomach and ileum), skeletal muscle and brain.

Since CP-dUrd is a nucleoside, it was reasoned that any manifestation of toxicity would likely affect the organs where this compound is metabolized or excreted. Therefore,
clinical parameters were chosen which would reflect toxic changes in the liver and the kidney. Serum was obtained by centrifugation of the whole blood. The parameters studied were serum alkaline phosphatase (ALK.Phos.), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum γ-glutamyltransferase (γ-GT) and glucose. Determination of clinical chemistry parameters was carried out by using Chemistry Analyzer Spectrum Series. Enzyme activities are expressed as U/L. Glucose values are given in mmol/L.

3.3.4.3 Statistical analysis of the data

The data of hematological and clinical chemistry parameters was analyzed using ANOVA. The statistics were performed on the raw data, comparing control versus experimental groups with $p < 0.05$ used as the level of significance.

3.4 RESULTS

3.4.1 Acute and delayed toxicity studies on HIV inhibitors and the antitherpes agents 5-methoxymethyl-2'-deoxycytidine and 5-methoxymethyl-2'-deoxyuridine

Generally, if a compound inhibits cell growth within the 4 days of exposure to the compound, it is considered to be due to acute toxicity. However, if a compound starts to retard the cell growth after 6 days of exposure and this effect is magnified on longer exposure to the test compound, it is considered that this toxic manifestation is due to delayed cytotoxicity of the drug.

3.4.1.1 Acute and delayed cytotoxicity of AZT, ddC, CP-AZT and CP-ddC in CEM cells

Previous studies have shown that among the ddNs tested, ddC produced potent delayed cytotoxic effects, while AZT didn’t (Chen et al., 1991). Therefore, ddC was used as
a positive control. AZT was included in toxicity assays for comparison purpose. ddC was tested at concentrations of 0.125, 0.25, 0.5, 1.0 and 5 μM and AZT was tested at concentrations of 2, 4, 8, 16 and 32 μM (only the 32 μM results are shown). The results of cytotoxicity studies in CEM cells using ddC and AZT are shown in Figures 3.1 and 3.2. The cells not exposed to drugs had a relatively stable doubling time (range 22~26 hrs) through the 14 days incubation period. In preliminary experiments, increase in doubling time of CEM cells on exposure to ddC was found to be dependent on concentration of the drug in the medium. Exposure to high concentrations of ddC (1 μM or higher) resulted in significant increase in doubling time within 4 days of exposure to the drug (acute toxicity). On the other hand, a concentration as low as 0.125 μM of ddC caused the doubling time of cells to increase after 10 days. At higher concentrations (0.25 μM and 0.5 μM) a very marked increase in doubling time of cells was observed from 6 days onward with ddC treatment. In contrast, AZT did not exhibit acute or delayed cytotoxicity after 14 days exposure at any of the concentrations tested. These results are in agreement with studies published previously (Chen et al., 1991).

CP-AZT and CP-ddC were tested at concentrations of 4, 16, 64, and 256 μM. Results of cytotoxicity studies of CP-AZT and CP-ddC are shown in Figure 3.2. CP-AZT did not exhibit acute or delayed cytotoxicity after 14 days exposure at any of the concentrations tested (only the 256 μM results are shown). In contrast, CP-ddC caused the doubling time of cells to increase on exposure at 4 μM after 6 days. The evidence of acute toxicity was indicated at this dose after 4 days exposure to the drug. At higher concentrations, a dose-dependent cytotoxicity (acute as well as delayed) was observed on treatment with CP-ddC. ddC (positive control) at concentration as low as 0.125 μM caused the doubling time of cells to increase after 10 days.
Figure 3.1 Delayed cytotoxicity of ddC in CEM cells. CEM cells (2 x 10^5 cells/ml) were incubated with various concentrations of ddC. The cell number was determined every 2 days. The doubling time was calculated.

Cell control (■■); ddC: 0.125 µM (▲▲), 0.25 µM (O-O), 0.5 µM (□□), 1.0 µM (●●) and 5.0 µM (◆◆).
Figure 3.2 Delayed cytotoxicity of CP-AZT, AZT, CP-ddC and ddC in CEM cells. CEM cells (2 x 10^5 cells/ml) were incubated with various concentrations of compounds. The cell number was determined every 2 days. The doubling time was calculated.
Panel A: Cell control (■■■); CP-AZT 256 μM (▼▼), and AZT 32 μM (O-O).
Panel B: Cell control (■■■); CP-ddC: 4 μM (□□), 16μM (O-O), 64μM (××) and 256 μM (▼▼); ddC: 0.125 μM (●●) and 0.25 μM (◆◆).
3.4.1.2 Acute and delayed cytotoxicity of CP-dThd, CP-dUrd, CP-dIno, MMdUrd and MMdCyd in CEM cells

CP-dUrd and CP-dIno were tested at concentrations of 100, 200, 400 and 800 μM (only the results at 800 μM are shown). CP-dThd was tested at concentration of 100 μM. These compounds did not affect cell growth during 14 days exposure at any of the concentrations tested. The results of cytotoxicity are shown in Figure 3.3. The viability of cells exposed to different concentration of CP-dThd, CP-dUrd and CP-dIno was the same as untreated cells. These results indicate that CP analogs of deoxyribonucleosides did not exhibit acute or delayed cytotoxicity.

MMdCyd and MMdUrd were tested at concentrations of 125, 250, 500 and 1000 μM and the results of cytotoxicity studies are shown in Figure 3.4 (only the 1000 μM results are shown). The two compounds did not exhibit any acute or delayed cytotoxicity after 14 days at concentrations tested. ddC at concentrations of 0.125 μM and 0.25 μM was included as positive control.

3.4.1.3 Comparison of the incorporation of dCTP, MMdCTP, dTTP and MMdUTP into template-primer complex by DNA polymerase γ

The results of MMdCTP and MMdUTP incorporation into primer-template complex by DNA polymerase γ are shown in Figure 3.5. As can be seen, DNA polymerase γ was able to utilize both analogs as substrates and incorporate them into DNA instead of dCTP and dTTP, respectively. The efficiency of incorporation and elongation of analogs were close to that of natural dNTP.
Figure 3.3 Delayed cytotoxicity of CP-dUrd, CP-dIno, CP-dThd and ddC in CEM cells. CEM cells (2 x 10^5 cells/ml) were incubated with various concentrations of tested compounds. The cell number was determined every 2 days. The doubling time was calculated.

Cell control (■-■), CP-dUrd 800 μM (□-□), CP-dIno 800 μM (△-△), CP-dThd 100 μM (○-○), ddC 0.125 μM (●-●) and ddC 0.25 μM (◆-◆).
Figure 3.4 Delayed cytotoxicity of MMdCyd, MMdUrd and ddC in CEM cells. CEM cells (2 x 10^5 cells/ml) were incubated with various concentration of compounds. The cell number was determined every 2 days. The doubling time was calculated. Cell control (■■); MMdCyd 1000 μM (▲▲); MMdUrd 1000 μM (○○); ddC 0.125 μM (●●) and ddC 0.25 μM (◆◆).
Figure 3.5 Comparison of the incorporation of analogues into template-primer complex by DNA polymerase γ
(a) Comparison of the incorporation of dCTP and MMdCTP into the template-primer complex. The sequence of the primer (22-mer) was: 5'-GTAATACGACGCTATGAACT-3'. The primer extension sequence is shown on the right. Lane 1, position of 5'-32P-labelled 22-mer primer; lane 2, primer extension by dCTP (1 μM); lanes 3 and 4, the same as lane 2, but in the presence of dGTP (1 μM) or dGTP (1 μM) and dATP (1 μM), respectively; lanes 5-7, primer extension by MMdCTP at concentrations of 0.25 μM, 1 μM and 5 μM, respectively; lanes 8 and 9, primer extension by MMdCTP (1 μM) in the presence of dGTP (2 μM) or dGTP (1 μM) and dATP (1 μM), respectively. (b) Comparison of the incorporation of dTTP and MMdUTP into template-primer complex. The sequence of the primer (17-mer) was: 5'-CCGTCATTTCCCTTAA-3'. The primer extension sequence is shown on the right. Lane 1, position of 5'-32P-labelled 17-mer primer; lane 2, primer extension by dTTP (1 μM); lanes 3 and 4, the same as lane 2, but in the presence of dATP (1 μM) or dATP (1 μM) and dCTP (1 μM), respectively; lanes 5-7, primer extension by MMdUTP at concentrations of 0.25 μM, 1 μM and 5 μM, respectively; lanes 8 and 9, primer extension by MMdUTP (1 μM) in the presence of dATP (2 μM) or dATP (1 μM) and dCTP (1 μM), respectively.
3.4.2 Effect of antiviral drugs (AZT, PFA), CP-dUrd and CP-dIno and deoxyribonucleosides (dThd, dUrd and dIno) on murine bone haematopoietic progenitor stem cells: CFU-GM.

Initially, the relative ability of antiviral drugs, AZT and PFA and deoxythymidine (dThd) to influence murine granulocyte-macrophage (CFU-GM) colony formation was investigated. Results are shown in Table 3.1 and Figures 3.6 – 3.10. Colonies of CFU-GM were reduced significantly on exposure to AZT. The concentrations of AZT, PFA and dThd required to inhibit CFU-GM colonies by 50% (IC₅₀) were 2.5 µM, 310 µM and 340 µM, respectively (Table 3.1). AZT was able to prevent colony formation almost completely (>90%) at 10 µM. Under similar assay conditions, concentrations of PFA and dThd required to inhibit CFU-GM colony by 90% (IC₉₀) was > 1 mM (highest concentration tested) and 900 µM, respectively. These results are in general agreement with values reported for AZT utilizing either normal human or murine marrow cells (Abraham, 1989; Blau et al., 1989; Dainiak et al., 1988; Faraj et al., 1994; Gallicchio et al., 1989; 1991; 1993a; Sommadossi et al., 1987; 1993; Sommadossi and Carlisle, 1987a).

Following completion of dose-escalation trials with approved anti-HIV drugs, studies on the inhibitory effects of CP-dUrd and CP-dIno on the haematopoietic progenitors CFU-GM were undertaken. The influence of dUrd and dIno (negative controls) on CFU-GM colony formation was also investigated simultaneously. Results of dose-escalation trials of CP-dUrd, CP-dIno, dUrd and dIno are shown in Figures 3.11 – 3.17. CP-dUrd and CP-dIno were moderate inhibitors of murine haematopoietic progenitors CFU-GM with IC₅₀ values of 250 µM and 160 µM, respectively. Deoxyinosine was devoid of toxicity towards CFU-GM up to 1 mM (highest concentration tested), whereas the IC₅₀ for dUrd was around 1 mM. The concentration required to inhibit CFU-GM colony formation by 90% (IC₉₀) for
Table 3.1 Relative potency of AZT, PFA, CP-dUrd, CP-dIno and deoxyribonucleosides on the murine haematopoietic progenitors CFU-GM

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>IC&lt;sub&gt;90&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>PFA</td>
<td>310</td>
<td>&gt; 1000&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>CP-dUrd</td>
<td>250</td>
<td>900</td>
</tr>
<tr>
<td>CP-dIno</td>
<td>160</td>
<td>420</td>
</tr>
<tr>
<td>Deoxyribonucleosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dIno</td>
<td>&gt; 1000&lt;sup&gt;4&lt;/sup&gt;</td>
<td>&gt; 1000&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>dThd</td>
<td>340</td>
<td>900</td>
</tr>
<tr>
<td>dUrd</td>
<td>1000</td>
<td>&gt; 1000&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. Assays for cloning CFU-GM were carried out using the modified bilayer soft-agar methodology (Sommadossi et al., 1987b and Warrington et al., 1985)
2. IC<sub>50</sub>, concentration required to reduce CFU-GM colony by 50%.
3. IC<sub>90</sub>, concentration required to reduce CFU-GM colony by 90%.
4. Highest concentration tested.
3.6 Photomicrograph of CFU-GM (cell control)
3.7 Photomicrograph of CFU-GM exposure to 0.1 μM AZT (non-toxic, upper photo) and 100 μM AZT (toxic, lower photo)
3.8 Photomicrograph of CFU-GM exposure to 10 μM PFA (non-toxic, upper photo) and 1000 μM PFA (toxic, lower photo)
3.9 Photomicrograph of CFU-GM exposure to 10 μM dThd (non-toxic, upper photo) and 1000 μM dThd (toxic, lower photo)
Figure 3.10. Dose-response effects of HIV inhibitors and deoxynucleosides on murine hematopoietic progenitors CFU-GM. Approximately 100 colonies/5 x 10^4 cells plated in culture media with GM-CSF (50 units/plate). AZT (● - ●), PFA (■ - ■) and dThd (◆ - ◆).
3.11 Photomicrograph of CFU-GM (cell control)
3.12 Photomicrograph of CFU-GM exposure to 10 µM CP-dUrd (non-toxic, upper photo) and 1000 µM CP-dUrd (toxic, lower photo)
3.13 Photomicrograph of CFU-GM exposure to 10 μM CP-dIno (non-toxic, upper photo) and 1000 μM CP-dIno (toxic, lower photo)
3.14 Photomicrograph of CFU-GM (cell control)
3.15 Photomicrograph of CFU-GM exposure to 10 µM dUrd (non-toxic, upper photo) and 1000 µM dUrd (small colony, lower photo)
Photomicrograph of CFU-GM exposure to 10 μM dIno (non-toxic, upper photo) and 1000 μM dIno (small colony, lower photo)
Figure 3.17 A and B. Dose-response effects of HIV inhibitors and deoxynucleosides on murine hematopoietic progenitors CFU-GM. Approximately 100 colonies/5 x 10^4 cells plated in culture media with GM-CSF (50 units/plate). Panel A: CP-dUrd (●-●) and CP-dlno (■-■) and Panel B: dUrd (●-●), dlno (■-■) and dThd (◆-◆).
CP-dUrd and CP-dIno was 900 µM and 420 µM, respectively. It is interesting to note that IC₉₀ values for CP-dUrd and deoxythymidine were similar (900 µM). Results are summarized in Table 3.1.

3.4.3 Acute Toxicity Studies of CP-dUrd in vivo

3.4.3.1 Acute toxicity

No mortality or abnormal clinical signs were observed in any of the control animals or in the treated animals after i.p. injection of 100 mg/kg CP-dUrd. Administration of CP-dUrd at 1000 mg/kg resulted in 50% mortality within 30-60 minutes after administration of CP-dUrd. Therefore, the LD₅₀ of CP-dUrd is about 1000 mg/kg. The other mice in the high dose group were morbid (relatively inactive) for 24 to 36 hour post administration and then recovered. Animals in the control group and low dose group appeared completely healthy throughout the experiment. The appetite and food consumption remained normal and no difference in the average weight was observed between the control and the treated group.

Gross examination of the tissues from the acute toxicity studies of mice given CP-dUrd at 100 or 1000 mg/kg did not show any drug-related lesions. Histopathological examination of tissues was not carried out.

3.4.3.2 Hematological and clinical chemistry

Results of hematological and clinical chemistry parameters are summarized in Tables 3.2 and 3.3. The overall results did not reveal any statistically significant differences between treated and control animal for the hematological and clinical chemistry parameters examined.
Table 3.2 Hematological parameters of control mice\(^1\) and mice given 100 mg/kg and 1000 mg/kg CP-dUrd\(^2\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control(^3)</th>
<th>100 mg/kg</th>
<th>1000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10(^9)/L)</td>
<td>8.70 ± 2.47(^4)</td>
<td>8.33 ± 3.03(^1)</td>
<td>9.67 ± 3.85(^1)</td>
</tr>
<tr>
<td>RBC (10(^12)/L)</td>
<td>6.65 ± 0.49(^1)</td>
<td>6.29 ± 0.86(^1)</td>
<td>5.66 ± 0.86(^1)</td>
</tr>
<tr>
<td>Hgb (g/L)</td>
<td>125 ± 9.73(^1)</td>
<td>119 ± 17.58(^1)</td>
<td>123 ± 15.70(^1)</td>
</tr>
<tr>
<td>HCT (L/L)</td>
<td>0.388 ± 0.03(^1)</td>
<td>0.357 ± 0.05(^1)</td>
<td>0.371 ± 0.04(^1)</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>58.4 ± 1.91(^1)</td>
<td>56.2 ± 2.56(^1)</td>
<td>55.7 ± 2.44(^1)</td>
</tr>
<tr>
<td>MCH (Pg)</td>
<td>18.87 ± 0.51(^1)</td>
<td>18.71 ± 1.0</td>
<td>18.47 ± 0.62(^1)</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>323 ± 8.73(^1)</td>
<td>333 ± 7.94(^1)</td>
<td>332 ± 7.21(^1)</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>11.9 ± 0.92(^1)</td>
<td>11.9 ± 0.95(^1)</td>
<td>12 ± 0.92(^1)</td>
</tr>
<tr>
<td>Proteins (g/L)</td>
<td>55.5 ± 3.87(^1)</td>
<td>48.4 ± 5.03(^1)</td>
<td>51.7 ± 8.59(^1)</td>
</tr>
</tbody>
</table>

1. Male White Swiss CD-1 mice, 17 – 20g.
2. CP-dUrd was administered by i.p. route.
3. Number of mice of each group: Control: n = 12; CP-dUrd (100 mg/kg): n = 12; CP-dUrd (1000 mg/kg): n = 7.
4. The values shown are mean ± SD.
Table 3.3 Clinical chemistry of control mice\(^1\) and mice given 100 mg/kg and 1000 mg/kg CP-dUrd\(^2\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control(^3)</th>
<th>100 mg/kg</th>
<th>1000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>15.9 ± 3.45</td>
<td>13.0 ± 2.70</td>
<td>14.8 ± 3.61</td>
</tr>
<tr>
<td>ALK PHOS (U/L)</td>
<td>165 ± 26.22</td>
<td>181 ± 48.92</td>
<td>158 ± 39.56</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>87 ± 35.92</td>
<td>115 ± 149.56</td>
<td>101 ± 45.0</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>34.1 ± 6.92</td>
<td>45.8 ± 63.01</td>
<td>31.6 ± 4.96</td>
</tr>
<tr>
<td>(\gamma)-GT (U/L)</td>
<td>0.17 ± 0.58</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

1. Male White Swiss CD-1 mice, 17 – 20g.
2. CP-dUrd was administered by i.p. route.
3. Number of mice of each group: Control: n = 12; CP-dUrd (100 mg/kg): n = 12; CP-dUrd (1000 mg/kg): n = 7.
4. The values shown are mean ± SD.
3.5 DISCUSSION

Antiviral nucleoside analogs (ANA), some of which are used to treat human immunodeficiency virus (e.g. dideoxynucleosides AZT, ddC, and ddl) and hepatitis B virus infection [e.g. FIAU (1-(2-fluoro-2'-deoxy-β-D-arabinofuranosyl)-5-iodouracil)], have been shown to exhibit unique multi-organ toxic effects on long-term therapy. Manifestations of toxicity suggest defective mtDNA due to inhibition of mtDNA polymerase γ.

Manifestations of ANA toxicities on long-term therapy in selected tissues is due to the combined effect of four principal factors: (i) the subcellular availability and abundance of the ANA in target tissue; (ii) the ability of cellular thymidine kinase/deoxycytidine kinase to phosphorylate ANA to monophosphate intracellularly and other cellular enzymes to convert ANA monophosphate to ANA triphosphate (via ANA diphosphate); (iii) the ability of ANA triphosphate to inhibit DNA polymerase γ either by serving as a competitive alternate substrate and incorporation into mtDNA or by terminating the nascent mtDNA chain (chain termination); and (iv) the metabolic requirements in the target tissues for oxidative phosphorylation. According to DNA polymerase γ hypothesis, the cellular enzymes (dThd kinase and dCyd kinase) play a critical role in mtDNA toxicity because these enzymes phosphorylate ANA to the monophosphate (Chen and Cheng, 1989; Lewis et al., 1992; Lewis and Dalakas, 1995; Parker and Cheng, 1994; Tsai et al., 1994; Wallace, 1992).

High concentrations of a drug like ddC produce acute toxicity in CEM cells and this may be due to incorporation of ddC into nuclear DNA. On the other hand, low concentrations of ddC resulted in depletion of mtDNA and induced delayed cytotoxicity in cell cultures (Chen and Cheng, 1989, 1992; Chen et al., 1991). This depletion of mtDNA may be responsible for the delayed toxicity of ddC observed in humans (Chen and Cheng, 1992).
The *in vitro* assay of delayed cytotoxicity by monitoring the doubling time has been shown to be useful for studying the delayed clinical toxicity of ddC and other ddNs (Chen *et al.*, 1991). It has been shown that CEM four day cell growth is not affected if only mtDNA synthesis is inhibited (Chen and Cheng 1989). The cell growth was retarded only after prolonged exposure to the drug. The delayed onset of the mitochondrial toxicity might be due to the presence of multiple copies of mtDNA in a cell (Robin and Wong, 1988). The ratio of C-IC$_{50}$/mt-IC$_{50}$ (C-IC$_{50}$: the concentration that inhibits cell growth by 50%; mt-IC$_{50}$: the concentration that inhibits mtDNA synthesis by 50%) was used as an indicator of the selective effects of compounds on mitochondrial toxicity. The larger this ratio, the more potent mitochondrial damage (and delayed cytotoxicity) relative to general cellular toxicity. The compounds with lower C-IC$_{50}$/mt-IC$_{50}$ ratio usually have limited toxicity against proliferating tissue, in contrast, the compounds with higher ratio (>>1) exhibit delayed organ toxicity upon long-term usage.

The results of delayed cytotoxicity indicated that ddC at high concentrations (5 µM) produced acute cytotoxicity (CEM four day cell growth inhibited). Under similar conditions, ddC at very low concentrations (0.125 µM) induced delayed cytotoxicity (cell growth was retarded after long exposure). In contrast, AZT did not exhibit delayed cytotoxicity in this assay system. These results are in agreement with earlier studies which indicated that ddC preferentially decreased the mtDNA content in human lymphoblastoid cell lines (Chen *et al.*, 1991; Chen and Cheng, 1989). In contrast, high concentration of AZT, well above the C-IC$_{50}$s was required to affect the mtDNA content of cells. It has been shown that in CEM cells, the ratio of C-IC$_{50}$/mt-IC$_{50}$ of ddC is 455 and that the value of AZT is 0.42 (Chen *et al.*, 1991). Therefore, our results of the delayed cytotoxicity of ddC and AZT support the mtDNA polymerase γ hypothesis.
CP-dIno, CP-dThd and CP-AZT did not exhibit delayed cytotoxicity when assayed using CEM cells. These CP analogs do not serve as substrates of gamma (γ) DNA polymerase, because cellular enzymes will not be able to phosphorylate these compounds to the triphosphate stage due to the presence of CP group at 5'-position. As a result, these compounds will not be incorporated into the mtDNA, an essential pre-requisite for permanent mtDNA damage. Further studies, however, are necessary to confirm this hypothesis using purified γ-DNA polymerase.

The fluorinated analog of uracil (FIAU) and cytosine [FIAC (1-(2-fluoro-2'-deoxy-β-D-arabinofuranosyl)-5-iodocytosine)] are potent inhibitors of HSV (Watanabe et al., 1979) and hepatitis virus (Staschke et al., 1994) replication. The clinical trials of FIAU against hepatitis B virus ended prematurely because of serious delayed toxicities (Macilwain, 1993; Parker and Cheng, 1994). The metabolic studies on FIAU using human cell lines suggest that the toxicity of FIAU is associated with its phosphorylation and incorporation into DNA (Klecker et al., 1994).

The compounds MMdCyd and MMdUrd are potent, selective antiherpes agents with low cytotoxicity (Aduma et al., 1990; Ayisi et al., 1980; 1986; Meldrum et al., 1980). My hypothesis is that MMdCyd and MMdUrd would be anabolized by viral and cellular enzymes in HSV-infected cells to their triphosphate form, which is the "active" form of the drugs responsible for antiviral activity. The lack of cytotoxicity against rapidly dividing cells indicates that MMdCyd and MMdUrd are not anabolized in uninfected cells because these nucleoside analogs most likely do not serve as substrates for cellular deoxycytidine kinase or thymidine kinase, respectively (Aduma et al., 1990; Ayisi et al., 1980). The data of present study with MMdCyd and MMUrd support this hypothesis. The results of enzyme studies showed that mtDNA polymerase γ was able to incorporate MMdCTP and MMdUTP into
DNA. The results of the incorporation studies were as expected since it has been previously shown that both MMdCTP and MMdUTP are good substrates for *Escherichia coli*, HSV-1 and human DNA polymerase α (Aduma *et al.*, 1991, 1992). On the basis of these results it would be expected that both MMdCyd and MMdUrd would exhibit delayed cytotoxicity. As shown in figure 3.5, this is not the case. The most likely reason for these observations is that in uninfected cells neither MMdCyd nor MMUrd is converted to the triphosphate form in significant amounts. As a result, little or no MMdCTP or MMdUTP builds up in the mitochondria and thus delayed toxicity is not observed.

In summary, the subtle differences in nucleoside structure obviously have a profound influence upon the anabolism, as well as pharmacological effects of the compounds.

The *in vitro* studies using human bone marrow cells also showed AZT has a direct, dose-dependent inhibiting effect on myeloid and erythroid progenitor cells. The IC$_{50}$ values for human CFU-GM (ranged from 1 to 50 μM) and BFU-E (0.005 μM) were reported for AZT (Abraham *et al.*, 1989; Blau *et al.*, 1989; Dainiak et al, 1988; Faraj *et al.*, 1994; Gallicchio *et al.*, 1991; 1993a; Sommadossi *et al.*, 1987a; 1993; Sommadossi and Carlisle, 1987b). These values correlate with the drug plasma levels (peak plasma concentration 4 ~ 6 μM in patients) at which cytotoxic effects are observed on human myeloid cells *in vivo* (Yarchoan *et al.*, 1986a).

The *in vitro* myelosuppressive effects on human bone marrow cells for ddC, ddI, d4T and fluorothymidine (FdThd) were also studied. FdThd was the most toxic compound while ddI and d4T were much less toxic than FdThd and AZT. The cytotoxicity of ddC was between the groups of FdThd, AZT and ddI, d4T (Faraj *et al.*, 1994; Gallicchio *et al.*, 1993a; Sommadossi, 1993). These findings are consistent with
clinical observations in that FdThd produced severe hematotoxicities while ddI and ddC had no substantial myelosuppressive effects in patients. Although ddC was toxic to bone marrow progenitor cells, the concentrations necessary for toxicity were much higher than the levels that are clinically achievable (Sommadossi, 1993). This is consistent with the findings that early clinical trials of ddC at higher doses led to the development of hematotoxic side effects (Yarchoan et al., 1988b). These observations also demonstrated that in vitro clonogenic assays are important prognostic tests for myelotoxicity in patients.

In the bone marrow toxicity studies, AZT was found to be toxic to the murine marrow progenitor cells. AZT in the range of 2.5 μM to 10 μM inhibited the colony formation of CFU-GM. These findings are consistent with published results (Abraham, 1989; Gallicchio et al., 1989; Sommadossi et al., 1987a). AZT inhibit the colony formation of the progenitor cells of granulocyte-macrophages (CFU-GM), erythrocyte (BFU-E: burst-forming unit of erythroid) and megakaryocyte (CFU-Meg) in vitro (Abraham, 1989; Gallicchio et al., 1989; Sommadossi et al., 1987a). The in vivo studies in mice have also shown that AZT is toxic to the hematopoietic progenitor cells of granulocyte-macrophages, erythrocytes and megakaryocytes of the bone marrow and spleen (Gallicchio et al., 1993a). In contrast to AZT, CP-dNs only showed moderate in vitro toxicity to the bone marrow progenitor cells. CP-dUrd and CP-dIno were 100-fold and 64-fold less toxic to murine CFU-GM compared to that of AZT. The concentration required to produce marrow toxicity of CP-dNs (IC₅₀: 160 ~ 250 μM) were much higher than the concentration required to inhibit HIV replication (ED₅₀: 0.23 μM by RT assay and 22 μM by FIA assay). These results indicated that CP-dUrd and CP-dIno have less potential to produce bone marrow toxicity compared to AZT.
AZT induced bone marrow toxicity is the result of several mechanisms, including inhibition of nuclear DNA synthesis following incorporation of AZT-TP into DNA of human bone marrow cells (Sommadossi et al., 1989); depletion of thymidine triphosphate and deoxycytidine triphosphate pools (Balzarini et al., 1987a; Frick et al., 1988; Furman et al., 1986; Hao et al., 1988) and the metabolism of AZT to 3'-amino-3'-deoxythymidine (AMT), which is three to seven times more toxic than AZT to bone marrow cells (Cretton et al., 1991a; 1991b; Faraj et al., 1994). The mechanisms of bone marrow toxicity of CP analogs were not investigated in this project. However, as discussed previously, cellular enzymes would not be able to phosphorylate CP analogs and as a result these compounds would not be incorporated into cellular DNA or inhibit the nuclear DNA synthesis. It is also unlikely, that these compounds would disturb the deoxynucleotide (dNTP) pools of the cells.

Boyum and colleagues reported that dThd at high concentrations was inhibitory to some mammalian cell lines (Boyum et al., 1988). It was reported that dThd as high as 100 μM had no inhibitory effects on human CFU-GM, however, 300 μM dThd may reduce the mice CFU-GM formation by 35% (Dainiak et al., 1988). In the assay system of murine bone marrow toxicity studies, the IC₅₀ for dThd was 340 μM; whereas dIno and dUrd were relatively non-toxic. The findings on dThd toxicity are in agreement with published results. The cytotoxicity induced by dThd may be purely due to the large load of dThd to the cells as the human plasma concentration of dThd is very low [0.1-0.8 μM, (Boyum et al., 1988)]. Thymidine is phosphorylated intracellularly to dTTP. The high concentration of dTTP would: (1) inhibit ribonucleoside diphosphate reductase, which is responsible for the conversion of CDP to dCDP and UDP to dUDP; (2) inhibit dCyd kinase, which is responsible for the phosphorylation of dCyd; and (3) inhibit dCMP
deaminase, which is responsible for the conversion of dCMP to dUMP. Therefore, high concentration of dThd most likely inhibit the formation of dCTP, thereby causing deficiency of one of the DNA precursors, which finally leads to a failure of cell division (Boyum et al. 1988; Smith et al., 1983). The marginal toxicity of dUrd to murine progenitor CFU-GM cells may be due to the large load of the dUrd or possibly due to disturbance of dNTP pools because of increased amount of formation of dTMP by the action of thymidylate synthase. Thymidine monophosphate can be further phosphorylated to dTTP which would inhibit the formation of dCyd and cause the toxicity to bone marrow cells by mechanisms discussed above.

In conclusion, the in vitro assays using murine bone marrow cells to evaluate the hematopoietic toxicities of CP analogs were chosen for the following reasons: (i) In vitro assay is an adequate model for investigating effects of antiviral compounds on bone marrow function; (ii) In vitro studies are rapid and economical compared to in vivo studies; and (iii) Murine bone marrow cells are readily available compared to the human bone marrow cells in laboratories. The data generated using AZT (control drug) indicates that our assay system was working well. These studies also indicate a low potential for bone marrow toxicity by CP-dUrd and CP-dIno. Further research on CP analogs for bone marrow toxicities should be carried out in mice and using human bone marrow cells in culture. The potential for bone marrow toxicity of CP-AZT should also be investigated.

The oral LD$_{50}$ values for AZT, ddC, ddI and d4T were > 3000 mg/kg, > 4000 mg/kg, > 3000 mg/kg and > 2000 mg/kg, respectively (Ayers, 1988; Lindstrom et al., 1990; Phillips et al., 1991). The LD$_{50}$ values of AZT were greater than 750 mg/kg in mice and rats when it was administered by i.v. route (Ayers, 1988). Preliminary toxicity studies carried out in
mice suggest that CP-dUrd has low mammalian toxicity. Considering the fact that absorption after i.p. administration is rapid and that the distribution of a drug by i.p. route is similar to that by i.v. route, it is reasonable to conclude that CP-dUrd is a fairly non-toxic compound. Further long term studies on CP-dUrd should be carried out by repeated administration of lower dosages to determine its toxicity potential.

A study conducted in mice using a 3-consecutive-day oral treatment protocol (dose 100-500 mg/kg) has shown that with the exception of ddI, all other ddNs (AZT, d4T, ddA and ddC) induced bone marrow toxicity at lower doses (Phillips et al., 1991). The bone marrow toxicity of AZT was manifested as erythrocytopenia, leukopenia and thrombocytopenia in subacute and subchronic toxicological studies carried out in mice, dogs and monkeys (Ayers, 1988; Mansuri et al., 1990). The gross pathological examination of tissues, and hematological and clinical chemistry parameters did not reveal any drug-induced abnormalities after CP-dUrd administrated. The histopathological examination of tissues and bone marrow smears should be carried out when technical support and expertise is available, because these studies may reveal changes (if any) induced by CP-dUrd in tissues and particularly to bone marrow.

In conclusion, the acute toxicity study reported in the thesis is only a preliminary toxicological study. From this study we can only obtain the very basic information about the toxicity potential of a compound. From this data, conclusions regarding safety profile of CP-dUrd cannot be made. Nonetheless, these investigations do suggest that CP-dUrd has low mammalian toxicity and this paves the way to design more extensive toxicological studies for this compound. In order to have more information on toxicological profile of CP-dUrd, a series of toxicological studies should be conducted in different species by different routes of administration of CP-dUrd. These investigations should be supplemented with histopathological examination of tissues and in particular the analysis of bone marrow samples.
4.0 PHARMACOLOGY OF
5'-CARBOXYPHOSPHONYL-DEOXYURIDINE

4.1 INTRODUCTION

5'-Carboxyphosphonyl-deoxyuridine (CP-dUrd) has good anti-HIV activity in vitro (Section 2.3.3). Compared to the other marketed RT inhibitors, CP-dUrd was found to have potentially low acute cytotoxicity and bone marrow toxicity (Section 2.3.4 and 3.4.2). In addition, CP-dUrd did not exhibit delayed toxicity and appears to have low mammalian toxicity (Section 3.4.1 and 3.4.3).

The in vivo antiviral effect is largely determined by two factors. First, the cellular uptake of a drug is critical, because HIV-infected cells can remain in the body and can be a continued source of new virions. Therefore, for optimal efficacy in vivo, it is important that the drug is able to kill free virus as well as virus-infected cells (Levy, 1998). Thus, the ability of a drug to enter and to reach appropriate concentration in these target cells is critical for its antiviral effects. Second, the pharmacokinetic properties in vivo play an important role in determining efficacy of the drug against HIV infection. The extent and rate of a drug's absorption, distribution, biotransformation and excretion determine the concentrations of the drug attained in the body/targeted organ. These factors are especially important for the clinical efficacy of an anti-HIV drug. Drug resistance is a common problem encountered with most drugs used for the treatment of AIDS (Condra et al., 1995; Deek et al., 1997; Gao et al., 1992; Goulden et al., 1996; Larde et al., 1989a; 1989b; Patick et al., 1996; Richman et al., 1992). In order to minimize the problem of drug resistance,
patients must strictly adhere to the therapeutic schedule to maintain the effective
centration of the drug in the body. In addition, whether a drug can affect HIV levels in
sanctuaries such as the brain is also very important in the treatment of AIDS. For instance,
protease inhibitors pass through the blood brain barrier poorly and this is a serious limitation
for their clinical use.

Therefore, studies on cellular uptake *in situ* using CEM cells and preliminary studies
on pharmacokinetic properties of CP-dUrd were undertaken. These studies are essential
prerequisite to determine its potential usefulness for the treatment of HIV infection. In this
chapter, results of cellular pharmacology and pharmacokinetic properties of CP-dUrd are
discussed.

4.2 MATERIALS AND METHODS

4.2.1 Cells and animals

Rapidly proliferating CEM cells were used for *in situ* metabolic studies. The source
and conditions for the growth of CEM cells have been described previously (Section 2.2.3).
The plasma for protein binding study was obtained from a 6 week old male pig (Yorkshire-
Landrace crossbred), and was kindly provided by Dr. Philip Willson of the Veterinary
Infectious Disease Organization, Saskatoon. Male White Swiss CD-1 mice were purchased
from the Animal Resources Centre of the University of Saskatchewan. Mice weighing 19 to
22 g were utilized for pharmacokinetic studies. Mice were acclimatized to the environment
for three days before experiments.

4.2.2 Drugs, chemicals, cell culture supplies and other materials

Labelled [6-³H]CP-dUrd was synthesized by Dr. Sashi Kumar and its chemical
purity (=98%) was confirmed by HPLC analysis. Specific activity of the final product was
1 mCi/m mole. The radioactivity of the dosing solution and counting efficiency was determined by external standardization using a Taurus automatic scintillation counter (ICN Biomedical, Inc. Costa Mesa, CA). The sources of media RPMI 1640, FBS and other tissue culture supplies were described previously (section 2.2.1). EcoLite scintillant was purchased from ICN Biomedical, Inc. (Costa Mesa, CA, USA). NCS-II tissue solubilizer. BCS-NA scintillant and aqueous counting scintillant (ACS) were obtained from Amersham Canadian Limited (Oakville, Ontario. Canada). Scintillation grade toluene and spectrapor II cellulose dialysis tubing with the molecular weight cutoff 12,000 to 14,000 dalton were purchased from Fisher Scientific, Nepean, Ontario, Canada. Glacial acetic acid, reagent grade benzoyl peroxide, Tris base and ammonium formate were obtained from Sigma Chemical Company, Oakville, Ontario. Canada. Filters were obtained from Hamilton Company, Reno, Nevada. C_{18} reverse-phase column was purchased from Supelco Company, Bellefonte, PA, USA. Whatman 3MM chromatography papers were obtained from Whatman Inc., Fairfield, NJ, USA. Scintillation vials and teflon tissue grinder were obtained from VWR Canlab, Ontario, Canada.

4.2.3 Cellular pharmacology

4.2.3.1 Uptake and efflux of CP-dUrd in CEM cells

CEM cells were grown using RPMI 1640 medium containing 5% FBS at 37°C in a 5% CO₂ humidified atmosphere. For drug uptake studies, 120 ml of exponential growing cells (5 x 10^5 cells/ml) were incubated with 50 μM [6-^{3}H] CP-dUrd. At indicated times, 20 ml of the medium was harvested and the cell number was determined using a Coulter counter. After centrifugation at 800g for 10 min, the supernatant and the cell pellet were stored at -20°C till analysis. For the efflux studies, 100 ml of cells (5 x 10^5 cells/ml) were
incubated with 50 μM [6-3H] CP-dUrd for 72 hours. Cell pellet was obtained by centrifugation as described above. The cells were washed three times by suspending each time in 30 ml of PBS (0.1M, pH 7.4) and centrifugation at 800g for 10 min. The cell pellet was collected, fresh medium was added to give a final concentration of 5 x 10^5 cells/ml. At periodic intervals, 20 ml of the culture mixture was removed and cells were collected after centrifugation. Cellular extracts were prepared immediately (see below). Media as well as cellular extracts were stored at -20°C till analysis.

Cellular extracts: The cell pellets were washed three times using PBS to remove drug from the surface of cells. The cell pellet was suspended in 0.3 ml of water, cells lysed by three cycles of freezing and thawing and the cellular debris was separated by centrifugation at 11,750g for 10 min. The supernatant (cellular extract) was collected for further analysis. For CEM cells, water space 0.8 μl per 10^6 cells has been reported (Mahony et al., 1992). This value was used to calculate the total concentration of CP-dUrd intracellularly.

The radioactivity of CP-dUrd was determined by counting 0.1 ml of culture medium or cell extract using 5 ml of EcoLite scintillation fluid. A series of standards were run simultaneously for calculating the concentration of the compound.

4.2.3.2 Metabolic studies

To 100 ml of the culture medium containing rapidly proliferating CEM cells (5 x 10^5 cells/ml), 50 μM of [6-3H] CP-dUrd was added. After 72 hours of incubation, the culture media was harvested and cells separated by centrifugation. For HPLC analysis, the media and cell pellet was processed as follows: (i) culture medium (20 ml) was lyophilized, residue was reconstituted in one ml of water. The cell pellet was suspended
in 0.5 ml of water and the cell extract was prepared using the procedure of freezing and thawing as described above. The samples were filtered through 0.45 μm and then 0.2 μm filters before analysis by HPLC.

The fate of CP-dUrd in CEM cells was analyzed by HPLC and using a UV detector. The running buffer for the C_{18} reverse-phase column was 0.1 M ammonium formate (pH 6.5). The flow rate was 1 ml/min. 1 ml fractions were collected for 60 min and the radioactive peaks of samples were identified by comparison with the UV profiles of standard CP-dUrd.

### 4.2.4 Protein binding studies

The method of equilibrium dialysis was used for determination of binding of CP-dUrd to plasma proteins. Radiolabeled CP-dUrd was dissolved in PBS (0.1M, pH 7.4), CP-dUrd (0.125 ml) was added to 2.5 ml of pig plasma to give the final concentration of 50, 100, 200, 400 and 600 μM. Plasma aliquots containing (2 ml) were transferred to dialysis tubings and were allowed to equilibrate with the same volume of PBS (0.1 M, pH 7.4) with slow shaking at 37°C for 36 hours. Care was taken to ensure that the height of the buffer in the test tube was equal to that of plasma in the dialysis tubing. Protein concentrations inside the dialysis tubing (compartment 1) and outside the dialysis tubing (compartment 2) were determined from the optical density at 280 nm. Post-dialysis plasma and buffer volumes were measured using a pipet. To determine the radioactivity, 0.1 ml of plasma or buffer was mixed with 0.3 ml of NCS-II tissue solubilizer and kept in 50°C water bath for 20 min. The mixture was then kept at 22°C in dark for 12 hours with slow shaking, to allow thorough digestion of the samples. 5 ml of ACS scintillant
was added to the mixture and the radioactivity was determined using Taurus automatic scintillation counter. Concentrations of CP-dUrd were determined using the external standard ratio method. All determinations were carried out in duplicate. Binding of CP-dUrd to plasma proteins was calculated using equation 1 (Boudinot and Jusko, 1984). The formula takes into consideration the fluid shift between the two compartments.

\[ D_B = (D_{Te} - D_F) \times V_{pe}/V_{pi} \]  

(Eq.1)

Where \( D_F \) and \( D_B \) are the free and protein-bound plasma drug concentrations, \( D_{Te} \) is the total plasma concentration at equilibrium, and \( V_{pi} \) and \( V_{pe} \) are the initial and equilibrium plasma volumes.

The fraction of drug bound to plasma proteins (\( F_B \)) was calculated using equation 2.

\[ F_B = \frac{D_B}{(D_B + D_F)} \]  

(Eq. 2)

When concentrations are available to determine the fractional binding, Eq.2 can be written as:

\[ F_B = \frac{[(C_P - C_B) \times V_{pe}/V_{pi}]/ \{[(C_P - C_B) \times V_{pe}/V_{pi}] + C_B\} }{ \} \]  

(Eq. 3)

Where \( C_P \) is the concentration in plasma and \( C_B \) is the concentration in buffer.

4.2.5 Pharmacokinetic studies

4.2.5.1 Treatment of animals

The pharmacokinetic studies on CP-dUrd in mice were carried out as follows. In the first experiment, pure [6-\( ^3 \)H]CP-dUrd (specific activity: 1 mCi/mmole) was used. The radiolabeled CP-dUrd was dissolved in deionized water and administered i.p. at 228.5 \( \mu \)mole/kg (76.8 mg/kg). The mice, in groups of three were euthanized using carbon dioxide at 0.5, 1, 1.5, 2, 3, 4 and 6 hours post injection. In second experiment, due to the shortage of radiolabeled compound, [6-\( ^3 \)H]CP-dUrd was mixed with the same
amount of cold CP-dUrd (so that the specific activity was 0.5 mCi/mmole) and injected at the same dosage (76.8 mg/kg). Two animals in each group were sacrificed at 12, 24, 48 and 72 hours after drug administration. Blood was collected from the heart as previously described (Section 3.3.4). After aliquots were taken for determination of radioactivity, blood was centrifuged to separate plasma. Immediately following sampling of blood, tissues were removed from each animal. The tissues removed were: brain, heart, lung, liver, kidney, spleen, stomach, intestine and muscle. Like tissues from the two or three animals in each group were combined, and chopped into small pieces with scissors. Urine and fecal samples from the mice in 24-, 48- and 72- hour groups were also collected. Control samples from the mice treated by water were also obtained. The samples were stored at –20°C till analysis. Blood, plasma and tissue samples were processed further for radioactivity determination as described below.

4.2.5.2 Preparation of samples for radioactivity counting

1. Blood samples: Because of the presence of haemoglobin, processing of blood samples require careful processing to minimize quenching of radioactivity counts. The procedure outlined below was used for processing of blood sample for radioactivity counting. Blood (0.1 ml) was mixed with an equal volume of water and placed in 7 ml scintillation vials. 1.2 ml of NCS-II tissue solubilizer was then added to each vial. The vials were capped and placed in water bath heated to 50°C for 20 min. The vials were removed from water bath, and shaken in dark for 72 hours at 22°C to allow the thorough digestion of the blood. Following this, 0.4 ml of warm solution of benzoyl peroxide in toluene (20% w/v) was added as a bleaching agent to reduce quenching and improve
efficiency of counting. The samples were shaken further in dark for 42 hours at 22°C. Scintillant (BCS-NA, 5 ml) was added to each vial, samples were shaken again for 24 hours in the dark to allow equilibration and radioactivity was determined using liquid scintillation counter. All determinations were carried out in duplicate.

2. Plasma samples: Plasma samples (0.1 ml) in duplicates were processed in a similar manner as blood samples, except 1 ml of NCS-II tissue solubilizer and 0.3 ml of 20% benzoyl peroxide were used to process the plasma samples.

3. Tissue samples: Duplicate samples of tissues (100 mg) were finely chopped with scissors and mixed with 0.1 ml of water. Tissue samples were processed in a similar manner as described above for plasma samples.

4. Fecal samples: 100 mg of feces were mixed with 0.2 ml of water and processed in the same manner as tissue samples.

5. Urine samples: The urine samples were centrifuged at 11,750g for 5 min to remove solids (food and feces). 0.1 ml of undiluted urine was mixed with 1 ml NCS-II tissue solubilizer and digested for 72 hours as described above. Glacial acetic acid (2 μl) was added to the mixture and slowly shaken at 22°C for 42 hours. 5 ml of aqueous counting scintillant was added to each sample for the determination of the radioactivity.

Appropriate controls were included for each experiment to allow for correction of the counts. The samples from control animals were processed in a similar manner (negative controls) as the samples obtained from the mice treated with [6-3H]CP-dUrd. The counts detected for these samples served as background. For positive controls, 10 μl of series of standard concentrations of [6-3H]CP-dUrd (in water) were added to blood, plasma and tissue sample of the negative control mice and then processed using identical
procedures. These counts were used to calculate the equivalent concentrations of [6-\(^3\)H]CP-dUrd in blood, plasma and different tissues of the mice.

4.2.5.3 Analysis of pharmacokinetic data

Pharmacokinetic data of CP-dUrd was processed by application of noncompartamental techniques using the nonlinear curve-fitting software package WINNONLIN (SCI, Lexington, KY, 1995). Terminal elimination rate constants (\(\lambda_2\)) were estimated by using only the terminal-phase (12, 24, 48 and 72 hours post dose) concentrations of CP-dUrd in blood and tissues. The elimination half-life of the terminal phase (\(t_{1/2}\)) was derived by dividing 0.693 by the terminal rate constant. Following administration of the i.p. dose, the maximum concentration (\(C_{\text{max}}\)) and the time to maximum concentration (\(T_{\text{max}}\)) were obtained from inspection of the blood/tissue concentration-versus-time curves.

The recovery rate of urine and feces was calculated from the ratio of total amount of CP-dUrd in the samples to the amount of CP-dUrd administered.

4.2.5.4 Analysis of [6-\(^3\)H]CP-dUrd and its metabolites from liver and urine samples

Most of the tissue samples as well as blood and plasma samples were used up for determination of total counts of radioactivity. Therefore, the characterization of metabolites of CP-dUrd was carried out only on liver samples and urine samples. Due to breakdown of the HPLC detector unit, a paper chromatography method was standardized for the separation of compounds.

1. Preparation of liver samples: Liver (500 mg) in 0.5 ml of water was homogenized using teflon tissue grinder, 0.5 ml of cold citrate-phosphate buffer, pH 3.0
was added, and after 15 min in ice, precipitate was removed by centrifugation. The supernatant was immediately neutralized (pH ≈ 7.0) using Tris .HCl buffer (1M, pH 8.0). The samples were lyophilized and dissolved in 300 µl water for chromatography.

2. Chromatographic analysis: 50 µl of urinary samples and 100 µl of liver samples were used to determine the fate of [6-3H]CP-dUrd. Chromatograms (Whatman 3MM paper) were developed using ethanol/1.0 M ammonium acetate, pH 7.5, 7/3 solvent system. Spots were located under UV illumination and approximate Rf values were used for designation of compounds in relation to the Rf values of standard compounds. The Rf values of standard compounds in this solvent system were: CP-dUrd, 0.29 ± 0.05, dUrd H-phosphonate, 0.67 ± 0.05 and deoxyuridine (dUrd), 0.78 ± 0.05. To determine the radioactivity of the liver and urinary samples, the 3MM papers were cut into 1 cm pieces and placed into 10 ml of EcoLite scintillation fluid for counting. Radioactivity was located and Rf values of spots were compared with reference standards.

4.3 RESULTS

4.3.1 Metabolic studies in situ

The penetration of CP-dUrd into cells from the medium was a slow process and limited amounts of the compound diffused intracellularly over a long period. The uptake of CP-dUrd into CEM cells appears to be bi-phasic and time dependant. The uptake of drug during the first 24 hours of incubation was relatively rapid after an initial lag phase of about 6 hours. This was followed by a plateau phase for the next 24 hours (24 ~ 48 hours) and then slower uptake for the next 24 hours. After 6 hours incubation of CEM
cells with 50 μM CP-dUrd, the intracellular concentration was only 0.02 pmole/10⁶ cells. At 72 hours post incubation, highest concentration (2.2 μM; 1.8 pmole/10⁶ cells) of CP-dUrd reached intracellular. This intracellular level of CP-dUrd is much less than the concentration of CP-dUrd in the culture medium (50 μM). The results of metabolic studies using tritium labelled CP-dUrd in CEM cells are shown in Figure 4.1A.

The clearance (efflux) of CP-dUrd accumulated in the CEM cells was also determined. The efflux profile of CP-dUrd appeared to follow the first-order rate process. The clearance of CP-dUrd was very slow, with the half-life approximately 25 hours. At 72 hours post removal of the drug from the medium, there were still approximately 13% of the compound inside the cells (Figure 4.1B).

In order to determine the metabolic fate of CP-dUrd, contents of the cell culture medium harvested at 72 hours were analyzed by HPLC. Only one radioactive peak was detected and the retention time (4 ~ 5 min period) of radioactive peak was the same as CP-dUrd (4.26 min) (Figure 4.2 A and B). These results indicated that CP-dUrd was stable in the culture medium during the entire incubation period. Furthermore, these results also suggest that very likely CP-dUrd was not metabolized because metabolites if formed should have been excreted into the medium. Unfortunately, due to the low levels of CP-dUrd present in cellular extracts, it was not possible to characterize compounds after HPLC separation because the radioactive counts were too low.

In conclusion, CP-dUrd penetrated very slowly into CEM cells and effluxed slowly from the CEM cells. CP-dUrd was the only compound identified in the
Figure 4.1A. Uptake of [6-\(^{3}\)H] CP-dUrd. CEM cells (5 x 10\(^5\)/ml) were incubated with 50 \(\mu\)M [6-\(^{3}\)H] CP-dUrd (1 mCi/mmole). At periodic intervals, samples were removed and analyzed for levels of CP-dUrd by radioactivity.
Figure 4.1B. Efflux of [6-^3^H]CP-dUrd. CEM cells (5 x 10^5/ml) were incubated with 50 µM [6-^3^H]CP-dUrd for 72 hours. Cells were washed with PBS and resuspended in drug-free medium for another 72 hours. At periodic intervals, samples were removed and analyzed for levels of CP-dUrd by radioactivity.
Figure 4.2A HPLC profiles of UV absorbance of extracellular culture medium. CEM cells (5 x 10^5 cells/ml) were incubated with 50 μM [6-^3^H]CP-dUrd for 72 hours. Culture medium was processed for HPLC analysis. The retention time of CP-dUrd is 4.26 min.
Figure 4.2B Radioactivity profiles of extracellular culture medium. CEM cells (5 x 10^5 cells/ml) were incubated with 50 μM [6-^3^H]CP-dUrd for 72 hours. Culture medium was processed for HPLC analysis. Fractions were collected for radioactivity determination.
extracellular medium by HPLC and radioactivity. It is uncertain at this time whether or not CP-dUrd was metabolized intracellularly.

4.3.2 Plasma protein binding of CP-dUrd

Post dialysis, the average concentration of protein in compartment 1 (plasma) and compartment 2 (buffer) were 23.4 ± 0.68 and 3.1 ± 1.62 mg/ml (mean ± SD), respectively. The small amount of proteins detected in buffer (compartment 2) may be due to the penetration of proteins or peptides with smaller molecular weight below 12,000 during dialysis from compartment 1 to compartment 2.

The results of the study on CP-dUrd binding to plasma proteins are summarized in Table 4.1 and Figure 4.3. The concentrations of CP-dUrd in two compartments were essentially equal, which indicated that CP-dUrd did not bind to plasma proteins at the concentrations tested. Except at 400 µM, the Fb values of CP-dUrd were lower than zero. The Fb value of CP-dUrd was greater than zero (+ 3.4%) at 400 µM. However, the difference between the concentrations of CP-dUrd in two compartments post dialysis was so small (143.1 versus 146.5 µM, respectively) that this difference is most likely due to the normal variation of the methodology. This data also support the contention that there was no protein binding of CP-dUrd to plasma proteins.

4.3.3 Pharmacokinetic studies on CP-dUrd in mice

The results of pharmacokinetic studies using labelled CP-dUrd are presented in this section. The chromatographic analysis of liver and urinary samples indicated that CP-dUrd was metabolized in the body (See section 4.3.3.4). Therefore, the description
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<th>Original concentration (μM)</th>
<th>Postdialysis Concentration (μM)</th>
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1. Equilibrium dialysis method was used for protein binding studies.
2. Fb: Fraction of drug bound to plasma proteins.
Figure 4.3 Concentration of $[^{6-3}\text{H}]$CP-dUrd in plasma and buffer. Plasma (●-●) and buffer (□-□).
of the results is based on the non-specific total radioactivity measurement. The unique pharmacokinetic profile of labelled CP-dUrd could be due to the intrinsic properties of the parent compound or the impact of its metabolite(s).

4.3.3.1 Concentration of CP-dUrd in blood and plasma

The equivalent concentrations of CP-dUrd in blood and plasma at different time intervals after i.p. administration of labeled compound are shown in Figure 4.4. Based on the total radioactivity, the blood and plasma have the similar concentration-time profiles for the drug. The plasma/blood ratio for CP-dUrd ranged from 0.80 to 1.21. The minor differences of the CP-dUrd concentrations between plasma and blood are most likely due to the normal variation of the methodology.

The profile of CP-dUrd in blood is unique because there are two peak concentrations in the blood post injection. The blood concentration versus time curve of CP-dUrd basically consists of three phases: (i) absorption phase; from the time of administration to 1.5 hours after i.p. injection; (ii) distribution and reabsorption phase; from 1.5 to 12 hours post injection; and (iii) elimination phase; from 12 to 72 hours post injection.

CP-dUrd was slowly absorbed and the concentration of the drug increased in the blood as a function of time during the first phase. The concentration of CP-dUrd in the blood reached 100 μM after 30 min post administration. The first peak blood level (177 μM) was attained after 1.5 hours post injection. The blood levels of the drug then started to drop slowly. At 3 hours post administration, blood levels had dropped to approximately half of the maximal concentration (46%). This decrease in blood levels is most likely due to distribution of the CP-dUrd from the central compartment (systemic
Figure 4.4 Equivalent concentrations of [6-\textsuperscript{3}H]CP-dUrd in blood and plasma of mice following intraperitoneal administration of a single dose of labelled compound at 228.5 \textmu mole/kg (76.8 mg/kg). Blood (●-●), plasma (◆-◆).
circulation) to the peripheral compartments. Interestingly and unexpectedly, the concentration of CP-dUrd in the blood then started to increase, from 95 μM at 3 hours to reach the second peak (174 μM) at 12 hours post injection. This peak is probably due to (1) the reabsorption of the drug from the other untested tissues of the body which happened to have sequestered significant amounts of administered drugs, such as gallbladder (entero-hepatic cycle), and possibly other tissues, such as skin, bone and fat, etc; or (2) the efflux of the drug from the peripheral compartments into the blood. The CP-dUrd then started to be slowly eliminated from the body with the terminal half-life of 38.4 hours. Approximately 73% of the drug was eliminated at 72 hours post injection.

4.3.3.2 Tissue distribution of [6-3H]CP-dUrd

Concentration of CP-dUrd in tissues - liver, spleen, kidneys, heart, lung, muscle, stomach, intestine and brain were determined by measurement of total radioactivity. Time-equivalent CP-dUrd levels in tissues at different time intervals are shown in Table 4.2 and Figure 4.5. CP-dUrd appeared to be distributed to all parenchymatous organs, with concentrations ranging from 23 to 414 nmole/g. Most tissues had similar concentration biphasic profiles, which are resembles the patterns of CP-dUrd distribution in blood and plasma. In general, CP-dUrd rapidly distributed into tissues after peritoneal administration. Within 30 min after i.p. injection, high levels of CP-dUrd were found in liver, kidneys and spleen (200 ~ 348 nmole/g). Heart, lung and muscle contained lower CP-dUrd levels (80 ~ 122 nmole/g), whereas brain had the lowest amount of CP-dUrd (42 nmole/g). The first peak levels of CP-dUrd in liver, spleen, kidneys, heart and lung were obtained at one hour post injection, which were 30 minutes earlier than that in the blood. Brain, muscle, stomach and intestine had a relatively flat peak concentrations between 1 to 1.5 hours post administration.
Table 4.2 Maximum concentrations ($C_{\text{max}}$), minimum concentrations ($C_{\text{min}}$) and half-lives of CP-dUrd in blood, plasma and tissues of mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$C_{\text{max}1}$ (µM)</th>
<th>$C_{\text{max}2}$ (µM)</th>
<th>$C_{\text{min}}$ (µM)</th>
<th>$t_{1/2}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>177 (1.5)</td>
<td>174 (12)</td>
<td>59 (72)</td>
<td>38.4</td>
</tr>
<tr>
<td>Plasma</td>
<td>170 (1.5)</td>
<td>194 (12)</td>
<td>48 (72)</td>
<td>30.9</td>
</tr>
<tr>
<td>Liver</td>
<td>414 (1)</td>
<td>193 (12)</td>
<td>57 (72)</td>
<td>36.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>321 (1)</td>
<td>273 (6)</td>
<td>81 (72)</td>
<td>39.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>328 (1)</td>
<td>186 (12)</td>
<td>58 (72)</td>
<td>36.2</td>
</tr>
<tr>
<td>Lung</td>
<td>216 (1)</td>
<td>239 (6)</td>
<td>68 (72)</td>
<td>39.1</td>
</tr>
<tr>
<td>Heart</td>
<td>129 (1)</td>
<td>199 (12)</td>
<td>53 (72)</td>
<td>33.3</td>
</tr>
<tr>
<td>Muscle</td>
<td>147 (1.5)</td>
<td>182 (6)</td>
<td>43 (72)</td>
<td>31.3</td>
</tr>
<tr>
<td>Stomach</td>
<td>146 (1)</td>
<td>88 (12)</td>
<td>23 (72)</td>
<td>31.6</td>
</tr>
<tr>
<td>Intestine</td>
<td>221 (1.5)</td>
<td>219 (6)</td>
<td>52 (72)</td>
<td>50.8</td>
</tr>
<tr>
<td>Brain</td>
<td>105 (1.5)</td>
<td>196 (12)</td>
<td>42 (0.5)</td>
<td>49.3</td>
</tr>
</tbody>
</table>

1. Labelled [6-$^3$H]CP-dUrd 228.5 µmole/kg (76.8 mg/kg) was administrated intraperitoneally.
2. $C_{\text{max}1}$: Maximum concentration of CP-dUrd reached during first peak. $C_{\text{max}2}$: Maximum concentration of CP-dUrd reached during second peak.
3. $C_{\text{min}}$: Minimum concentration.
4. $t_{1/2}$: Terminal half-life.
5. The number presented in brackets is the time (hours) when maximum or minimum levels were reached.
Figure 4.5A. Equivalent concentrations of [6-\textsuperscript{3}H]CP-dUrd in tissues of mice following intraperitoneal administration of a single dose of labelled compound at 228.5 \textmu mole/kg (76.8 mg/kg). Liver (●●), kidney (■■) and spleen (◆◆).
Figure 4.5B and 4.5C. Equivalent concentrations of [6-^3^H] CP-dUrd in tissues of mice following intraperitoneal administration of a single dose of labelled compound at 228.5 μmole/kg (76.8 mg/kg).
Panel B: heart (○-○), lung (■-■), muscle (◆-◆);
Panel C: stomach (○-○), intestine (■-■) and brain (◆-◆).
(Table 4.2). The concentration of CP-dUrd in tissues then started to decrease during the 2 to 4 hours post injection. However, the extent of decline CP-dUrd varied among different tissues. The levels of CP-dUrd in liver, kidneys and stomach dropped to approximately 70% of their corresponding first peak levels, whereas those of heart, lung and spleen declined to approximately 50%. The concentrations of CP-dUrd in various tissues reached a second peak at 6 or 12 hours post administration, which were earlier or same as that of blood (the second peak of blood was 12 hours post injection). The second peak levels of CP-dUrd in liver, kidneys and spleen were lower than their corresponding first peak levels (47 ~ 85% of first peak levels), whereas the concentrations of CP-dUrd in heart and brain were higher than the first peak levels (154 ~ 187% of the first peak levels). The levels of CP-dUrd in lung, muscle, stomach and intestine were essentially similar to those found during the first peak.

The maximum concentrations (C_{max}) of CP-dUrd in different tissues are summarized in Table 4.2. The highest levels of the compound were reached in the liver (414 nmole/g), followed by kidneys (328 nmole/g) and spleen (321 nmole/g). The levels of CP-dUrd in other tissues ranged from 180 to 240 nmole/g.

CP-dUrd was eliminated from the tissues by a first-order rate process during the terminal phase (12 ~ 72 hours post injection). The majority of tissues showed half-lives of 30 to 40 hours. However, the rate of elimination from brain and intestine over this period was relatively slower (t_{1/2}: 49 and 51 hours, respectively). Interestingly, a significant amount of radioactivity was present in tissues at 72 hours post administration of CP-dUrd, the levels of equivalent CP-dUrd in tissues ranged from 23 to 81 nmole/g at 72 hours post injection. By this time, however, approximately 82% to 86% of the drug had been eliminated from the kidneys, liver and stomach, and slightly lower (72% to
76%) from other tissues (spleen, lung, heart, muscle and intestine). The efflux of the drug from the brain was at the slowest rate. The levels in the brain at 72 hours were still around 40% of the maximum levels.

**Brain/blood ratios**

As described above, CP-dUrd was able to cross the blood brain barrier and was present in the brain of mice. However, the concentration-time curve of the compound in brain was unique in that the brain levels increased slowly and then declined very slowly (Figure 4.6). These results indicate slower penetration of CP-dUrd into the CNS and more importantly slow efflux of CP-dUrd from the brain. The brain levels of CP-dUrd, although initially low (42 nmole/g at 30 min post injection), reached as high as 196 nmole/g at 12 hours post injection. Interestingly, the amount of CP-dUrd in brain was considerably higher than corresponding plasma levels 24 hours onward post administration and these levels were maintained up to 72 hours (Fig 4.6 and Table 4.3). In this respect, the behaviors of this compound is quite unique as compared to other anti-HIV agents.

4.3.3.3 Excretion of CP-dUrd in mice

The results of radioactivity recovered from urine and fecal samples are summarized in Table 4.4. The renal excretion appears to be the major route for elimination of CP-dUrd. Radioactivity eliminated by renal excretion accounted for approximately 58.5% of the total administered radioactivity. The majority of the dose was excreted during the first 24 hours and was virtually complete within 48 hours after administration. The recovery rate of \[^{3}H\]CP-dUrd in feces was very low (2.8 %),
Figure 4.6 Equivalent concentrations of \( [6-^3\text{H}]\text{CP-dUrd} \) following intraperitoneal administration of a single dose of labelled compound at 228.5 \( \mu\text{mole/kg} \) (76.8 mg/kg) in brain (•-• nmole/g) and blood (■-■ nmole/ml) in mice.
Table 4.3 Equivalent concentration of [6-^3^H]CP-dUrd in brain and blood of mice following i.p. administration of a single dose of labelled compound at 228.5 μmole/kg (76.8 mg/kg)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Brain (nmole/g)</th>
<th>Blood (nmole/ml)</th>
<th>Brain/blood (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>42.5</td>
<td>99.7</td>
<td>0.43</td>
</tr>
<tr>
<td>1</td>
<td>99.3</td>
<td>150</td>
<td>0.66</td>
</tr>
<tr>
<td>1.5</td>
<td>104.6</td>
<td>176.9</td>
<td>0.59</td>
</tr>
<tr>
<td>2</td>
<td>85.8</td>
<td>110.3</td>
<td>0.78</td>
</tr>
<tr>
<td>3</td>
<td>77.8</td>
<td>95.2</td>
<td>0.70</td>
</tr>
<tr>
<td>4</td>
<td>81.9</td>
<td>143.8</td>
<td>0.57</td>
</tr>
<tr>
<td>6</td>
<td>109.4</td>
<td>141.9</td>
<td>0.77</td>
</tr>
<tr>
<td>12</td>
<td>196.1</td>
<td>174.0</td>
<td>1.13</td>
</tr>
<tr>
<td>24</td>
<td>154.9</td>
<td>120.7</td>
<td>1.28</td>
</tr>
<tr>
<td>48</td>
<td>122.7</td>
<td>71.8</td>
<td>1.71</td>
</tr>
<tr>
<td>72</td>
<td>81.0</td>
<td>58.7</td>
<td>1.38</td>
</tr>
</tbody>
</table>
Table 4.4 Urine and fecal recovery of equivalent CP-dUrd after i.p. administration of labelled CP-dUrd 228.5 μmole/kg (76.8 mg/kg) in mice

<table>
<thead>
<tr>
<th>Time (hour)</th>
<th>Urinary Recovery (%)</th>
<th>Fecal Recovery (%)</th>
<th>Total recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24</td>
<td>55.4</td>
<td>0.3</td>
<td>55.7</td>
</tr>
<tr>
<td>24-48</td>
<td>3.1</td>
<td>0.4</td>
<td>3.5</td>
</tr>
<tr>
<td>48-72</td>
<td>0</td>
<td>2.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

1. Urine and fecal samples were collected at 24, 28 and 72 hours after i.p. administration of [6-³H]CP-dUrd. Samples were processed for radioactivity determination as described in section 4.2.5.2. The recovery rate of urine and feces was calculated from the ratio of total amount of CP-dUrd in the samples to the amount of CP-dUrd administrated.
indicating that this is only a minor route of excretion for this compound.

4.3.3.4 Preliminary studies on metabolism of [6-\textsuperscript{3}H]CP-dUrd

Because of the lack of availability of sufficient amount of plasma and other tissues, studies described in this section were carried only on liver samples. Adequate amounts of liver samples available for indicated time periods were analyzed for radioactivity. The fate of [6-\textsuperscript{3}H]CP-dUrd was determined from pooled liver samples collected at 1, 12 and 72 hour post administration of the labeled compound. The results of 1 hour liver samples showed that 100% of CP-dUrd was metabolized to dUrd H-phosphonate (R\textsubscript{f} vaule of 0.67 ± 0.05). No radioactive spots were found from 12 and 72 hour samples.

Chromatographic analysis of urine samples collected during the first 24 hours after CP-dUrd dosing gave four radioactive spots with R\textsubscript{f} values corresponding to CP-dUrd (R\textsubscript{f}: ≈ 0.29 ± 0.05), dUrd H-phosphonate (R\textsubscript{f}: ≈ 0.67 ± 0.05), deoxyuridine (R\textsubscript{f}: ≈ 0.78 ± 0.05) and an unidentified metabolite (R\textsubscript{f}: ≈ 0.11 ± 0.05). Based on radioactivity, the approximate amounts were: CP-dUrd (12%), dUrd H-phosphonate (14%), dUrd (71%) and unidentified product (3.5%). Results are summarized in Table 4.5.

4.4 DISCUSSION

The cellular pharmacological studies on [6-\textsuperscript{3}H]CP-dUrd using rapidly proliferating CEM cells indicate that the uptake of CP-dUrd in CEM cells was slow and limited. Only a low level of CP-dUrd inside the cell (1.8 pmol/10\textsuperscript{6} cells) was achieved even after CEM cells were incubated with high concentration of the compound (50 μM) for a long period (72 hours). Similarly, the process of efflux of CP-dUrd from the CEM cells was also a very slow
Table 4.5 Identification of [6-\textsuperscript{3}H]CP-dUrd metabolites in the liver and urine of mice\textsuperscript{1}

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>R\textsubscript{f} (x 100)</th>
<th>Liver (%)</th>
<th>Urine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-dUrd</td>
<td>29</td>
<td>ND\textsuperscript{2}</td>
<td>11.6</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>78</td>
<td>ND</td>
<td>71.2</td>
</tr>
<tr>
<td>dUrd H-Phosphonate</td>
<td>67</td>
<td>100</td>
<td>13.7</td>
</tr>
<tr>
<td>Metabolite x</td>
<td>11</td>
<td>ND</td>
<td>3.5</td>
</tr>
</tbody>
</table>

1. The one hour liver sample and the 24 hour urinary samples were collected following intraperitoneal administration of a single dose of labelled CP-dUrd at 228.5 \(\mu\)mole/kg (76.8 mg/kg). The samples were processed for chromatographic analysis as described in section 4.2.5.4. For metabolic analysis, the samples were developed on Whatman 3MM paper using ethonal/1.0 ammonium solvent system. The radioactivities of the samples on the paper were located and R\textsubscript{f} values of spots were compared with reference standards.

2. ND: Not detected.
process. The presence of two negative charges on the CP-dUrd molecule, most likely results in the electrostatic repulsion between the compound and the exterior of the cell membrane. The poor cell permeability of this molecule is consistent with the chemical structure of CP-dUrd. Similar results have been reported for acyclic nucleoside phosphonates such as PMEA (Aduma et al., 1995; Palu et al., 1991). PMEA carries two negative charges on phosphonomethoxyethyl group. The studies on cellular pharmacology of PMEA have shown that the maximal levels in Vero cells were reached after 12 hour incubation with 25 μM of PMEA (Aduma et al., 1995). The intracellular levels of PMEA and its metabolites PMEA monophosphate (PMEAp) and PMEA diphosphate (PMEApp) were 1.3, 0.08 and 0.34 pmole/10^6 cells, respectively. PMEA and its metabolites were cleared from the cells with a half-life of 4.9 hours (Aduma et al., 1995). In contrast, AZT, which is less polar than CP-dUrd, permeates the cell membrane easily by passive diffusion (Zimmerman et al., 1987). The concentration of AZT and its metabolites in H9 cells reached 665 pmole/10^6 cells after incubation with 50 μM of the drug for 24 hours, which was 369 fold higher than that of CP-dUrd. The efflux of AZT was also rapid, only 2.5% of AZT and its metabolites remained inside the cell 4 hours after removal of the drug (Furman et al., 1986). In contrast, substantially higher concentration of CP-dUrd (13%) was still present inside the CEM cells at 72 hours after removal of the compound. Compared to AZT, the uptake of ddC by cells is even faster. In Molt 4 cells, the intracellular levels of drug were found in the range of 13 to 50 pmole/10^6 cells 6 hours after exposure to concentrations 2 μM and 10 μM of ddC (Starnes and Cheng, 1987). These studies showed that the transport of ddC is dependent on a nucleoside carrier (Cooney et al., 1986; Ullman et al., 1988). ddC is also rapidly cleared from the cells, with half-life of 5.7 hours in Molt 4 cells (Starnes and Cheng, 1987). In contrast, the half-life of CP-dUrd in CEM cells was 25 hours, indicating the slow efflux of
this compound.

The poor permeability of CP-dUrd across the cell membrane and the low intracellular level is the most likely reason for the low anti-HIV activity observed for this series (carboxyphosphonyl analogs) of compounds when lymphoblast cell lines are used for assays (Section 2.3.3). The poor permeability characteristics of CP-dUrd may also be a contributing factor for low acute cytotoxicity to lymphoblast cell lines, progenitor bone marrow cells and lack of delayed toxicity observed for this compound. The possible reason for delayed cytotoxicity in CEM cells at low concentration is the rapid uptake and rapid processing of ddC by the cells to its active metabolite ddC 5'-triphosphate which can interfere with metabolic functions of mitochondrial DNA polymerase (Balzarini, 1994a). Parallel studies on AZT indicate that AZT-MP accumulates inside the cells and only a small portion of AZT was converted to AZT-TP (Balzarini, 1994a). Therefore, in contrast to ddC, although AZT diffuses into the cells easily, it does not show delayed cytotoxicity even at high concentrations.

The metabolic fate of CP-dUrd transported into CEM cells was not characterized in these investigations, although it is unlikely that the metabolites of CP-dUrd were formed intracellularly in significant amounts, however, further studies need to be carried out when more radiolabelled CP-dUrd preferably with higher specific activity becomes available, to either confirm or deny it.

There was no binding of CP-dUrd to pig plasma proteins. In this respect, the behavior of CP-dUrd is similar to MMdUrd and d4T, but different from AZT. MMdUrd did not bind to rat plasma proteins (Meldrum et al., 1981). The studies in humans have shown that ddI and ddC also have poor affinity for plasma proteins [(protein binding less than 5%), Dudley, 1995]. In contrast, approximately 21-23% of AZT was found to bind to plasma proteins of human and rats (De Miranda et al., 1990; Dudley, 1995).
For pharmacokinetic studies, intraperitoneal (i.p.) administration of drug in mice has two advantages over intravenous (i.v.) administration in rats: (1) ease of administration and rapid absorption although not as instantaneous as i.v. (2) Cost of animals and their maintenance. Furthermore, in order to obtain similar pharmacokinetic data from rats, much larger amount of radiolabelled drug would be required. Therefore, it was decided to investigate the pharmacokinetic properties of CP-dUrd in mice by i.p. administration of the radiolabelled CP-dUrd.

However, when a large amount of drug is metabolized in the liver, the first-pass effects has to be considered when the drug is administration by i.p. route. Therefore, the equivalent CP-dUrd concentrations of total radioactivity were used to express the concentration of CP-dUrd. The noncompartment approach was selected to calculate pharmacokinetic parameters of CP-dUrd. The major advantage of this model-independent method is that no assumption for a specific compartment model is required to analyze the data (Shargel and Yu, 1993).

The pharmacokinetic studies showed that CP-dUrd was slowly absorbed, well distributed in all tissues, extensively metabolized in the body and slowly eliminated from the body by renal excretion. CP-dUrd has unique pharmacokinetic properties. Two major differences between CP-dUrd and other nucleoside analogs are: (1) CP-dUrd had two peak levels in the blood and tissues versus time concentration curves. The second peak levels were almost as high as the first peak. (2) This compound had relatively long half-lives of elimination from the blood and the tissues. In contrast, the ddN analogs in general are rapidly absorbed, well distributed throughout the body, biotransformed to phosphorylated products and rapidly eliminated from the body (See table 1.3).

CP-dUrd was slowly absorbed after i.p. administration. A relatively high
concentration reached in blood (100 μM) at first time point of detection (30 min post injection). However, it took approximately 1.5 hours (T\text{max}) for CP-dUrd to reach the first peak level in blood. In contrast, the peak plasma concentration was achieved within 10 min after i.p. administration of MMdUrd in mice (Meldrum et al., 1981). The T\text{max} for AZT was 25 min post oral administration of AZT in rats (De Miranda et al., 1990).

CP-dUrd was widely distributed in all tissues. In this respect, CP-dUrd was similar to other nucleoside analogs. In humans, AVD values ranging from 0.5 to 3.9 L/kg have been reported for ddNs, which implies that ddNs are well distributed throughout the body. The studies on AZT in mice and rat showed that AZT is distributed in all tissues, including the liver, kidneys, spleen, heart, lung, muscle, stomach, intestine, contents of gastrointestinal tract, thymus, lymph nodes, bone, bone marrow, skin and brain. (Ahmed et al., 1991; De Miranda et al., 1990). MMdUrd was also well distributed to the parenchymatous organs tested in mice (Meldrum et al., 1981). However, the pattern of tissue distribution of CP-dUrd is quite different from AZT and MMdUrd. Firstly, CP-dUrd was homogeneously distributed in all tissues and concentrations approaching 200 nmole/g of equivalent CP-dUrd were reached in tissues (except stomach). The differences in concentrations noted among tissues for CP-dUrd were time-dependent and were approximately within 3 to 4 fold range. For example, the first peak concentrations of CP-dUrd ranged from 100 to 400 nmole/g, and the second peak concentrations ranged from 88 to 273 nmole/g for tissues (Table 4.2). In contrast, AZT levels found in the gastrointestinal tract and kidneys were approximately 67 to 110 fold higher than other tissues (Ahmed et al., 1991; De Miranda et al., 1990). The brain levels of CP-dUrd were relatively high (100 - 200 nmole/g), whereas concentrations of AZT and MMdUrd in the brain were considerably lower than other tissues (Meldrum et al.,
1981; De Miranda et al., 1990). Secondly, the concentrations versus time profiles of CP-dUrd in tissues were similar to that of CP-dUrd blood levels versus time curves. The first peak levels of CP-dUrd in tissues reached between 1 and 1.5 hours post injection. The terminal half-lives of CP-dUrd in different tissues ranged from 31 to 51 hours. These results suggest that the efflux of CP-dUrd from tissues occurred slowly. In contrast, MMdUrd was rapidly distributed in tissues after i.p. administration. The maximum levels of MMdUrd were attained 20 to 30 min post injection. The half-lives of MMdUrd in tissues were reported in the range of 40 to 60 min (Meldrum et al., 1981). Similarly, AZT was also rapidly eliminated from tissues. At 24 hours post i.v. administration to mice, AZT was almost completely eliminated from the animal (Ahmed et al., 1991).

Another unique characteristic of CP-dUrd distribution was the pattern of influx of this compound in the brain. CP-dUrd penetrated the brain more slowly and its efflux from this tissue was also more gradual than other tissues examined. This was most likely due to the effect of blood brain barrier, which is a major obstacle for the passage of most drugs into the brain from the blood (Benet and Sheiner, 1995). Similar findings have been reported for AZT and MMdUrd (Meldrum et al., 1981; De Miranda et al., 1990). In patients treated with AZT, relatively high ratios of cerebrospinal fluid (CSF)/plasma (0.15~1.35) were found. However, further studies showed that AZT, like some other nucleosides, does not penetrate the blood-brain barrier (BBB) readily. Instead, it was transported into the CSF through the choroid plexus (Terasaki et al., 1988). This may explain why low levels of AZT were found in the brain in animal studies (Ahmed et al., 1991; De Miranda et al., 1990). In contrast, CP-dUrd appears to cross the BBB and reach high concentration in the brain. The highest level of CP-dUrd was 196 nmole/g
and the highest brain/blood ratio was 1.71. Further studies should be pursued to elucidate mechanisms involved in the transport of CP-dUrd into the brain. Studies should also be carried out to characterize compounds present in the brain. These studies would be helpful in deciding on the possible utility of CP-dUrd for the treatment of HIV infection of the CNS system.

CP-dUrd showed poor membrane permeability in the pharmacological studies using CEM cells. In contrast, CP-dUrd was well distributed in the body. One of the possible explanations is that in tissues such as liver and kidneys, there are various types of cells. Some of these cells have active transporter mechanisms of organic molecules. Therefore, in viva, CP-dUrd could have entered the cells by utilizing active transporter mechanisms.

The pharmacokinetic studies on CP-dUrd revealed the presence of two distinct peaks in blood/tissue drug concentration curves. The secondary peaks were as high as the first peak for blood and most tissues. Intestine (duodenum portion) had a high content of radioactivity for the entire period. Although CP-dUrd was administered to mice by i.p. route, small amount of radioactivity was also recovered in feces. These findings suggest that CP-dUrd or its metabolites utilized entero-hepatic cycle for re-entry into blood circulation in the body. CP-dUrd was most likely absorbed via mesenteric vessels to the hepatic portal vein and then distributed to the liver. CP-dUrd was then secreted into the bile and became highly concentrated in the gallbladder. From there, CP-dUrd was excreted into the duodenum. Subsequently, CP-dUrd was reabsorbed into the blood. Since fecal excretion amounted to only 2.8% (Table 4.4), this can not be a major pathway of excretion, i.e. most of the metabolite and/or CP-dUrd was reabsorbed back into blood circulation. The declines of CP-dUrd concentrations in blood/tissue during the 2-4 hours post injection may have been due
to the secretion of the compound or its metabolite(s) into gallbladder which was not measured, and the emergence of second peaks could be due to the reabsorption via the entero-hepatic cycle. In addition, it is a general rule that drugs with molecular weights between 300 and 500 are excreted both in bile and urine, and drugs excreted into bile usually require a strongly polar group (Shargei and Yu, 1993). CP-dUrd meets both these criteria. Thus, the complicated kinetic profiles observed for CP-dUrd may be explained by the entero-hepatic cycle theory. However, direct measurements of CP-dUrd in the bile would have to be carried out to validate this hypothesis when more radiolabelled compound is available.

Like CP-dUrd, AZT is also secreted into the bile. The studies of AZT in mice showed that the drug accumulates in high concentration in the gallbladder and then eliminated into the intestinal contents after 4 hours post i.v. injection (Ahmed et al., 1991). Another study carried out in rats showed that at least 7% of parenteral dose of AZT was secreted in the bile and the major metabolite in the bile was 3'-azido-3'-deoxy-5'--glucuronylthymidine (GAZT) (De Miranda et al., 1990).

Possible routes for the biotransformation of CP-dUrd in the body are shown in Chart 4.1. CP-dUrd may undergo decarboxylation to yield dUrd H-phosphonate, which upon further oxidation would result in the formation of a natural metabolite deoxyuridine monophosphate (dUMP). Decarboxylases are present ubiquitously in the body, whereas oxidative enzymes are primarily located in the liver. dUMP can enter the metabolic pool by conversion to deoxythymidine monophosphate (dTMP). This is the de novo pathway for the synthesis of thymine nucleotides in mammalian cells. Catabolism of dUMP and dTMP will result in the formation of corresponding deoxyribonucleosides and ultimately uracil and thymine. Phosphatases and nucleoside phosphorylases are widely distributed in the body.
Chart 4.1 Proposed routes for the metabolism of CP-dUrd in vivo.
CP-dUrd appears to undergo metabolic transformations in the liver. Decarboxylation of CP-dUrd results in the formation of dUrd H-phosphonate, as indicated by analysis of 1 hour liver samples. Surprisingly, no other metabolites were found in these liver samples. Although it is conceivable, that the methodology used was not sensitive enough to detect other metabolites because these were produced in very small amounts. Interestingly, the chromatographic analysis of 24 hours urinary samples revealed the presence of several radioactive spots. Based on radioactivity measurements and mobility on paper chromatograms (R_f values), tentatively the metabolic profile was characterized as follows: CP-dUrd (12%), dUrd H-phosphonate (14%), fast moving spot with R_f value similar to dUrd (71%) and the metabolite X [slow moving spot, Rf value similar to phosphorylated products (3.5%)]. On the basis of these preliminary studies the following conclusions can be drawn: (i) CP-dUrd is decarboxylated in the liver to give dUrd H-phosphonate. (ii) dUrd H-phosphonate is then oxidized to dUMP (presumably by the microsomal enzymes present in the liver). (iii) dUMP then enters the metabolic pool and is converted to phosphorylated products (minor route) as well as degraded to deoxyribonucleosides (major pathway) and (v) Some CP-dUrd (6.7% administered dose) is excreted unchanged. The formation of metabolites found in the liver and urinary samples can be rationalized on the basis of the proposed metabolic pathways for CP-dUrd shown in Chart 4.1.

In conclusion, the introduction of the carboxyphosphonyl (CP) group at the 5'-position of dUrd conferred a unique pharmacokinetic profile for this pharmacophore in vivo. Further studies on the metabolism of CP-dUrd should be pursued in animals to understand more thoroughly its metabolic fate in the body. These studies should include analysis of plasma and tissue samples collected at different time intervals. Plasma
studies are particularly important because these investigations will reveal the proportion of CP-dUrd and its metabolites in the circulation. Emphasis should also be placed on characterization of drug profiles in brain samples because these investigations will provide information on the possible utility of CP-dUrd in the treatment of CNS infection due to HIV.
5.0 GENERAL DISCUSSION

AIDS is a lethal "multi-system" disease that has become a major health problem worldwide. The life cycle of HIV, etiological agent of AIDS, can be theoretically interrupted by *pharmacophores* at various steps of virus replication starting from the initiation of infection (adsorption) to the release of virus particles (budding) from the infected cell. The discovery of drugs that have the ability to inhibit virus replication by targeting two unique functions of HIV, namely, viral enzymes RT and protease have opened the era of the chemotherapeutic approach to combat HIV infection. The combined use of HIV inhibitors in combination chemotherapy has been found to dramatically decrease the virus load in AIDS patients and thus slow down the disease progress and prolong the life-span of people infected with HIV. However, AIDS remains 'incurable' because the HIV can not be completely *eradicated* from the body by chemotherapeutic regimens. The rebound of HIV replication following withdrawal of drugs is a common phenomenon seen in patients taking drugs. The emergence of drug resistant strains and toxicity to both RT and protease inhibitors are additional limitations for the clinical use. Therefore, there is a need for the development of drugs with greater selectivity against HIV infections because treatment has to be given for life-long to AIDS patients.

The toxicity induced by RT inhibitors (AZT, ddC, d4T, ddI and 3TC) are mainly due to their interaction with the host cellular DNA polymerases. Observations that HIV strains resistant to one ddN drug may be susceptible (or hypersensitive) to other ddNs
(which have similar mechanisms of action) indicates that even a minor change in the chemical structure confers useful therapeutic attributes. This is the basis for the use of ddC and ddI in patients who become refractory to AZT. The rationale for the synthesis of carboxyphosphonyl analogs of ddN’s and deoxynucleoside analogs was discussed in section 1.7. Because of the presence of carboxyphosphonyl (CP) group at 5'-position of the deoxyribose moiety, these compounds can not be phosphorylated to 5'-triphosphates, and therefore it was reasoned that CP analogs should have lower toxicity profile compared to presently approved drugs. It was also hypothesized that CP analogs should inhibit HIV drug-resistant strains because the mechanisms of action of these compounds would be different from ddNs. Results presented in this thesis provide ample evidence to support these hypotheses.

Two classes of CP analogs, namely, CP-ddNs (CP-AZT, CP-ddC, CP-ddI and CP-d4T) and CP-dNs (CP-dUrd, CP-dThd and CP-dlno) were synthesized. The antiviral activity of CP analogs and other drugs was evaluated by RT assay and MTT assay against several HIV strains using different lymphoblast cell lines and HT4-6C cells (FIA assay) in cell culture. The apparent ED$_{50}$ and CC$_{50}$ values of compounds tested varied considerably, depending on the virus strain, cell line and the assay method used. These findings are in general agreement with previous investigations on HIV inhibitors (Table 3 of appendix). RT assay using cord blood mononuclear cells (CBMC) or monocyte derived macrophages was the most sensitive method for determining antiviral activity of CP-analogs. The endpoints for the measurement of antiviral activity are reduction of infected cells and cell protection, respectively, when focal immunoassay (FIA) and cytopathic inhibition assay (MTT assay) are used. Therefore, intracellular levels attained by the drug are critical for the anti-HIV activity when FIA and MTT assays are used for
the determination of antiviral activity. In contrast, the extent of viral replication is indicated by the RT activity in the culture medium (supernatant) when RT assay is used. Therefore, one possible explanation for the potent anti-HIV activity in the RT assay is that the virus replicating in the medium was exposed to relatively higher concentrations of these compounds. Furthermore, on the basis of chemical structure of CP analogs, it is logical to suggest that most likely these compounds inhibited HIV replication by interfering with RT activity (product analog inhibitor). Therefore, levels of RT activity were significantly reduced in the presence of CP inhibitors. Therefore, on the basis of these results, it is obvious that RT assay is the best method for evaluating activity of compounds of this series.

The results of anti-viral screen and cytotoxicity data indicate that carboxyphosphonyl analogs of deoxyribonucleosides (CP-dNs) were selective HIV inhibitors. CP-dNs exhibited potent antiviral activity against HIV-1 IIIB in CBMC cells. However, these compounds were only moderate inhibitors of HIV replication in FIA nd MTT assays. Interestingly, CP-dNs were active against AZT resistant virus replicates. CP analogs of deoxyribonucleosides were found to have low cytotoxicity. The selectivity indices (SI) of all CP-dNs were greater than 1000 when CBMC were used for antiviral assays. SI values of CP-dUrd and CP-dIno were higher than AZT and PFA in HT4-6C cells. In contrast, among the CP-ddN analogs, only CP-AZT showed good anti-HIV activity. All other CP-ddNs were less potent than their corresponding parent compounds. Of considerable interest was the finding that cytotoxicity of CP-ddNs was significantly less compared to ddNs.

ddC induces neuropathy in AIDS patients. The molecular basis of this neurological disorder has been suggested to be inhibition of gamma (γ) DNA
polymerase (Lewis and Dalakas, 1995). Several investigators have proposed that potential of adverse effects of a drug (specifically nucleoside analogs) can be predicted to some extent by undertaking delayed cytotoxicity studies using lymphoblast cell lines in vitro (Chen, et al., 1991; Chen and Cheng, 1989). The studies carried out using CEM cells indicated that CP-dNs as well as CP-AZT were essentially devoid of delayed cytotoxicity. Compared to ddC, these compounds were at least 800 – 8,000 folds less toxic to CEM cells (Figure 3.1-3.3). These results are interesting and suggest that the probability of delayed cytotoxicity arising from CP-dUrd, CP-dIno and CP-AZT is lower, if these compounds are used for the treatment of HIV infection.

Bone marrow toxicity is a serious dose-related side effect of AZT commonly observed in AIDS patients. CP-dUrd and CP-dIno were not very toxic to bone marrow progenitors CFU-GM cells. The inhibitory concentration (IC₅₀) for these compounds for bone marrow cells are approximately 1500 fold higher than the amount required for inhibiting HIV replication. In summary, although these in situ assays do not take into consideration all aspects of in vivo metabolism, nonetheless, these results suggest that neither CP-dUrd nor CP-dIno or CP-AZT is likely to produce serious side effects of neuropathies and bone marrow toxicity manifestations commonly observed when ddN’s are used for the treatment of AIDS.

Review of the chemical structures of potent RT inhibitors indicates that modification of the structure of 2’-deoxyribose group of naturally deoxynucleosides, for example, omitting the 3’-OH group (ddC and ddI), introduction of the azido group to the sugar ring (AZT) and even opening of the ribose ring (PMEA), confers the anti-RT activity. The synthesis of carboxyphosphonyl (CP) analogs was accomplished by introducing the CP group to the 5’-position of deoxyribonucleosides. At present, the
mechanism(s) by which CP analogs elicit antiviral activity remains unknown. However, based on the chemical structure of CP analogs, it is logical to suggest that these compounds inhibit HIV replication most likely by inhibiting the RT activity of the virus. These 5′-CP analogs are structurally similar to the diphosphate forms of deoxynucleosides or dideoxynucleosides. Thus these compounds should be able to interact with HIV RT directly, that is without further metabolic activation. This is possibly one of the mechanisms responsible for anti-HIV activity of CP-analogs. Further studies to support this hypothesis should be carried out using purified RT. Effects of CP-analogs on other unique enzymes, such as, integrase and protease of HIV should also be investigated.

The uptake and efflux of [6-3H]CP-dUrd by CEM cells were very slow. The influence of two negative charges (carboxylate ion) of the CP group in CP-dUrd were most likely the major contributory factors for the poor cellular permeability of this molecule. The in situ metabolic studies of CP-dUrd in CEM cells indicate that CP-dUrd was not metabolized in CEM cells. Thus, the anti-HIV activity observed for CP analogs appears to be intrinsic to the molecule.

Preliminary pharmacokinetic and toxicological studies on CP-dUrd were carried out in mice. CP-dUrd showed a very unique pharmacokinetic profile-characterized by slow absorption, two peak levels in blood and tissues and slow excretion. Most importantly, CP-dUrd was well distributed in the body and high levels of the compound were reached in the brain. If levels attained and maintained in the brain were those of CP-dUrd (uncharged form), then this compound may prove to be useful for the treatment of CNS HIV infection. Administration of a single dose of labeled CP-dUrd (228.5 µmole/kg, 76.8 mg/kg, i.p.) in mice, resulted in the maximum equivalent concentrations
of the compound 194 μM (nmole/ml) and 196 μM (nmole/g) in plasma and brain, respectively. These values are significantly higher than the concentration required to inhibit HIV (ED₅₀: 0.23 μM in RT assay and 22 μM in FIA assay). Preliminary toxicological studies on CP-dUrd indicate that this compound has relative low mammalian toxicity. No mortality or undesirable side effects were observed after administration of CP-dUrd up to 100 mg/kg in mice i.p. LD₅₀ (i.p.) of CP-dUrd in mice is around 1000 mg/kg. Haematological parameters after administration of a single dose of CP-dUrd (1000 mg/kg) were normal. At this stage, one should be very careful to make any final conclusion from this preliminary study, regarding toxicity potential of this compound. Nonetheless, these results are encouraging and suggest that CP-dUrd may have a favorable toxicity profile for in vivo administration on long term basis.

In summary, on the basis of results of acute toxicity and pharmacokinetic studies, the following potential inference can be drawn: (1) Moderate doses of CP-dUrd may be administered on long term basis without inducing toxicities, and (2) Adequate concentrations of the drug (higher than ED₅₀ values) should be readily attained to inhibit viral replication in the body. Thus, CP-dUrd may prove to be an effective and safe anti-HIV agent in vivo.

This conclusion is based on the assumption that CP-dUrd is not metabolized in the body. However, the preliminary analysis of the fate of CP-dUrd indicates that CP-dUrd most likely is decarboxylated and undergoes further phase-I metabolic transformation and then enters the metabolic pool of naturally occurring deoxyribonucleosides in the body. These findings are interesting and can be rationalized on the basis of the chemical structure of the molecule. The analysis of urine samples
showed that approximately 12% of CP-dUrd was excreted in unchanged form. These results suggest that, a certain amount of CP-dUrd escapes phase-I transformation and remains in blood and tissues in active form. The levels of 'active' form of the drug and the duration for which efficient levels are maintained in the plasma and important tissues such as lymphoid tissue and brain are critical for the in vivo efficacy of this compound as an anti-HIV agent. Therefore, further studies on the metabolic fate of CP-dUrd in plasma and tissues at different times post administration should be carried out in animals. In addition, the metabolic pathway and the extent of metabolism of a compound may differ in different species. Thus, further studies of disposition of CP-dUrd should be conducted in other animals, such as primates.

In summary, carboxyphosphonyl derivatives of nucleosides are a novel class of potent and selective anti-HIV agents. Results of biological studies presented in this thesis indicate that this class of compounds has favorable toxicological profiles compared to presently approved drugs, for the treatment of HIV infection. The following studies should be undertaken to determine further therapeutic potential of this class of compounds.

1. In vitro antiviral activity of CP analogs
   (i) Combination chemotherapy: The antiviral activity of CP analogs in combination with RT inhibitors and protease inhibitors should be evaluated.
   (ii) The possibility of development of resistance to CP analog in cell cultures should investigated.

2. Studies on mechanism(s) of anti-HIV activity of CP analogs in situ should be carried out.
3. Further studies on the pharmacokinetic properties of CP-dUrd, especially its metabolic fate in blood, plasma and tissues of mice and other animals should be pursued.

4. Further toxicological studies: subacute and chronic (low dosage administration) should be undertaken to evaluate its safety profile.

5. Development of new anti-HIV compounds: The chemical structure should be modified to improve the cellular permeability and metabolic degradation in vivo.
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APPENDIX

Table 1. Cell lines: properties and sources

<table>
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<tr>
<th>Cell</th>
<th>Description</th>
<th>Source</th>
<th>Reference</th>
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<tbody>
<tr>
<td>HT4-6C (HeLa CD4)</td>
<td>HeLa cells infected with a retroviral vector expressing CD4 and neomycin resistance. This cell line expressed human CD4 protein on the cell surface and can be infected by most HIV-1 isolates.</td>
<td>Dr. Chesebro</td>
<td>Chesebro &amp; Wehrly, 1988</td>
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<tr>
<td>H9</td>
<td>Single cell clone derived from a specific HUT-78 cell line. H9 was selected for high yield permissive growth with HIV-1.</td>
<td>NIH¹</td>
<td>Mann et al., 1989; Popovic et al. 1984a; 1984b</td>
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<tr>
<td>HUT 78</td>
<td>Human cutaneous T-cell lymphoma derived from PBMCs of a patient with Sezary syndrome.</td>
<td>ATCC²</td>
<td>Gazdar et al., 1980</td>
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<tr>
<td>MT-2</td>
<td>Human T cell leukemia cells isolated from cord blood lymphocytes and cocultured with cells from patients with adult T-cell leukemia.</td>
<td>ATCC</td>
<td>Haertle et al., 1988; Harada et al., 1985</td>
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<tr>
<td>MT-4</td>
<td>Human T cells isolated from a patient with adult T-cell leukaemia. HTLV-1-transformed.</td>
<td>NIH</td>
<td>Larder et al., 1989a; Pauwels et al., 1987</td>
</tr>
<tr>
<td>CBMC</td>
<td>Human cord blood mononuclear cells HIV-seronegative donors</td>
<td>Salomon et al., 1994</td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>Human blood monocyte macrophages HIV-seronegative donors</td>
<td>Lazdins et al., 1990</td>
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<tr>
<td>CEM</td>
<td>T lymphoblastoid cell line isolated from peripheral blood buffy coat of a 4-year-old female with acute lymphoblastic leukemia.</td>
<td>Dr. Warrington</td>
<td>Foley et al., 1965</td>
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2. ATCC: American Type Culture Collection, 1992
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<th>Virus</th>
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<td>HIV-1</td>
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<tr>
<td>ADA-M monocyto-</td>
<td>From seropositive AIDS patient</td>
<td>Dr. Gendelman</td>
<td>Gendelman et al., 1988; 1989</td>
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<tr>
<td>tropic virus</td>
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<td></td>
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<tr>
<td>RF</td>
<td>From PBMC</td>
<td>MRC AIDS¹ Reagent Project</td>
<td>Popovic, M. et al., 1984a; 1984b</td>
</tr>
<tr>
<td>IIIB (HTLV-IIIB/H9)</td>
<td>Concentrated culture fluids of peripheral blood or bone marrow from several patients with AIDS or related disease were used to establish a permanent productive infection in H9 cells.</td>
<td>NIH²</td>
<td>Popovic, M. et al., 1984a; 1984b</td>
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<td>MO</td>
<td>From an AIDS patient.</td>
<td>The Center for Disease Control, Ottawa, Ontario, Canada</td>
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<td>1075</td>
<td>Clinically derived AZT intermediate resistant strain. Genotype: RT site 215 mutant. AZT IC₅₀: 0.22 µM³</td>
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<td>Larder &amp; Kemp. 1989b</td>
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<td>Larder &amp; Kemp. 1989b</td>
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<tr>
<td>RTMC (ADP/141)</td>
<td>AZT resistant strain. Genotype: RT site 67, 70, 215, 219 mutant</td>
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<td>EHO</td>
<td>From AIDS patients.</td>
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<td>Rey et al., 1989</td>
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<td>ROD</td>
<td>From West African AIDS patients</td>
<td>MRC AIDS Reagent Project</td>
<td>Clavel et al., 1986</td>
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1. Medical Research Council AIDS Reagent Project, National Institute for Biological Standards and Control, United Kingdom.
2. NIH: National Institutes of Health, AIDS Research and Reference Reagent Program Catalog, 1998
3. IC₅₀: Values were obtained by plaque-reduction assay with HT4-6C cells.
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<th>Compound</th>
<th>Cell type/Virus strain (Method)</th>
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<th>CC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Selectivity index</th>
<th>References</th>
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<td>&gt;187</td>
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<td>JM/HIV-1-GBB (p24)</td>
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<td>1.039</td>
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<td>U937/HIV-1U455(synctium formation)</td>
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<td>&gt;22,000</td>
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</table>

| ddC               | MT-4 /HIV-1 IIIB (CPE)                              | 0.06                  | 37                   | 616              | 1          |
|                   | MT-4 /HIV-1 IIIB (CPE-trypsin blue)                | 0.046                 | 9.1                  | 128              | 8          |
|                   | ATH8 /HIV-1 LAV (CPE)                              | 0.2                   | 35                   | 175              | 3          |
|                   | ATH8 /HIV-1 LAV (CPE)                              | 0.2                   | 20                   | 100              | 9          |
|                   | MT-4 /HIV-1 IIIB (MTT)                             | 0.3                   | 356                  | 1,187            | 10         |
|                   | PBM /HIV-1 LAV (RT)                                | 0.01                  | >100                 | >10,000          | 5          |
|                   | MT-4/HIV-1RF (RT)                                  | 0.001                 | 86                   | 86,000           | 13         |
|                   | JM/HIV-1-GBB(synctium formation)                   | 0.007                 | 7                    | 1,000            | 13         |
|                   | JM/HIV-1-GBB (p24)                                 | 0.002                 | 7                    | 3,500            | 13         |
|                   | U937/HIV-1U455(synctium formation)                  | 0.05                  | 5                    | 100              | 13         |
|                   | U937/HIV-1U455 (p24)                               | 0.05                  | 5                    | 100              | 13         |
|                   | H9/HIV-1 IIIB (synctium formation)                  | 0.013                 | 13                   | 1,000            | 13         |
|                   | H9/HIV-1 IIIB (p24)                                | 0.003                 | 13                   | 433              | 13         |
|                   | C8166/HIV-1 RF (synctium)                          | 0.13                  | 48                   | 369              | 13         |
|                   | C8166/HIV-1 RF (p24)                               | 0.9                   | 48                   | 53               | 13         |
|                   | C8166/HIV-1RF (RT)                                 | 0.03                  | 48                   | 1,600            | 13         |
|                   | CEM/HIV-2 ROD (synctium formation)                  | 0.013                 | 8                    | 615              | 13         |
|                   | PBL/HIV-1 IIIB (p24)                               | 0.003                 | 292                  | 97,333           | 13         |
|                   | PBL/HIV-1 LAV (p24)                                | 0.096                 | 292                  | 3,042            | 13         |
|                   | PBL/HIV-1 MN (p24)                                 | 0.04                  | 292                  | 7,300            | 13         |
|                   | PBL/HIV-1 RF (p24)                                 | 0.009                 | 292                  | 32,444           | 13         |
|                   | PBL/HIV-1 U455 (p24)                               | 0.02                  | 292                  | 14,600           | 13         |
|                   | HT4-6C/HIV-1 LAV<sub>anu</sub> (FIA)                | 0.2                   | -                    | -                | 15         |
|                   | HT4-6C/HIV-2 LAV<sub>anu</sub> (FIA)                | 0.35                  | -                    | -                | 15         |
|                   | HT4-6C/HIV-1 NL4-3 (FIA)                           | <0.2                  | -                    | -                | 16         |

<p>| ddl               | ATH8/HIV-1 LAV (CPE)                               | 8                     | 1,000                | 125              | 9          |
|                   | PBM /HIV-1 LAV (RT)                                | 4.3                   | &gt;100                 | &gt;23              | 5          |
|                   | MT-4/HIV-1RF(MTT)                                  | 8.5                   | 833                  | 98               | 13         |
|                   | MT-4/HIV-1RF (RT)                                  | 0.68                  | 833                  | 1,225            | 13         |
|                   | JM/HIV-1-GBB(synctium formation)                   | 3.2                   | 1,111                | 347              | 13         |
|                   | JM/HIV-1-GBB (p24)                                 | 4.4                   | 1,111                | 252              | 13         |
|                   | U937/HIV-1U455(synctium formation)                  | 11.6                  | 392                  | 34               | 13         |
|                   | U937/HIV-1U455 (p24)                               | 4.5                   | 392                  | 87               | 13         |
|                   | H9/HIV-1 IIIB (synctium formation)                  | 0.21                  | 1,001                | 4,767            | 13         |
|                   | H9/HIV-1 IIIB (p24)                                | 0.08                  | 1,001                | 12,512           | 13         |
|                   | C8166/HIV-1 RF (synctium)                          | 2.3                   | 967                  | 420              | 13         |
|                   | C8166/HIV-1RF (p24)                                | &gt;21                   | 967                  | -                | 13         |
|                   | C8166/HIV-1RF (RT)                                 | 0.62                  | 967                  | 1,560            | 13         |</p>
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*The values indicated in the following tables are: ED_{50}: The concentration required to reduce 50% of viral replication. CC_{50}: the concentration required to inhibit 50% cell growth. SI: selectivity index: CC_{50}/ED_{50}.

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13. J.A. Coates et al., Antimicrobial Agents and chemotherapy 1992;36:733-739
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