IN VITRO CHARACTERIZATION OF
LAMIVUDINE (3TC) RENAL TRANSPORT

by

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A thesis submitted in conformity with the requirements for the
degree of Master of Science
Graduate Department of Pharmaceutical Sciences
Faculty of Pharmacy
University of Toronto

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3TC, a cytidine dideoxynucleoside analog, is commonly used in combination with other antiretroviral drugs in the treatment of HIV-1 infection. In humans, 3TC is highly (~70-80%) excreted unchanged in urine via glomerular filtration and tubular secretion. We hypothesized that similar to other dideoxynucleoside analog drugs (i.e., zidovudine and zalcitabine), that the renal transport of 3TC may involve multiple membrane carrier systems (i.e., organic cation, anion, and/or nucleoside systems). The objectives of this work were i) to characterize the transport properties of 3TC by a continuous renal epithelial cell line (i.e., LLC-PK₁) grown as a monolayer on an impermeable plastic surface and ii) to explore the possible involvement of P-glycoprotein (P-gp) in the renal tubular transport of 3TC. The uptake of 3TC (5 μM) by LLC-PK₁ monolayer cells was temperature dependent, saturable ($K_m = 4.6 \pm 0.7$ mM and $V_{max} = 2042 \pm 172$ pmol/mg protein/ml/min), and Na⁺-independent. Although 3TC uptake by the monolayer cells was not significantly altered in the presence of various purine and pyrimidine nucleosides and nucleoside transport inhibitors, a number of endogenous and exogenous cation probes and inhibitors (i.e., choline, guanidine, spermine, thiamine, amantadine, NMN, mepiperphenidol, nicotine, quinine, TEA, TMP, and verapamil) significantly inhibited 3TC uptake by the monolayer cells. In addition, an outwardly directed proton gradient generated by a standard NH₄Cl protocol significantly enhanced 3TC uptake suggesting the involvement of an organic
cation/H⁺ exchanger. Dideoxynucleoside analogs, including 3TC, did not significantly alter the accumulation of vinblastine, a well-known P-gp substrate, by CHR/C5 monolayer cells known to highly express P-gp. In summary, 3TC renal transport appears to be mediated by an organic cation/proton exchanger but does not involve P-gp and nucleoside transporters.
This thesis is dedicated to:

My family,

especially,

My Mom, Kit-Yee Tam,

My Sister, Flossie Leung

and

My Fiancée, Janet Albright
ACKNOWLEDGMENTS

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<th><strong>Abbreviation</strong></th>
<th><strong>Full Name</strong></th>
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<tr>
<td>3TC</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ARC</td>
<td>AIDS related complex</td>
</tr>
<tr>
<td>BBM</td>
<td>Brush border membrane</td>
</tr>
<tr>
<td>BLM</td>
<td>Basolateral membrane</td>
</tr>
<tr>
<td>CH\text{\textsuperscript{R}}C5</td>
<td>Chinese hamster ovary colchicine resistant cell line</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>$D$</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>DADs</td>
<td>Dideoxynucleoside Analog Drugs</td>
</tr>
<tr>
<td>d4T</td>
<td>Stavudine</td>
</tr>
<tr>
<td>ddI</td>
<td>Didanosine</td>
</tr>
<tr>
<td>ddC</td>
<td>Zalcitabine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>env</td>
<td>envelope</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>gag</td>
<td>Group antigen</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatographic</td>
</tr>
<tr>
<td>$IC_{50}$</td>
<td>50% Inhibitory concentration</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Affinity constant</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>LLC-PK\textsubscript{1}</td>
<td>Porcine pig kidney cell line</td>
</tr>
<tr>
<td>NBMPR</td>
<td>Nitrobenzylthioinosine</td>
</tr>
<tr>
<td>NMN</td>
<td>$N^1$-Methylnicotinamide</td>
</tr>
<tr>
<td>NRTIs</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
</tr>
</tbody>
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## ABBREVIATIONS (con’t)

<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>NNRTIs</td>
<td>Non-nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>NT</td>
<td>Nucleoside Transport</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic anion transport</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transport</td>
</tr>
<tr>
<td>OK</td>
<td>Opossum kidney cell line</td>
</tr>
<tr>
<td>PAH</td>
<td>p-aminohippurate</td>
</tr>
<tr>
<td>pKₐ</td>
<td>Ionization constant</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PGL</td>
<td>Progressive generalized lymphadenopathy</td>
</tr>
<tr>
<td>PIs</td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SMX</td>
<td>Sulfamethoxazole</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>Vₘax</td>
<td>Maximum capacity</td>
</tr>
<tr>
<td>ZDV</td>
<td>Zidovudine</td>
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CHAPTER 1

GENERAL INTRODUCTION

1.1. HIV-1 INFECTION

Human immunodeficiency virus-1 (HIV-1) is now known to be the primary cause of one of the most devastating diseases of mankind, AIDS (Acquired Immunodeficiency Syndrome) and/or ARC (AIDS related complex). The first documentation of this disease in the US appeared in the Morbidity and Mortality Weekly Report from the Centers for Disease Control and Prevention in the 1980’s and subsequently increasing number of cases of AIDS have been reported. It was estimated that more than 30 million people were infected with HIV-1 worldwide and more than 44,000 cases of this infection occurred in the US in 1998 (Kahn and Walker 1998). The HIV-related mortality rate is estimated to be 7,000 persons per day. Since the advent of AIDS epidemic, an immense research effort has been undertaken to understand the pathogenesis of HIV-1 infection and explore the feasibility of HIV-1 eradication in infected patients. Highly active antiretroviral therapy (HAART) has become the key factor in the reduction of HIV-1 related mortality and morbidity, hospitalizations, and opportunistic infections.

1.1.1. THE BASIC STRUCTURE OF HIV-1

HIV-1 belongs to a family of retrovirus called Retroviridae in which all members possess the unique enzyme, reverse transcriptase, to facilitate the replication of deoxyribonucleic acid (DNA) copies from their ribonucleic acid (RNA) genomes (Schoub 1995). HIV-1 appears as a
spherical shape of medium size (100-150 nm in diameter) and consists of an outer lipid cell membrane envelope and an inner viral core (Figure 1.1). The outer envelope, a lipid bilayer, consists of 72 external viral protein "spikes" that project into the external medium. Each spike is composed of a knob-like protein called gp120 on the outside and a smaller spike-like protein called gp41 embedded in the membrane. These proteins play a crucial role in the initial attachment of the virus to its target cells (Greene 1997; Schoub 1995). The HIV-1 lipid bilayer is also studded with a number of host proteins (i.e., class I and II histocompatibility antigens). Underneath the outer envelope lies layers of gag proteins (i.e., p17, p9, p24, and p7). The hollow and truncated cone shaped inner core is made of gag p24, which plays an important role in the clinical detection of HIV-1 infection. The inner viral core also encloses two separate copies of viral RNA genome with various preformed viral enzymes (i.e., the reverse transcriptase, integrase, protease, and ribonuclease (Greene 1997; Schoub 1995).

1.1.2. THE GENOME OF HIV-1

Each single-stranded RNA genome (~ 9.2 kilobases) of HIV-1 is composed of three structural genes (i.e., gag, pol, and env) and six regulatory genes (i.e., tat, rev, nef, vif, vpr, and vpu) (Figure 1.2). The gag and env genes encode for the inner nucleocapsid proteins (i.e., p24, p17, p7, and p6) and the outer envelope glycoproteins (i.e., gp120 and gp41) respectively (Greene 1997; Schoub 1995). The pol gene encodes for four functional enzymes (i.e., ribonuclease, integrase, reverse transcriptase, and protease) which are responsible for the cleavage of RNA, integration of viral DNA into host cell’s chromosome, reverse transcription of viral RNA genome, and splitting of proteins into functional smaller fragments (Greene 1997; Schoub 1995). At either end of each HIV-1 genome located a segment of nucleic acid termed
Figure 1.1. Cross-Sectional Schematic Diagram of HIV-1 Virion. Each virion expresses 72 glycoprotein projections composed of gp120 and gp41. Gp41 is a transmembrane molecule that crosses the lipid bilayer of the envelope. Gp120 is noncovalently associated with gp41 and serves as the viral receptor for CD4 on host cells. The viral envelope also contains some host-cell membrane proteins such as class I and class II MHC molecules. Within the envelope is the viral core, or nucleocapsid, which includes a layer of a protein called p17 and an inner layer protein called p24. The HIV-1 genome consists of two single stranded ssRNA, which are associated with two molecules of reverse transcriptase p64 and nucleoid proteins p10, a protease, and p32, an integrase.

(Adapted from Greene 1997; Schoub 1995).
Figure 1.2. Genomic structure of HIV-1.
The nine known genes of HIV-1 are shown, and their recognized primary functions are summarized. The 5' and 3' long terminal repeats (LTRs) containing regulatory sequences recognized by various host transcription factors are also depicted (Adapted from Greene 1997; Schoub 1995)
the long terminal repeat (LTR) which is involved in the regulation of protein expression of the three structural genes.

The positive regulatory genes, *tat, rev, vif,* and *vpu,* when activated, act as a viral transcription activator, a genetic switch to a latent state virus, virion maturation and infectivity promoter, and virion budding enhancer respectively (Greene 1997; Schoub 1995). The negative regulatory gene, *nef,* becomes the target for research because this gene and its product act on the LTR segment to downregulate viral replication by blocking the expression of the structural genes. The remaining negative regulatory gene, *vpr,* may play a role in inhibiting the viral transcription (Frankel and Young 1998).

1.1.3. REPLICATION OF HIV-1

The HIV-1 life cycle follows a sequence of steps, which are regulated by both viral and cellular proteins. The precise ordering of the steps has not been definitively determined and multiple steps are likely to occur in concert. The first event to occur in the replication of HIV-1 is the attachment of the virus to the resting CD4+ T-lymphocyte through the binding of the envelope surface proteins, gp120, and activation of a group of chemokine co-receptors (*i.e.*, CXCR4). Subsequently, a conformational change occurs in gp120 and exposes the gp41 proteins, which mediates the fusion between the viral envelope and the host cell membrane and allows entry of the inner viral core. Once internalized, the viral core breaks open and releases the two single-stranded viral RNA genomes that are then transcribed by the HIV-1 reverse transcriptase to produce viral DNAs. This step is one of the targets for nucleoside and non-nucleoside analog drugs (*Figure 1.3*). The newly formed viral DNA is then incorporated into the host nuclear DNA through the action of integrase (Greene 1997). The integrated viral DNA
Figure 1.3. Schematic diagram of HIV-1 life cycle and indications of target sites for antiretroviral drugs
(NRTIs = Nucleoside reverse transcriptase inhibitors; NNRTIs = Non-nucleoside reverse transcriptase inhibitors; PIs = Protease inhibitors)
(Adapted from Barry et al. 1999)
serves as the template for the synthesis of viral mRNAs which are expressed from the HIV-1 promoter located at the 5’ long terminal repeat (LTR), with tat proteins enhancing the rate of transcription (Frankel and Young 1998). After transcription, the viral mRNAs are transported through the regulation of the rev domain proteins from the nucleus to the cytoplasm where translation occurs (Figure 1.3). The viral protein precursors, localized at the host cell membrane, are later assembled with other domains to give rise to an immature virion, which subsequently obtains its outer membrane envelope, composed of surface gp120 and transmembrane gp41 proteins, by budding through the host cell membrane (Frankel and Young 1998). To become infective, each immature HIV-1 virion undergoes a series of proteolytic cleaving processes mediated by the HIV-1 protease enzyme which can be inhibited by protease inhibitors (Figure 1.3).

1.1.4. TRANSMISSION OF HIV-1

Since the early 1980’s of the AIDS epidemic, sexual promiscuity was alleged to be the major factor in the rapid transmission of HIV-1 infection in the male homosexual community and later heterosexual contact became the most common route of transmission in the Western countries. HIV-1 can be transmitted from person to person through various means (i.e., unprotected sexual contact, sharing of infected needles among intravenous illicit drug users, infected blood products, needle-stick injuries in health care practitioners, vertical transmission during pregnancy and childbirth, and breast-feeding) (Greene 1997; Schoub 1995).
1.1.5. CLINICAL MANIFESTATIONS OF HIV-1

HIV-1 infection is a slow progression disease in humans. HIV-1 attacks mainly two systems of the body, the immune and the central nervous systems, and the disease manifestations are principally the deterioration of these two systems. In the immune system, CD4+ T-lymphocytes are the primary cellular target for HIV-1 with a high affinity constant of 4 nM (Frankel and Young 1998; Pantaleo and Perrin 1998; Greene 1997). The virus also attacks other mammalian cells (i.e., monocytes-macrophages, microglial cells, intestinal cells, and bone marrow progenitors) (Castro et al. 1998; Pantaleo and Perrin 1998; Greene 1997). It has been reported that infected monocytes may serve as a reservoir for HIV-1, allowing the spread of this virus to the brain and other parts of the body (Castro et al. 1998).

The clinical stages of HIV-1 infection include: i) the acute HIV-1 infection, ii) the latent stage, iii) progressive generalized lymphadenopathy (PGL), and iv) AIDS and/or ARC (Schoub 1995). Acute HIV-1 infection often refers to as “seroconversion” illness as the serum of the infected individual starts making HIV-1 antibodies which are associated with high titer HIV-1 replication and induction of an immunological response. It is estimated that 40% to 90% of newly infected individuals will develop this type of illness, which occurs two to four weeks after infection and lasts about one to two weeks (Kahn and Walker 1998; Schoub 1995). Table 1.1 summarizes the symptoms associated with the acute HIV-1 infection. This syndrome is often undiagnosed or misdiagnosed, as HIV-1 antibodies are usually not detectable during the early phase of infection. Early identification of acute HIV-1 infection with pertinent serologic tests (i.e., p24 antigen and viral RNA levels) and early initiation of antiretroviral therapy can prevent the spread of this infection and slow the progression of HIV-1 (Kahn and Walker 1998).
Table 1.1. Frequency of symptoms associated with acute HIV-1 infection

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>% of patients</th>
</tr>
</thead>
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<tr>
<td>Fever</td>
<td>&gt; 80-90</td>
</tr>
<tr>
<td>Fatigue</td>
<td>&gt; 70-90</td>
</tr>
<tr>
<td>Rash</td>
<td>&gt; 40-80</td>
</tr>
<tr>
<td>Headache</td>
<td>32-70</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>40-70</td>
</tr>
<tr>
<td>Pharyngitis</td>
<td>50-70</td>
</tr>
<tr>
<td>Myalgia or arthralgia</td>
<td>50-70</td>
</tr>
<tr>
<td>Nausea, vomiting, or diarrhea</td>
<td>30-60</td>
</tr>
<tr>
<td>Night sweats</td>
<td>50</td>
</tr>
<tr>
<td>Aseptic meningitis</td>
<td>24</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>10-20</td>
</tr>
<tr>
<td>Genital ulcers</td>
<td>5-15</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>45</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>40</td>
</tr>
<tr>
<td>Elevated hepatic-enzyme levels</td>
<td>21</td>
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</table>

(Adapted from Kahn and Walker 1998)
After the symptoms of the acute HIV-1 infection subside, infected individuals enter the latent stage where the immune system appears to function normally. At this stage, the patients may be asymptomatic for up to an average of ten years before the progression to AIDS (Schoub 1995). As the disease progresses, the serum viral RNA levels increase significantly and the immune function becomes severely suppressed. The patients begin to experience clinical signs and symptoms (i.e., lymphadenopathy and opportunity infections), corresponding to the PGL stage. However, many HIV-1 infected individuals may bypass the PGL stage and experience directly the later stage of this disease – AIDS (Schoub 1995). As HIV-1 disease progresses and the decline in CD4⁺ T-lymphocytes becomes significant, patients are increasingly more susceptible to suffer from a variety of opportunistic infections. Table 1.2 lists the common infections seen in HIV-1 infected patients.

1.1.6. TREATMENT OF HIV-1 INFECTION

The assessment of HIV-1 disease progression and therapeutic efficacy has been shifted from the traditional clinical end points (i.e., disease progression and/or mortality rate) to surrogate markers (i.e., viral load/serum HIV-1 RNA assays, CD4⁺ cell count, and p24 antigen). Currently, HIV-1 clinical trials have been relying on a reduction in the viral load as an important indicator of therapeutic efficacy. The updated recommendations for initiating antiretroviral treatment suggest that antiretroviral therapy should be administered in patients with a serum HIV-1 RNA level higher that 5,000 to 20,000 copies/ml, regardless the CD4⁺ counts (Carpenter et al. 1998; Guidelines 1998). Effective HIV-1 treatment should reduce a patient’s viral load by at least 10 fold after three to four weeks of treatment. The current therapeutic goal is to decrease
Table 1.2. Clinical manifestations commonly observed in HIV-1 Infected Patients

<table>
<thead>
<tr>
<th>Disease</th>
<th>Risk of Occurring</th>
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<tbody>
<tr>
<td>Pneumocystis carinii pneumonia</td>
<td>75%</td>
</tr>
<tr>
<td>Mycobacterium avium complex</td>
<td></td>
</tr>
<tr>
<td>Disseminated Tuberculosis</td>
<td>40%</td>
</tr>
<tr>
<td>Toxoplasmosis</td>
<td>25%-50%</td>
</tr>
<tr>
<td>Cerebral</td>
<td></td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>25%-40%</td>
</tr>
<tr>
<td>Retinitis</td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td></td>
</tr>
<tr>
<td>Colitis</td>
<td></td>
</tr>
<tr>
<td>Candidiasis</td>
<td></td>
</tr>
<tr>
<td>Oral (thrush)</td>
<td></td>
</tr>
<tr>
<td>Esophagus</td>
<td></td>
</tr>
<tr>
<td>Vaginitis (women)</td>
<td></td>
</tr>
<tr>
<td>Cryptococcus meningitis</td>
<td>5%-10%</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td></td>
</tr>
<tr>
<td>Chronic Dysentery</td>
<td></td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>27%</td>
</tr>
<tr>
<td>Disseminated</td>
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<td>Herpes simplex</td>
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</tr>
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<td>Oral (fever blisters)</td>
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<tr>
<td>Shingles</td>
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<tr>
<td>Epstein-Barr virus</td>
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<tr>
<td>Oral lesion</td>
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</tr>
<tr>
<td>Kaposi's sarcoma</td>
<td></td>
</tr>
<tr>
<td>Lymphomas</td>
<td></td>
</tr>
</tbody>
</table>

(Adapted from Horowitz et al. 1998)
serum HIV-1 RNA levels as much as possible (< 50 copies/ml), ideally to undetectable levels, four to six months after initiation of a new regimen (Carpenter et al. 1998; Guidelines 1988).

Increasing array of therapeutic agents and effective antiretroviral regimens have become available to HIV-1 patients and clinicians in recent years. Three distinct classes of medications have been well recognized to be the components of initial combination options. These include the dideoxynucleoside analog reverse transcriptase inhibitors (NRTIs), the non-nucleoside reverse transcriptase inhibitors (NNRTIs), and the protease inhibitors (PIs) (Table 1.3). Combination HAART has proven to be the most effective approach to treat HIV-1 infection. Triple drug therapy has now become the standard of care and is far more superior to dual- and monotherapy in delaying the progression of HIV-1 disease. In asymptomatic or initially diagnosed with advanced disease stage patients who have not been exposed to antiretroviral therapy, the preferred initial regimen involves the use of 2 NRTIs and 1 PI with the first alternative therapy being 1 NNRTI instead of 1 PI (Carpenter et al. 1998; Guidelines 1998; Hammer and Yeni 1998). For instance, combinations, such as zidovudine, lamivudine, and indinavir or zidovudine, zalcitabine, and saquinavir, have shown a profound viral suppression in HIV-1 patients (Gulick et al. 1997; Hammer et al. 1997; Collier et al. 1996). Table 1.4 lists the options of initial regimen and its alternative following treatment failure. Although the choices for initial potent, combination regimens are increasing, the regimen choices are relatively limited as a result of cross-resistance, toxicities, tolerance, antagonism, drug interactions and other practical considerations. Intracellular pharmacologic antagonism has been reported with zidovudine and stavudine (Hoggard et al. 1997). As for NNRTs, drug resistance emerges very rapidly (as early as 1 week after initiation of therapy) if they are used alone or with only 1 NRTI. As for PIs, HIV-1 that is resistant to indinavir and ritonavir shows an almost complete cross
Table 1.3. Antiretroviral agents used in the treatment of HIV-1 infection

<table>
<thead>
<tr>
<th>Generic Name (abbreviation)</th>
<th>Trade Name</th>
<th>Standard Dosage</th>
<th>Common Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIDEOXYNUCLEOSIDE ANALOG REVERSE TRANSCRIPTASE INHIBITORS (DADs or NRTIs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abacavir (1592U89)</td>
<td>Ziagen</td>
<td>300 mg twice daily</td>
<td>Hypersensitivity</td>
</tr>
<tr>
<td>Didanosine (ddI)</td>
<td>Videx</td>
<td>200 mg twice daily (125 mg twice daily if patient weighs &lt; 60 kg)</td>
<td>Peripheral neuropathy, pancreatitis</td>
</tr>
<tr>
<td>Lamivudine (3TC)</td>
<td>Epivir</td>
<td>150 mg twice daily</td>
<td>Nausea, headache, fatigue, diarrhea, abdominal pain, dyspepsia</td>
</tr>
<tr>
<td>Stavudine (d4T)</td>
<td>Zerit</td>
<td>40 mg twice daily (30 mg twice daily if patient weighs &lt; 60 kg)</td>
<td>Peripheral neuropathy, pancreatitis (rare)</td>
</tr>
<tr>
<td>Zalcitabine (ddC)</td>
<td>Hivid</td>
<td>=&gt; 60 kg: 0.75 mg 3 times daily &lt; 60 kg: 0.375 mg 3 times daily</td>
<td>Peripheral neuropathy, stomatitis pancreatitis (uncommon)</td>
</tr>
<tr>
<td>Zidovudine (ZDV)</td>
<td>Retrovir</td>
<td>200 mg 3 times daily or 300 mg twice daily</td>
<td>Anemia, neutropenia, nausea, headache</td>
</tr>
<tr>
<td>NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nevirapine</td>
<td>Viramune</td>
<td>200 mg twice daily</td>
<td>Rash</td>
</tr>
<tr>
<td>Delavirdine</td>
<td>Rescriptor</td>
<td>400 mg 3 times daily</td>
<td>Rash</td>
</tr>
<tr>
<td>Efavirenz (DMP-266)</td>
<td>Sustiva</td>
<td>Pending</td>
<td>Rash</td>
</tr>
<tr>
<td>PROTEASE INHIBITORS (PIs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saquinavir</td>
<td>Invirase (hard gel) Fortovase (soft gel)</td>
<td>600 mg 3 times daily 1200 mg 3 times daily</td>
<td>Gastrointestinal side effects, hyperglycemia, lipodystrophy</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>Norvir</td>
<td>600 mg twice daily</td>
<td>Nausea, vomiting, diarrhea, anorexia, and abdominal pain; Peripheral and circumoral paraesthesias, altered taste; increased serum levels of triglycerides; elevated liver enzymes</td>
</tr>
<tr>
<td>Indinavir</td>
<td>Crixivan</td>
<td>800 mg every 8 hours</td>
<td>Nephrolithiasis; hyperbilirubinemia, hyperglycemia, lipodystrophy</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>Viracept</td>
<td>750 mg 3 times daily</td>
<td>Diarrhea, hyperglycemia, lipodystrophy</td>
</tr>
<tr>
<td>Amprenavir (141W94)</td>
<td>(investigational)</td>
<td>Pending</td>
<td>Rash</td>
</tr>
</tbody>
</table>

(Adapted from Hovanessian 1999; Havlir and Lange 1998; Maenza and Flexner 1998)
### Table 1.4. Initial combination options of antiretroviral agents

<table>
<thead>
<tr>
<th>Initial Regimen</th>
<th>Alternative Following Treatment Failure</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| NRTI₁ + NRTI₂ + PI₁ | NRTI₁ + NRTI₄ + PI₂  
NRTI₁ + NRTI₄ + PI₂ + NNRTI  
PI₂ + PI₁ + NRTI₂/NRTI₄ + NNRTI | Most experience  
Current first choice  
Applicable to all viral load levels | Regimens complex  
Adherence a challenge  
Failure can compromise future options  
? Long-term toxicities |
| NRTI₁ + NRTI₂ + NNRTI | NRTI₁ + NRTI₄ + PI₁  
PI₁ + PI₂ + NRTI₁/NRTI₄ | Permits deferral of PI  
Reasonable alternative | Potency compared to PI-containing regimen uncertain |
| PI₁ + PI₂ + NRTI₁/NRTI₂ | NRTI₁ + NRTI₂ (or NRTI₁ + NRTI₄) + PI₁ + NNRTI  
NRTI₁ + NRTI₂ (or NRTI₁ + NRTI₄) + PI₁ + PI₂ + NNRTI | Exploits pharmacokinetic interactions  
Potency > PI + 2 NNRTIs?  
Twice-daily regimens | Potential for broad PI resistance  
? Long-term toxicities |
| NRTI₁ + NRTI₂ + NNRT₁ + PI₁ | NRTI₂ + NRTI₁ (or NRTI₁ + NRTI₄) + PI₂ + PI₁ | May improve potency  
Multiple target attack | Multidrug and class resistance at risk |
| NRTI₁ + NRTI₂ + NRTI₃ | PI₁ + PI₂ + NRTI₄  
PI₁ + PI₂ + NNRTI  
PI₁ + PI₂ + NRTI₄ + NNRTI | Single target attack  
Defers use of PIs and NNRTIs | Data still limited regarding potency  
Multi-ddN resistance a risk |

NRTI, NNRTI, and PI indicate nucleoside reverse transcriptase inhibitor, Non-nucleoside reverse transcriptase inhibitor, and protease inhibitor respectively. Numeric subscript (i.e., 1, 2, 3, and 4) corresponds to one particular antiretroviral agent.
(Adapted from Carpenter et al. 1998; Hammer and Yeni 1998)
resistance to saquinavir and nelfinavir (Condra et al. 1996 and 1995). Other factors to consider in choosing an initial combination are listed in Table 1.5.

Until recently, the CDC recommends a 4-week course of zidovudine/lamivudine for most significant postexposure prophylaxis for individuals who had experienced an incident of occupational exposure to HIV-1 (Hovanessian 1999; Carpenter et al. 1998; Guidelines 1998). The addition of a PI to the regimen occurs in relatively high-risk situations (i.e., percutaneous, mucous membrane or skin exposure to HIV-1 infected body fluids containing higher viral titers). Despite the fact that zidovudine is the only antiretroviral drug that has been shown to prevent HIV-1 transmission in humans, didanosine or zalcitabine could be used as an alternative in ZDV-resistant or intolerant patients (Connor et al. 1994).
Table 1.5 Factors involved in the selection of an antiretroviral agent combination

<table>
<thead>
<tr>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient's disease stage</td>
</tr>
<tr>
<td>Clinical status, CD4+ cell count, and HIV-1 RNA plasma level</td>
</tr>
<tr>
<td>Prior treatment history, if any, or chance of acquisition of resistant virus</td>
</tr>
<tr>
<td>Underlying medical conditions</td>
</tr>
<tr>
<td>Patient's commitment to treatment</td>
</tr>
<tr>
<td>Concomitant medications</td>
</tr>
<tr>
<td>Available agents and access</td>
</tr>
<tr>
<td><em>In vitro</em> data concerning synergy or antagonism of combinations under consideration</td>
</tr>
<tr>
<td>Clinical trial research</td>
</tr>
<tr>
<td>Potency of regimen</td>
</tr>
<tr>
<td>Potential for cross-resistance to previously administered or future agents</td>
</tr>
<tr>
<td>Toxicity profile of agents under consideration</td>
</tr>
<tr>
<td>Potential effect on patient's quality of life</td>
</tr>
<tr>
<td>Philosophy of treatment and its goal</td>
</tr>
<tr>
<td>Cost</td>
</tr>
</tbody>
</table>

(Adapted from Hammer and Yeni 1998)
1.2. DIDEOXYNUCLEOSIDE ANALOG DRUGS (DADS)

In the past few years, the focus of the research in our laboratory has been to characterize the membrane transport properties of DADs using in vitro cell culture models. These drugs include: zidovudine (ZDV), didanosine (ddI), zalcitabine (ddC), lamivudine (3TC), stavudine (d4T) and the recently approved abacavir (Figure 1.4). DADs are the first group of agents shown to be effective in the treatment of HIV-1 infection which is characterized by high rates of viral turnover (less than 48 hours) and CD4+ T-cells depletion throughout the disease progression (Ho et al. 1995; Wei et al. 1995). The viral target for this class of drugs is HIV-1 reverse transcriptase, an RNA-dependent DNA polymerase. Standard dosing regimens, common side effects, and pharmacokinetic properties of these agents are summarized in Tables 1.3 and 1.6. The primary focus of this project is to characterize the renal transport properties of 3TC.

1.2.1. LAMIVUDINE (3TC) PHARMACOLOGICAL PROPERTIES

Mechanism of action

3TC, the (-)-enantiomer of the racemic 2'-deoxy-3'-thiacytidine, is a dideoxycytidine analog with a sulfur atom in place of the 3' carbon ribose ring (Figure 1.4) (Coates et al. 1992a). 3TC has been shown to be effective in inhibiting the replication of HIV-1 and HIV-2 in vitro (Coates et al. 1992a; Coates et al. 1992b; Schinazi et al. 1992) and is also potent in suppressing the ZDV resistant HIV-1 isolates (Rooke et al. 1991; Soudeyns et al. 1991) and hepatitis B virus in vitro and in humans (Dienstag et al. 1995; Chang et al. 1992a; Chang et al. 1992b; Doong et al. 1991). 3TC has been shown to be a potent and selective inhibitor of the HIV-1 and HIV-2 infected human peripheral blood lymphocytes, various T-lymphoblastoid cell lines, monocytes, and macrophages by assays that measure HIV-induced syneytium formation, p24 production,
Figure 1.4. Chemical Structures of Dideoxynucleoside Analog Drugs
Table 1.6. Summary of pharmacokinetic properties of dideoxynucleoside analog drugs for HIV-1 infection in humans

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bioavailability (%)</th>
<th>Vd (L/kg)</th>
<th>CSF-Serum Ratio</th>
<th>Half-Life (hr)</th>
<th>Total Body Clearance</th>
<th>Renal Clearance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abacavir (1592U89)</td>
<td>76-100</td>
<td>1.9</td>
<td>0.18</td>
<td>0.98-1.17</td>
<td>nd</td>
<td>20 ml/min/Kg</td>
<td>Kumar et al. 1999; Huges et al. 1999; Ravitch et al. 1998</td>
</tr>
<tr>
<td>Didanosine (ddI)</td>
<td>25-43</td>
<td>1.0</td>
<td>0.21 (adults); 0.46 (children)</td>
<td>1.4</td>
<td>800 ml/min</td>
<td>400 ml/min</td>
<td>Hartman et al. 1990; Yarchoan et al. 1986</td>
</tr>
<tr>
<td>Lamivudine (3TC)</td>
<td>82-88</td>
<td>1.3</td>
<td>0.06</td>
<td>5-7</td>
<td>400 ml/min</td>
<td>260 ml/min</td>
<td>Yuen et al. 1995;</td>
</tr>
<tr>
<td>Stavudine (d4T)</td>
<td>80</td>
<td>0.5</td>
<td>0.55</td>
<td>1.6</td>
<td>594 ml/min</td>
<td>240 ml/min</td>
<td>Browne et al. 1993; Dudley et al. 1992</td>
</tr>
<tr>
<td>Zidovudine (ZDV)</td>
<td>65</td>
<td>1.3 to 1.6</td>
<td>0.15 to 1.35</td>
<td>1.1</td>
<td>1.3 l/min/70 kg</td>
<td>400 ml/min/70 kg</td>
<td>Blum et al. 1988; Klecker et al. 1987</td>
</tr>
<tr>
<td>Zalcitabine (ddC)</td>
<td>80-88</td>
<td>0.6</td>
<td>0.2</td>
<td>1.0-3.0</td>
<td>227 ml/min/m²</td>
<td>190 ml/min/m²</td>
<td>Gustavson et al. 1990; Hartman et al. 1990; Klecker et al. 1987</td>
</tr>
</tbody>
</table>

nd = not determined
HIV-1 reverse transcriptase activity and cell viability (Coates et al. 1992a; Coates et al. 1992b; Schinazi et al. 1992). In vitro mean 50% inhibitory concentration (IC₅₀) of 3TC against a number of strains of HIV-1 and HIV-2 infected CD4⁺ lymphocyte cell lines has been reported to range from 4 nM to 0.67 μM (Coates et al. 1992b). However, 3TC has displayed a more potent inhibition against a range of HIV-1 strains in peripheral blood lymphocytes (mean IC₅₀ = 2.5 to 90 nM) (Coates et al. 1992b).

3TC is phosphorylated intracellularly by deoxycytidine kinase (Shewach et al. 1993; Hart et al. 1992) and subsequently by other human cellular kinase enzymes (Cammack et al. 1992; Hart et al. 1992) to 5' mono-, di- and triphosphate from which the later one being the pharmacologically active form of 3TC (van Leeuwen et al. 1992; Cammack et al. 1992). 3TC 5'-triphosphate inhibits the viral reverse transcriptase by competing with one of its natural substrates, 2'-deoxycytidine-5'-triphosphate, for incorporation into viral DNA. The competitive inhibition constant of 3TC 5'-triphosphate to RNA-dependent DNA polymerase is approximately 10 μM in vitro (Hart et al. 1992). However, 3TC 5'-triphosphate is a relatively weak competitive inhibitor with respect to 2'-deoxycytidine-5'-triphosphate to mammalian DNA polymerases alpha, beta, and gamma (Gray et al. 1995; Hart et al. 1992). In vitro studies with combinations of different nucleoside analogs showed that 3TC at various concentrations (0.06 to 60 μM) did not affect the phosphorylation of 0.02 μM ZDV in freshly isolated peripheral blood mononuclear cells (Veal et al. 1996). In contrast, ddC phosphorylation was significantly impaired by 3TC in these cells and in monocytic and T-lymphoblastoid cell lines (U937 and MOLT-4, respectively) without altering the intracellular ddC accumulations (Veal et al. 1996). The intracellular elimination half-life of 3TC triphosphate is between 10.5 to 15.5 hours in HIV-1 infected cells suggesting that 3TC is not a substrate for cytidine/2'-deoxycytidine deaminase.
(Cammack et al. 1992). Since 3TC triphosphate lacks the 3'-hydroxyl group required for nucleic acid replication, viral DNA chain elongation is terminated, thus preventing HIV-1 replication.

**Resistance**

Although 3TC is a potent inhibitor of ZDV resistant HIV-1 isolates (Rooke et al. 1991; Soudeyns et al. 1991), HIV-1 resistance to 3TC does occur rapidly in vivo with a genomic mutation at codon 184 (Met184 → Val) being detected during both mono- and dual therapy (Bartlett et al. 1996; Goulden et al. 1996; Kuritzkes et al. 1996; Staszewski et al. 1996; Eron et al. 1995; Tisdale et al. 1993). This mutation leads to a 500 to 1000 fold increase in the $IC_{50}$ of 3TC, as well as displays some extent of cross-resistance to both ddI and ddC (Gu et al. 1992). Therefore knowledge of resistance and cross-resistance is vital in the choice of initial and subsequent therapy following treatment failure.

### 1.2.2. LAMIVUDINE (3TC) PHARMACOKINETIC PROPERTIES

**Absorption and Bioavailability**

In humans, following the oral administration of 2 mg/kg, 3TC is well absorbed from the gastrointestinal tract and reaches a peak plasma concentrations of $1.5 \pm 0.5 \mu g/ml$ at 1 to 1.5 hours (Glaxo 1995; van Leeuwen 1992). The bioavailability of 3TC in adults ranges from 82% to 88% for oral dosages of 0.25 to 8.0 mg/kg (van Leeuwen et al. 1992; Yuen et al. 1995). However, in infants and children, the bioavailability of 3TC has been reported to be lower with a mean value of 68% after single oral doses of 0.5 to 10 mg/kg (Lewis et al. 1996). In adults, the bioavailability of 3TC has not been found to be altered by food (Angel et al. 1993).

21
Distribution

3TC is distributed equally into total body water with an apparent volume of distribution of 1.31 L/kg and a total body clearance of 400 ml/min (van Leeuwen et al. 1992). The extent of 3TC serum protein binding is low (less than 34%) (Glaxo 1995). Significant levels of 3TC can be detected in major body organs, including the liver, kidney, and intestine (Perry and Faulds 1997). In HIV-1 infected patients, 3TC can also be detected in the cerebrospinal fluid (CSF). Two hours after an oral administration (8.0 to 20.0 mg/kg/day), the mean CSF-to-serum ratio is 0.06 (0.04 to 0.08) with plasma concentrations ranging from 0.41 to 1.43 μM (van Leeuwen et al. 1995). Similar CSF-to-serum ratios of 3TC have also been reported in HIV-1 infected children (Mueller et al. 1998; Lewis et al. 1996). In a nonhuman primate model predictive of CSF drug penetration in humans, the mean lumbar CSF-to-serum ratio of 3TC (0.41) is reported to be significantly higher than the mean ventricular CSF-to-serum ratio (0.079), suggesting the involvement of an efflux mechanism in the transport of 3TC from the ventricular CSF (Blaney et al. 1995). 3TC can also be detected in amniotic fluid, umbilical cord, and neonatal serum during labor and in breast milk 1-week post partum (Johnson et al. 1999; Moodley et al. 1998).

Metabolism and Elimination

In humans, 5% to 10% of 3TC is metabolized to a pharmacologically inactive trans-sulphoxide metabolite, which has been identified in urine through high-performance liquid chromatographic assay (Plumb et al. 1996). In asymptomatic HIV-1 infected male patients, approximately 70%-80% of 3TC has been reported to be excreted unchanged in the urine (van Leeuwen et al. 1992). 3TC unbound renal clearance (i.e., 210 to 384 ml/min) exceeds the glomerular filtration rate implying that an active secretory process is involved in 3TC clearance (Johnson et al. 1998; van Leeuwen et al. 1992). The mean terminal half-life of 3TC in HIV-1
infected patients was 11.5 hours after a single 300 mg oral dose (Heald et al. 1996), 8.4 to 9.1 hours after various oral formulations of a single 100 mg dose (Yuen et al. 1995) and only 2.5 hours after a single oral dose (range: 0.25 - 8.0 mg/kg) (van Leeuwen et al. 1992). Decreased renal clearance of 3TC in HIV-1 infected patients has been reported to be associated with a decrease in renal function (Johnson et al. 1999; Heald et al. 1996).

The renal disposition of 3TC has been investigated in freshly isolated perfused rat kidney (Sweeney et al. 1995). In this study, the renal clearance of 3TC was found to be nonlinear between 500 ng/ml and 5000 ng/ml and the excretion ratio decreased from 3.67 to 2.49 consistent with a decrease in 3TC secretion. In this model, ZDV, ddI, and ddC did not exert a significant effect on 3TC elimination, but trimethoprim (TMP) induced a reduction of the renal clearance and the excretion ratio of 3TC from baseline (1.25 versus 3.06 and 1.43 versus 3.67, respectively) (Sweeney et al. 1995). In humans, coadministration of trimethoprim-sulfamethoxazole (TMP/SMX) significantly increased the area under the curve and decreased the renal clearance of 3TC by 43% and 35%, respectively (Moore et al. 1996).

1.2.3. CLINICAL USE OF LAMIVUDINE (3TC)

In a phase I/II study, 3TC monotherapy (0.5 to 20 mg/kg/day) had improved the surrogate markers of HIV-1 infection in asymptomatic or symptomatic HIV-1 infected patients with CD4+ counts ≤ 400 cells/μl for up to 12 weeks (Ingrand et al. 1995). Similarly, in another dose range study of 3TC in patients with advanced HIV-1 infection (mean CD4+ count at entry 128 cells/μl) showed that CD4+ cells significantly increased up to 4 weeks when the drug was given in higher doses (8 and 12 mg/kg/day) (Pluda et al. 1995). Moreover, in all treatment groups, significant reductions in p24 antigen levels occurred during the first 4 weeks and continued to decline below
the baseline values up to 32 weeks. Despite the moderate improvement in HIV-1 disease progression from 3TC monotherapy, combinations of antiretroviral drugs have been documented to be superior to monotherapy (de Jong et al. 1996).

Combination regimens of HAAT are now well established as preferable to monotherapy to inhibit viral replication completely and to prevent or delay the emergence of drug resistant HIV-1 mutants. The drug of choice among these DADs depends on the activation state of the infected cells. It has been reported that ZDV and d4T are more efficacious in replicating HIV infected peripheral blood mononuclear cells whereas 3TC, ddC, and ddI are more effective in resting cells (Merrill et al. 1996; Gao et al. 1994). 3TC (150 or 300 mg twice daily) in combination with ZDV (200 mg three times daily) has shown to produce a greater improvement in CD4+ cell counts, decreased serum HIV-1 RNA and p24 antigen levels than ZDV alone or ZDV plus ddC (Bartlett et al. 1996; Katlama et al. 1996; Staszewski et al. 1996; Eron et al. 1995). 3TC has also been administered with other antiretroviral agents (i.e., other nucleoside analog drugs, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors) to manage HIV-1 infection (Pialoux et al. 1998; Gulick et al. 1997). Three drug combination regimens (i.e., indinavir, ZDV, and 3TC or saquinavir, ZDV, and 3TC) showed a significant reduction in HIV-1 RNA load (<50 copies/ml) in both treatment groups at week 24 (Cohen et al. 1999). Gulick and colleagues (1997) have demonstrated that three drug therapy (i.e., indinavir, ZDV, and 3TC) and monotherapy (i.e., indinavir) decreased HIV RNA levels to less than 500 copies/ml in 90% and 43%, respectively in HIV-1 infected patients at week 24. In the ZDV and 3TC group, no change in HIV-1 RNA load was observed.
1.3. Renal Drug Elimination

1.3.1. General Function of the Kidney

Since the primary interest of this work is to investigate the renal tubular transport of 3TC, a brief review on the general function of the kidney and on renal organic ion transport will follow. The primary function of the kidney involves the excretion of end-products metabolites, reabsorption of various nutrients \((i.e.,\text{ amino acids, glucose, and nucleosides})\), and regulation of serum electrolyte concentrations and extracellular fluid volume. Different segments of the nephron are involved to a variable degree in the reabsorption and secretion of nutrients and ions. Approximately, 98% of all reabsorption and secretion processes occur in the renal proximal tubule via multiple facilitated diffusion and active transport systems. A number of physiological substances \((i.e.,\text{ glucose, amino acids, proteins, and nutrients})\) are almost completely reabsorbed by active transport processes at the brush border membrane of the renal proximal tubule. These transport processes may also be involved in drug renal elimination.

Drug transport processes can occur across cell membranes of a number of organs, in particular the kidney. These processes can be mediated by transport proteins that can play a significant role in renal drug elimination \((i.e.,\text{ tubular secretion and reabsorption})\), drug pharmacological effect, drug-drug interactions and drug nephrotoxicity. Transcellular flux of ions in the renal tubules involves the movement of solutes across the basolateral membrane (BLM), accumulation within the tubular cell, and subsequent efflux across the brush border membrane (BBM) into the lumen. Transcellular flux of ions and substrates can involve a saturable carrier mediated transport system \((i.e.,\text{ binding of the substrate to a membrane bound carrier protein})\) and/or a passive nonionic diffusion process. Other factors \((i.e.,\text{ plasma protein})\)
binding, drug $pK_a$, relative lipophilicity, urine pH, and urine flow) influence the overall renal elimination of drugs, particularly the passive reabsorption process (Bendayan 1996).

1.3.2. Renal Tubular Heterogeneity of Organic Ion Transport

In the mammalian kidney, two major types of nephrons exist: i) superficial and ii) juxtamedullary nephrons. The proximal tubular portion of the nephron can be divided into three segments S1, S2, and S3 (Figure 1.5). Each segment of the nephron has the ability to transport an organic ion at a different rate and one type of nephron can transport an organic ion differently from another type (Kosoglou and Vlasses 1989; Berry 1982). It has been reported that the secretion rate of the organic anion, p-aminohippurate (PAH), is five fold greater in the S2 segment than in the S1 and S3 segments in both the superficial and juxtamedullary nephrons (McKinney 1982). The order of transport rate of the organic cation, procainamide, in superficial nephrons is $S_1 > S_2 > S_3$, whereas in juxtamedullary nephrons is $S_1 = S_2 > S_3$ (McKinney 1982). Membrane transporters for organic anions have been suggested to be localized in the S2 segments of the superficial and juxtamedullary nephrons (Shimomura et al. 1981). In superficial nephrons, the organic cation transporters are primarily found in the $S_1$ segment, whereas in juxtamedullary nephrons, they are located in $S_1$ and $S_2$ segments (McKinney 1982).
Figure 1.5. A schematic diagram depicting a juxtamedullary, long-looped nephron (left) and a superficial, short-looped nephron (right). The numbers identify the various nephron segments as follows: 1-renal corpuscle; 2-early proximal convoluted tubule; 3-late proximal convoluted tubule; 4-proximal straight tubule; 5-descending thin limb; 6-ascending thin limb; 7-distal straight tubule (medullary thick ascending limb); 8-cortical thick ascending limb; 9-distal convoluted tubule; 10-connecting tubule; 11-cortical collecting tubule (duct); 12-outer medullary collecting duct; 13-inner medullary (papillary) collecting duct.
(Adapted from Kriz and Bankir 1988)
1.3.3. **Organic Cation Transport Systems**

Organic cation secretion by the mammalian kidney was first reported by Rennick *et al.* (1947) and Sperber (1948). Subsequently, various methods have been developed to investigate the renal transport mechanisms of organic ions. These methods include *in vivo* and *in vitro* clearance techniques, the Sperber technique, stop-flow analysis, renal slices, isolated renal tubule suspensions, *in vivo* micropuncture and microperfusion, *in vitro* microperfusion, purified membrane vesicles isolated from whole animal kidney cortex, tissue cell culture, and molecular cloning and functional expression techniques (Bendayan 1996; Zhang *et al.* 1998).

Many endogenous compounds including pharmacological agents and endogenous metabolites are primary, secondary, tertiary, or quaternary amines which are positively charged according to their $pK_a$ values and the physiological pH. Collectively, these compounds are termed organic cations and they include a broad range of commonly used clinical agents (*i.e.*, amantadine, cimetidine, nicotine, procainamide, quinine, quinidine, tetraethylammonium [TEA], TMP, and verapamil) and endogenous agents (*i.e.*, choline, guanidine, $N^1$-methylnicotinamide [NMN], and thiamine) (Bendayan 1996; Zhang *et al.* 1998). Since many of these agents are polar and positively charged at physiologic pH, membrane transporters generally are involved in the absorption, distribution, and elimination of these compounds. Recently, several organic cation transporters, members of the organic cation transport (OCT) protein family, have been cloned from various species, in particular the epithelia of the kidney and they are summarized in Table 1.7.

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Table 1.7.

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Table 1.7. Cloning and characterization of OCT transport proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Cloned Origin</th>
<th>pH Dependence</th>
<th>Membrane Potential Dependence</th>
<th>Transport Substrates</th>
<th>Location</th>
<th>Tissue Distribution</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCTN1</td>
<td>human fetal liver</td>
<td>+</td>
<td>-</td>
<td>TEA</td>
<td>apical</td>
<td>liver, kidney, lung and bone marrow skeletal muscle, prostate, lung, pancreas placenta, heart uterus, spleen and spinal cord lung carcinoma A459, colorectal adenocarcinoma SW480 myelogenous leukemia K-562, HeLa cell S3</td>
<td></td>
</tr>
<tr>
<td>rOCT1</td>
<td>rat kidney</td>
<td>-</td>
<td>+</td>
<td>TEA</td>
<td>basolateral</td>
<td>kidney (high in cortex), liver, intestine</td>
<td></td>
</tr>
<tr>
<td>rOCT2</td>
<td>rat kidney</td>
<td>-</td>
<td>+</td>
<td>TEA</td>
<td>basolateral</td>
<td>kidney only (high in medulla)</td>
<td></td>
</tr>
<tr>
<td>rOCT3</td>
<td>rat placenta</td>
<td>+ ?</td>
<td>(expressed in HeLa cells)</td>
<td>TEA</td>
<td>nd</td>
<td>placenta (abundantly) intestine, heart and brain (moderately) kidney and lung (low); liver (undetectable)</td>
<td></td>
</tr>
<tr>
<td>rOCT1A</td>
<td>rat kidney</td>
<td>nd</td>
<td>nd</td>
<td>TEA</td>
<td>nd</td>
<td>kidney cortex and medulla, and intestine</td>
<td></td>
</tr>
<tr>
<td>OCT2p</td>
<td>LLC-PK1 cells</td>
<td>-</td>
<td>-</td>
<td>TEA</td>
<td>apical</td>
<td>kidney and central nervous region</td>
<td></td>
</tr>
<tr>
<td>rbOCT1</td>
<td>rabbit kidney</td>
<td>-</td>
<td>+</td>
<td>$^{3}$H-MPP$^+$</td>
<td>apical</td>
<td>kidney, liver, and intestine</td>
<td></td>
</tr>
<tr>
<td>hOCT1</td>
<td>human liver</td>
<td>-</td>
<td>+</td>
<td>$^{3}$H-MPP$^+$</td>
<td>nd</td>
<td>liver (primarily)</td>
<td></td>
</tr>
<tr>
<td>hOCT2</td>
<td>human kidney</td>
<td>-</td>
<td>+</td>
<td>$^{3}$H-MPP$^+$</td>
<td>apical</td>
<td>kidney (distal tubule)</td>
<td></td>
</tr>
</tbody>
</table>

*: dependent; -: independent; nd: not determined  
$^{3}$H-MPP$^+$: $^{3}$H-1-methyl-4-phenylpyridinium
Organic cation transport (i.e., TEA or NMN) across the renal tubular BLM can involve a carrier mediated process which is primarily driven by an intracellular negative electrical potential (Figure 1.6) (Besseghir et al. 1990; Sokol and McKinney 1990; Jung et al. 1989; Montrose-Rafizadeh et al. 1989; Besseghir and Roch-Ramel 1987; Wright and Wunz 1987; Takano et al. 1984; Holohan and Ross 1981; Kinsella et al. 1979). A basolateral bicarbonate facilitated organic cation transport has also been demonstrated for amantadine using purified renal cortical rat tubules (Escobar et al. 1994). At the BBM, the efflux of organic cations (i.e., TEA, NMN, cimetidine, and procainamide) into the tubular lumen has been suggested to be coupled to an outwardly directed proton gradient generated by the Na\(^+\)/H\(^+\)-antiporter system (Bendayan et al. 1994 and 1990; Dantzler et al. 1989; Jung et al. 1989; Maegawa et al. 1988; Hsyu and Giacomini 1987; Wright and Wunz 1987; Hori et al. 1985; Inui et al. 1985; McKinney and Kunnemann 1985; Sokol et al. 1985; Wright 1985; Takano et al. 1984; Holohan and Ross 1980). The major role of this antiporter is to regulate the intracellular pH by exchanging sodium ions with protons (Alpern and Chambers 1986; Yoshitomi and Fromter 1984; Kinsella and Aronson 1980; Murer et al. 1976). In addition, the maintenance of the intracellular pH is also linked to the basolateral Na\(^+\)/HCO\(_3\)- and Cl\(^-\)/HCO\(_3\)- cotransporters (Akiba et al. 1987; Verkman and Alpern 1987) and the apical H\(^+\)-ATPase pump (Bendayan et al. 1994; Jehmlich et al. 1991). Intracellular sequestration of organic cations in acidic cytoplasmic organelles (i.e., endosomes, lysosomes, and golgi vesicles) has also been suggested (Pritchard et al. 1994; Miller et al. 1993; Van dyke 1990).
Figure 1.6. Model depicting organic cation (NMN\(^{+}\)) transport across the renal proximal tubular cell from the basolateral membrane (BLM) to the brush border membrane (BBM).

(Adapted from Bendayan 1996)
1.3.4. **Organic Anion Transport Systems**

Renal membrane transport of organic anions has been studied extensively using various *in vivo* and *in vitro* models (Ullrich and Rumrich 1993; Moller and Sheikh 1983). Organic anions include PAH, lactate, penicillin, salicylic acid, sulfate, urate, and probenecid, a standard organic anion transport inhibitor (Ullrich and Rumrich 1993; Moller and Sheikh 1983; Sheikh and Maxild 1978; Weiner et al. 1960). Using PAH as substrate, various *in vitro* studies have demonstrated that the uptake of organic anions at the BLM is mediated by a tertiary active transport mechanism which is ultimately stimulated by Na⁺/K⁺-ATPase (Figure 1.7) (Dantzler 1989; Shimada et al. 1987; Sheikh and Maxild 1978). Furthermore, the uptake of PAH at the BLM may involve the exchange of a dicarboxylate (*i.e.*, α-ketoglutarate) which is transported into the cell through a coupling to the electrochemical Na⁺ gradient (Makhuli et al. 1995; Pritchard 1995; Pritchard 1988; Pritchard 1987; Shimada et al. 1987). At the BBM, the efflux of organic anions is mediated by an organic anion exchanger which is sensitive to probenecid, a potent organic anion transport inhibitor (Kinsella et al. 1989; Steffens et al. 1989; Tune et al. 1969). A basolateral PAH sensitive organic anion transport protein termed rOAT1 has recently been cloned from rat kidney (Sekine et al. 1997; Sweet et al. 1997).

Although exogenous substrates, such as drugs and their metabolites, are excreted distinctly by these two systems (*i.e.*, organic cation and organic anion), several studies have revealed the existence of cross reactivity between these transporters (Bendayan et al. 1994; Brandle and Greven 1992; Gisclon et al. 1989; Hsyu et al. 1988). For instance, cimetidine, a known substrate and inhibitor of the organic cation system, can interact *in vitro* with the organic anion system at the BLM (Brandle and Greven 1992). Probenecid has also been shown to decrease the renal excretion of cimetidine in humans (Gisclon et al. 1989).
Figure 1.7. Model depicting organic anion (PAH\(^{-}\)) transport across the proximal tubular cell from the basolateral membrane (BLM) to brush border membrane (BBM). (Adapted from Bendayan 1996)
1.3.5. Nucleoside Transport Systems

Numerous nucleoside transport (NT) proteins have been identified in various tissues and the plasma membranes of specific cells such as erythrocytes (Kwong et al. 1988; Plagemann and Wohlhueter 1980), intestinal (Huang et al. 1993; Jarvis and Griffith 1991; Roden et al. 1991; Jarvis 1989; Vijayalakshmi and Belt 1988; Jakobs and Paterson 1986), lymphocytes (Darnowski et al., 1987), macrophages (Plagemann and Aran 1990), choroid plexus (Wu et al. 1994 and 1992) and a number of tumor cells (Belt et al. 1993; Crawford and Belt 1991; Crawford et al., 1990). NT systems have also been identified in the kidney of various mammalian tissues and cell types and their functional activities have been studied extensively (Brett et al. 1993; Doherty and Jarvis 1993; Gutierrez and Giacomini 1993; Griffith et al. 1992; Gutierrez et al. 1992; Williams and Jarvis 1991; Doherty and Jarvis 1990; Williams et al. 1989; Le Hir and Dubach 1985 and 1984; Angielski et al. 1983; Kuttesch and Nelson 1982). Two major mechanisms for NT have been identified: an equilibrative (facilitated diffusion) and a concentrative (Na+-dependent). The equilibrative systems have been characterized as NBMPR (nitrobenzylthioinosine) sensitive (es) or insensitive (ei) (Wang et al. 1997; Cass 1995; Belt et al. 1993). The es transporters exhibit a high affinity for NBMPR (Kd ~ 0.1-1.0 nM) whereas the ei carriers are insensitive to NBMPR or are inhibited only by high (>10 μM) concentrations of NBMPR (Cass 1995). Both es and ei NT systems transport a diverse group of purine and pyrimidine nucleosides as permeants and are also inhibited by low concentrations of dipyridamole and dilazep (0.1-100 nM) (Cass 1995). Conversely, at least five concentrative NT systems have been identified and classified as: N1 (purine and uridine selective), N2 (pyrimidine and adenosine selective), N3 (purine and pyrimidine nucleosides selective), N4, found only in human kidney (pyrimidine nucleosides, guanosine and adenosine sensitive), and
N5 (both purine and pyrimidine selective as well as NBMPR sensitive) (Wang et al. 1997; Cass 1995). These concentrative systems generally transport nucleosides into the cells by a Na⁺-cotransport process which is driven by the Na⁺/K⁺-ATPase (Wang et al. 1997; Griffith and Jarvis 1996; Cass 1995). The Classification of nucleoside transporters is summarized in Table 1.8.

1.3.6. P-Glycoprotein (P-gp)

Clinical resistance to chemotherapeutic drugs is a major barrier in the success of eradicating cancerous cells and controlling the progression of cancer disease. One form of drug resistance, termed multidrug resistance (MDR), is defined by the ability of cells exposed to a single drug to develop resistance to a wide range of structurally and functionally unrelated drugs. One of the major MDR mechanisms was first proposed in Chinese hamster ovary (CHO) cell mutants to be the increased expression of a cell surface glycoprotein, termed P-glycoprotein (P-gp) which acts as an efflux pump to decrease colchicine intracellular accumulation (Juliano and Ling 1976). Since then, P-gp has been identified in various highly resistant MDR cultured tumor and lymphoblastoid cell lines which show a decrease in cellular accumulation of a number of cytotoxic drugs (i.e., actinomycin D, colchicine, doxorubicin, daunomycin, vincristine and vinblastine (Fardel et al. 1996; Ford and Hait 1990).

P-gp is a 170-180 kDa energy dependent efflux membrane-bound glycoprotein (Doige and Sharom 1992; Hamada and Tsuruo 1988; Horio et al. 1988; Chen et al. 1986; Gerlach et al. 1986). MDR P-gp is encoded by MDR1 gene in humans (Roninson et al. 1986; Ueda et al. 1986; Riordan et al. 1985) and mdr1 and mdr3 in rodents (Gros et al. 1988, 1986a, and 1986b). Structural analyses of MDR using cDNA of P-gp reveal that P-gp consists of four distinct domains: two highly hydrophobic, homologous integral membrane domains and
Table 1.8. Classification of nucleoside transporters

<table>
<thead>
<tr>
<th>Nucleoside Transporter</th>
<th>ex</th>
<th>ei</th>
<th>cif (N1)</th>
<th>cit (N2)</th>
<th>cit (N3)</th>
<th>cit (N4)</th>
<th>cs (N5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBMPR (&lt;1 nM)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NBMPR (&gt;10 μM)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dipyridamole (0.1-100 nM)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dilazep (0.1-100 nM)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sodium Dependency</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

| Substrate Specificity  |    |    |          |          |          |          |          |
| Adenosine              | +  | +  | +        | +        | +        | +        | +        |
| Uridine                | +  | +  | +        | +        | +        | +        | nd       |
| Thymidine              | +  | +  | -        | +        | +        | +        | nd       |
| Formycin B             | +  | +  | +        | -        | +        | -        | -        |
| Inosine                | +  | +  | +        | -        | +        | -        | nd       |
| Guanosine              | +  | +  | +        | -        | +        | +        | nd       |

c = concentrative
e = equilibrative
s = sensitive to NBMPR
i = insensitive to NBMPR
f = purine selective
t = pyrimidine selective
b = broad specificity
+ = permeant
- = not permeant
nd = not determined

(Adapted from Cass 1995)
two hydrophilic nucleotide-binding domains located at the cytoplasmic side of the inner membrane (Chen et al. 1986; Gros et al. 1986b and 1986c) (Figure 1.8). The integral membrane domains function as the binding site(s) and pathway for translocating substrates. Each P-gp domain is made up of six membrane-spanning α-helices linked together by a hydrophilic loop, a total of 12 transmembrane segments (~1280 amino acids) per P-gp (Higgins et al. 1997). It has been suggested that the integral domain of P-gp may possess at least two distinct drug binding sites: one for P-gp substrates and one allosterically-coupled site for P-gp modulators to interact (Higgins et al. 1997). Sharing 30%-40% amino acid sequence, the two nucleotide binding domains of P-gp are responsible for the binding and hydrolysis of ATP and may also be involved in ATP hydrolysis-induced conformational changes of P-gp. The specific functional activity of each P-gp transmembrane segment remains to be elucidated.

Immunohistochemical staining techniques using monoclonal antibodies against P-gp have demonstrated a high protein expression in normal human tissues (i.e., blood brain barrier, adrenal glands, kidney and placenta) (Cordon-Cardo et al. 1990; Sugawara et al. 1988). At a cellular level, P-gp is localized at the biliary canalicular surface of hepatocytes, apical surface of the columnar epithelial cells of jejunum and colon, small pancreatic ductules, and the endothelial cells of the blood brain barrier (Cordon-Cardo et al. 1989; Thiebaut et al. 1987). P-gp has also been found to be expressed in the kidney, especially at the apical membrane of the renal proximal tubular cells (Ernest et al., 1997; Ernest and Bello-Reuss 1995; Lieberman et al., 1989; Thiebaut et al., 1987). Fojo and colleagues (1987) reported an abundant expression of the MDR1 gene in the adrenal, kidney, jejunal, rectal, liver and lung tissues and low levels in the brain and prostate. These findings suggest that P-gp may play a significant role in the clearance of a
Figure 1.8. A primary structure model of MDR transmembrane protein, P-gp. P-gp consists of four distinct domains: two highly hydrophobic integral membrane domains (IMD1 and IMD2) and two hydrophilic nucleoside-binding domains (NBD1 and NDB2). OUT and IN refer to the outside and inside of the cytoplasmic membrane, respectively (Higgins et al. 1997).
number of xenobiotics and in protecting the system from the entry of potential toxic compounds. More importantly, despite the introduction of more potent and effective anticancer drugs and improved chemotherapeutic strategies, the overexpression of P-gp in human cancerous cells remains to be one of the leading factors of chemotherapy failure.

Exogenous agents including anticancer drugs (i.e., vinblastine, doxorubicin, vincristine, and daunomycin), immunosuppressants (i.e., cyclosporin A and FK506), cardiac glycoside (i.e., digoxin) and certain antiretroviral drugs (i.e., protease inhibitors) are among the therapeutic agents that have been identified as being P-gp substrates and/or inhibitors (Lee et al. 1988; Saeki et al. 1993; Tanigawara et al. 1992; Ford and Hait 1990). Data provided from photolabelling studies suggest that P-gp expressed in the human kidney, adrenal glands, and MDR resistant KB cells may possess a similar drug binding site for several structural unrelated organic compounds (i.e., cimetidine and ciprofloxacin) (Ichikawa et al. 1991). P-gp has also been shown to be expressed in a number of cell culture systems including LLC-PK1, CHR5C5, and Caco-2 cells from porcine pig kidney, Chinese hamster ovary and human colorectal adenocarcinoma (Kim et al. 1998; Cavet et al. 1997; Ito et al. 1993; de Lannoy et al. 1992; Tanigawara et al. 1992; Horio et al. 1990). P-gp not only mediates the MDR in tumor cells to a variety of structural unrelated anticancer drugs, but it also appears to participate in normal physiologic processes, including renal and biliary clearance of drugs and their metabolites. Blockade of the transport properties of P-gp could also have a significant impact on drug pharmacokinetic and pharmacodynamic properties. A search for specific inhibitors of P-gp, one of the logical and therapeutic approaches to overcome MDR, has been undertaken to modulate the functional activity of P-gp. Numerous structurally unrelated pharmacologically active agents (i.e., verapamil and cyclosporin A) termed
modulators that are unrelated to cancer therapy have been tested in animals and humans to inhibit the pumping action of P-gp (Lum et al. 1992; Ford and Hait 1990; Kerr et al. 1986).

1.4. TRANSPORT OF DADS ACROSS CELL MEMBRANES

The degree of recovery of unchanged DADs in the urine after an intravenous dose is the highest for 3TC (70-80%) followed by ddC (60%), ddl (55%), d4T (35-40%), ZDV (17-30%) and abacavir (13%) (Beach 1998; Foster and Faulds 1998). In purified renal tubular membrane vesicles isolated from rat kidney cortex, ZDV renal transport is mediated by the organic anion system at the basolateral membrane (Griffiths et al. 1992) and the organic cation system at the brush border membrane of the renal proximal tubule respectively (Griffiths et al. 1991). By measuring the in vivo renal transcellular transit time of ZDV in the presence of cimetidine and probenecid, Aiba and colleagues (1995) also observed that ZDV was transported by the anion and cation systems at the basolateral and brush border membranes. Previously, our lab had also demonstrated the involvement of an organic cation transporter in the renal transport of ZDV (Bendayan et al. 1995). Early reports on ZDV and other 2',3'-dideoxynucleosides have documented that these analogs are primarily transported across various cell membranes (i.e., human erythrocytes and lymphocytes) through non-facilitated transport processes (Domin et al. 1988; August et al. 1991). However, a number of reports have also documented that several dideoxynucleosides including ddl permeate human erythrocyte membranes via multiple transporters (i.e., nucleoside and nucleobase) (Domin et al. 1993). The transport of carbovir, a carboxylic analog of 2',3'-dideoxyguanosine that exhibits in vitro activity against HIV-1 in human erythrocytes, was also shown to be primarily mediated by a nucleobase carrier (nucleoside transport was found to be minor) (Mahony et al. 1992; Gati et al. 1992). In addition,
recombinant Na⁺ nucleoside transporter (rCNT1) expressed in *Xenopus laevis* oocytes has been shown to mediate the intestinal and renal uptake of ZDV, ddC, and uridine (\(K_m = 0.5\) mM, 0.5 mM, 37 \(\mu\)M, respectively) (Yao *et al.* 1996; Huang *et al.* 1994). This transport protein exhibited the transport characteristics of the nucleoside transport system cit (selective for pyrimidine nucleosides and adenosine). ZDV was also found to inhibit uridine transport in oocytes (\(I_{C50} < 1\) mM) and its transport was 77% Na⁺-dependent (Huang *et al.* 1994). A newly cloned renal Na⁺-dependent human nucleoside transporter (hCNT1), which is 83% identical to rCNT1, has also recently been shown to mediate the transport of ZDV (Ritzel *et al.* 1997). Subsequently, the renal transport of ddI had been shown to occur by another cloned Na⁺ dependent human nucleoside transporter (hCNT2) which was 83% and 72% identical to rCNT2 and hCNT1, respectively (Ritzel *et al.* 1998). The expression of hCNT2 could be detected in the kidney and intestine, but not in the liver.

In Caco-2 cells, human erythrocytes and lymphocytes, the transport of ZDV, ddI and d4T occurred primarily by passive diffusion (Sinko *et al.* 1995; Hu 1993; August *et al.* 1991; Zimmerman *et al.* 1987). It has also been reported that d4T intestinal transport could be mediated by an N3 (Na⁺ dependent, formycin B and thymidine selective) nucleoside transporter (Waclawski and Sinko 1996). In the brain, the efflux of ZDV and ddI had been reported to occur via a probenecid sensitive carrier (Takasawa *et al.* 1997).
1.5. GOAL

The goal of this project was to characterize the renal transport properties of 3TC using *in vitro* tissue cell culture systems and to identify, predict and quantitate its renal drug-drug interactions.

1.6. HYPOTHESIS

Since multiple membrane transport systems (*i.e.*, organic cation, nucleoside, and P-gp) have been shown to be involved in the renal transport of DADs (*i.e.*, ZDV and ddC), we hypothesized that these transport systems could also be involved in the renal transport of 3TC. If this is the case, competition and interactions for a common carrier could occur between 3TC and other renally eliminated drugs.

1.8. OBJECTIVES

a) To investigate the renal transport properties of 3TC by a continuous renal epithelial cell line (*i.e.*, LLC-PK1)

b) To determine the involvement of P-gp in the renal transport of 3TC.
CHAPTER 2

METHODS AND MATERIALS

2.1. Tissue Cell Culture

Porcine pig kidney (LLC-PK1) and opossum kidney (OK), two continuous renal epithelial cells, were originally obtained from American Type Culture Collection (Rockville, MD). Multidrug-resistant (CH^{R}C5) Chinese hamster ovary (CHO) cells originally selected from the parent line for resistance to colchicine, were kindly provided from Dr. Victor Ling (Toronto and Vancouver, Canada). Cells were cultured as monolayers on an impermeable surface as previously described (Lui and Bendayan 1998). LLC-PK1 and OK cells were fed with Dulbecco’s Modified Eagle medium (Gibco) containing L-glutamine and D-glucose, 10% fetal bovine serum, and 0.5% penicillin/streptomycin suspension. CH^{R}C5 cells were fed with α-minimum essential medium (Gibco) supplemented with 10% fetal bovine serum, and 0.5% penicillin/streptomycin suspension. Cells were incubated at 37 °C, 95% humidified air and 5% CO₂. When the cells grew to confluency, they were subcultured with 0.05% trypsin-EDTA.

2.2. Uptake Studies

Drug uptake and/or accumulation experiments were performed on confluent monolayer cells (LLC-PK1, 4 days; OK, 7 days; CH^{R}C5, 5 days) seeded on Sarstedt 24 well plastic plates (2 cm² growth area; Figure 2.1) as described previously (Lui and Bendayan, 1998). Cells were seeded at a density of 4×10⁴, 2×10⁴, 5×10⁴ cells/well for LLC-PK1, CH^{R}C5, and OK, respectively. Experiments were initiated by first washing the cells and pre-incubating them for
Uptake Studies

Radiolabelled probes

[Diagram of cell monolayer with apical and basolateral membranes]

**Figure 2.1.** Diagram illustrates the impermeable support (uptake studies). Once confluency is reached, the cells form a monolayer with the apical membrane facing the culture medium and the basolateral membrane attaching to the bottom of each well. For uptake studies, the apical side of the cellular membrane is exposed to various radiolabelled probes.
30 minutes with 0.5 ml of an Earle's balanced saline solution (EBSS) buffer containing (in millimolar concentrations): 1.8 CaCl₂, 5.4 KCl, 0.8 MgSO₄, 138 NaCl, 1.0 Na₂HPO₄, 5.6 D-glucose, 20.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and trizma base as needed to bring the pH to 7.4. Following 30-minute pre-incubation, each uptake or accumulation experiment was initiated by adding to each well 0.5 ml of the same buffer containing 0.5 μCi/ml of [³H]3TC and its unlabelled compound at the desired concentration or 0.1 μCi/ml of [¹⁴C]TEA or 0.08 μCi/ml of [³H]vinblastine. At the specific time interval, the uptake process was terminated by adding 2 ml of ice-cold 0.16 M sodium chloride (stop solution). After removal of the stop solution, the cells were solubilized with 1 ml of 1N sodium hydroxide solution for 30 minutes and were then transferred to scintillation vials containing 0.5 ml of 2N hydrochloric acid solution. To test for uptake specificity, a potential inhibitor was added in the standard incubation medium and compared to control (i.e., inhibitor-free medium). The amount of cell radioactivity was quantitated by standard liquid scintillation techniques using a Beckman LS5000TD scintillation counter. For intracellular acidification experiments, a standard ammonium chloride (NH₄Cl) protocol that generates an outwardly directed proton gradient was used as previously described (Bendayan et al., 1994; Jans et al., 1987). LLC-PK₁ cells were first pre-incubated in EBSS (pH 7.4) and were subsequently pre-incubated in EBSS containing 30 mM NH₄Cl (pH 7.4) for 15 minutes. Immediately after the removal of the NH₄Cl solution, uptake measurements were then undertaken as described above. For studies examining membrane potential effects, the potassium ionophore valinomycin (1μM) was added to EBSS buffer containing 138 mM KCl. Because valinomycin stock solution was prepared in ethanol, we verified the ethanol concentration (i.e., < 0.1%) did not affect the cell viability and 3TC uptake. Non-specific uptake was determined by measuring [³H]3TC uptake in the presence of an excess concentration (5
mM) of the most potent 3TC transport inhibitor we could identify (i.e., quinine). Protein concentrations in cell monolayers were determined by a colorimetric method using Bio-Rad reagent and bovine serum albumin as the standard (Bradford 1976). The counts were corrected for "zero time" uptake and background radioactivity. 3TC uptake by cultured cells was expressed in pmoles/mg protein/ml.

2.3. Metabolism of 3TC

Metabolism of 3TC by LLC-PK₁ cells was assessed by a modified thin layer chromatography (TLC) method as previously described for deoxycytidine and 2',3'-dideoxycytidine (Arner and Eriksson 1993). LLC-PK₁ monolayer cells were incubated for 1, 10, and 30 minutes with 5 μM 3TC (100 μCi/ml) at 37°C. The reaction was terminated as described above. 3TC and cytidine were used as the standards. The cell extracts were chromatographed for 6 hours on silica gel precoated plates (silica gel 60), 250 μm thick (Sigma Chemical, St. Louis, MO) impregnated with a fluorescent indicator. Butan-1-ol saturated with water was used as the solvent. After drying the plate, the zones bearing standards (1 mM) were detected and marked under ultraviolet light. The Rₓ values were 0.5, 0.29, and 0 for 3TC, cytidine, and 3TC nucleotides respectively. Radioactivity associated with these zones was determined by standard liquid scintillation counting techniques.

2.5. Data Analysis

Results are expressed as means ± standard deviation and are the average of at least two separate experiments undertaken in cells pertaining to different passages. Experimental points in an individual experiment were performed in quadruplicate. The kinetic parameters (Kₘ and
and the diffusion coefficient \( (D) \) for 3TC uptake were determined by a non-linear least squares analysis using the computer program Enzyme Fitter 1.0 (Elsevier Biosoft). The \( IC_{50} \) values and other analyses in the Results of Chapter 3 and 4 are estimated using a nonlinear regression analysis program (Sigma Plot 4.0). Student's \( t \) test for unpaired data was applied to compare any two groups in a particular experiment. Analysis of variance (ANOVA) test was applied to determine the significance of the inhibitory effects of various treatments on the uptake of 3TC by the cells. Linear correlation between two parameters was determined by least-squares regression analysis. A \( p \)-value of \(< 0.05 \) was considered statistically significant.

2.6. Materials

\([^3]H\)3TC (12.1 Ci/mmol) was a generous gift from GlaxoWellcome Biochem Inc. (UK); \[^3\]H\)vinblastine (3.5 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA); \[^{14}C\)TEA (2.4 mCi/ml) was purchased from New England Nuclear Life Products (Boston, Mass.); ZDV and 3TC were also gifts from GlaxoWellcome, Inc. (Canada); ddI and ddC were kindly provided by Bristol-Myers Squibb (Syracuse, NY) and Hoffman-LaRoche, (Mississauga, ON) respectively; Bio-Rad reagent was purchased from Bio-Rad (Mississauga, ON). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and are the highest purity available.
2.7. **Tissue Cell Culture Methodologies for the Study of Renal Drug Transport**

Tissue cell culture methodologies have been used in the cellular and molecular studies of the processes involved in the growth and differentiation of mammalian cells (Handler et al. 1980). These techniques are widely used for the investigation of drug permeability, drug metabolism, and mechanisms of drug transport into specific cells and across specific biological barriers (Wilson 1990; Gstraunthaler 1988; Handler 1983). These barriers include the epithelia, a sheet of epithelial cells, found at many sites in the body, especially the kidney. One advantage of epithelial cell culture is that when grown on a permeable or impermeable surface the cells form monolayers of highly differentiated and polarized cells with apical microvilli, apical junctions and basolateral infoldings. Moreover, the epithelia retain many of the morphological and functional properties of intact cell layers that act as barriers to regulate movement of substances in and out of the target organ in situ (Wilson 1990; Gstraunthaler 1988; Handler 1983). Other advantages include minimal variability among experimental samples, relatively large amounts of homogenous material can be produced for experiments, cells can be kept frozen and reused at a later date, and development and study of one single cell type or mutant cell lines (Handler et al. 1980).

2.8. **LLC-PK₁ Cells**

An established continuous renal epithelial cell line, LLC-PK₁, has been widely used as a useful *in vitro* model for the study of renal drug transport and metabolism (Gstraunthaler 1988; Handler 1983). LLC-PK₁ cells are derived from an unknown site of the kidney of a 17-lb juvenile male Hampshire pig to serve initially as host for the growth of viruses (Hull et al. 1976). This continuous cell line has been characterized as epithelial-like in orientation with relatively
large, round to slightly oval shaped nuclei, most of which manifest one to three nucleoli or occasional binucleated expression (Hull et al. 1976). This stable epithelial-like pig kidney cell strain retains a diploid number of chromosomes (38 chromosomes) and has a generation time of 7.5 to 9.0 hr (split ratio of 1 to 15 per week) during its logarithmic growth phase (Hull et al. 1976). Compared to poor growth in a variety of agitated fluid suspension culture conditions, LLC-PK₁ cells can proliferate rapidly in monolayer cell cultures without the appearance of microbial and viral contaminants even at high passages as well as tumor free during the immunosuppressed laboratory animal studies (Hull et al. 1976). Because LLC-PK₁ cell strain is epithelial-like in nature, upon reaching confluency, it forms a monolayer consisting of highly polarized, differentiated epithelium with the basolateral membrane attaching to the growth surface and the apical membrane, consisting of numerous elongated microvilli, facing the culture medium (Figure 2.2a). The maintenance of epithelial cell polarity is partly attributed by the apical junctional complexes at which the apical and basolateral epithelial membranes connect and the cell polarity can be readily disrupted by the effect of chelation (Handler 1985). Another distinct characteristic of confluent LLC-PK₁ monolayer cells is the formation of domes or fluid-filled blisters, caused by a transepithelial vectorial apical to basolateral transport of solutes and water, being entrapped between the cultured cell layer and the water-impermeable culture dish (Figure 2.2b) (Gstraunthaler 1988; Hull et al. 1976). The diameter of the domes varies and is estimated to be in the range of 100-1000 μm and their number can extend from a few per culture to hundreds (Hull et al. 1976).
Figure 2.2. Morphological Studies of LLC-PK₁ Monolayer Cells
(a) Electron scanning micrograph illustrating LLC-PK₁ forms a monolayer consisting of highly polarized, differentiated epithelium with the basolateral membrane attaching to the growth surface and the apical membrane domain, consisting of numerous microvilli, facing the culture medium. (b) Optical micrograph illustrating the formation of domes or fluid-filled blisters once the cells reach confluence.
Confluent LLC-PK₁ monolayer cells express various differentiated properties of the renal proximal tubule, including apical sodium dependent glucose, amino acid, and phosphate cotranports (Rabito and Karish 1982; Rabito and Ausiello 1980), apical and basolateral enzyme marker activities (Rabito et al. 1984), and transepithelial fluid transport (Mullin and O'Brien 1986). LLC-PK₁ cells in confluent monolayers express a Na⁺/K⁺-ATPase activity which has been shown to be located exclusively at the basolateral membrane (Mills et al. 1979).

LLC-PK₁ cells have been a useful model for the study of the transport processes of organic cations (*i.e.*, cimetidine, procainamide, and TEA), nucleosides (*i.e.*, uridine), cardiac glycoside (*i.e.*, digoxin), and cytotoxic agents (*i.e.*, vinblastine; daunorubicin) (Lui and Bendayan 1988; Bendayan et al. 1994; Ito et al. 1993; de Lannoy et al. 1992; Griffith et al. 1992; Takano et al. 1992; Tanigawara et al. 1992; Fauth et al. 1988; Takano et al. 1984). Moreover, it has been suggested that the renal transport of antiretroviral agents (*i.e.*, ZDV and ddC) can be mediated by multiple membrane transporters (*i.e.*, organic cation, organic anion, and nucleoside) (Yao et al. 1996; Aiba et al. 1995; Griffiths et al. 1992 and 1991). The renal organic cation mediated ZDV transport has also been demonstrated in LLC-PK₁ cells grown on permeable membranes (Bendayan et al. 1995). In addition, a low organic anion activity has been detected in LLC-PK₁ cells suggesting that these cells are not a useful model for organic anion transport studies (Miller 1992; Rabito 1986).

### 2.9. Opossum Kidney (OK) Cells

OK cells were derived from kidney tissue of a female American opossum and were initially used for mammalian chromosome studies (Koyama et al. 1978). This continuous monolayer cell line has been described as epithelial-like in morphology. These cells have a
stable nondiploid chromosome number of 23 and have a mean doubling time of 18 hr. Once confluency is reached, these cells are extremely flat and form oval to polygonal shape with 20 to 40 μm in diameter (Koyama et al. 1978). Multinucleated OK cells with diameter up to 100 μm have also been observed. OK cells appear to be ruffling when confluent and domes appear at about 4 to 5 days in medium with or without serum (Leiderman et al. 1989). Ultrastructural examination on confluent OK cells revealed polarized monolayers with the presence of sparsely scattered microvilli and numerous desmosomes between adjacent cells (Koyama et al. 1978). The OK cell line provides an appropriate in vitro model system to study mechanisms of proximal tubular function, specifically in relation to parathyroid hormone sensitive phosphate transport (Leiderman et al. 1989; Caversasio et al. 1986). Transport of endogenous compounds (i.e., amino acid, glucose, sulfate) have also been demonstrated in OK cells (Van den Bosch et al. 1989; Malstrom et al. 1987).

Similar to LLC-PK1, OK cells have been a useful in vitro model for the study of organic cation and anion transport mechanisms. Organic cation transporter mediated uptake of organic cation probes (i.e., NMN and TEA) has been reported in OK monolayer cells (Yuen et al. 1991; McKinney et al. 1990). Carrier mediated PAH transport by OK monolayer cells has established this cell line suitable for the study of organic anion transport (Takano et al. 1994). Recently, a novel nucleoside-sensitive organic cation transporter has been reported to be expressed in OK cells (Chen et al. 1999).

2.10. Chinese Hamster Ovary Colchicine Resistant Cells (CHRC5)

CHRC5 monolayer cells were mutant cells derived from Chinese hamster ovary auxotrophic mutant AUXB1 cells (Ling and Thompson 1974). Compared to parental cells,
CH8C5 cells show a decreased permeability to a number of mitosis arrest agents (i.e., colchicine, vinblastine, and colcemid) (Julian and Ling 1976; Ling and Thompson 1974). Information pertaining to the morphological properties of this cell line is limited. However, these cells appear to be elongated and fibroblast-like in morphology and are considered as euploid with a chromosome number of 22 (Ling and Thompson 1974; Puck et al. 1958). The interest of this cell line began when a surface glycoprotein, now commonly known as P-gp, was discovered to play an important role in the efflux of a number unrelated hydrophobic agents (i.e., colchicine, puromycin, daunomycin, vinblastine, emetine, ethidium bromide and gramicidine D) (Juliano and Ling 1976; Bech-Hansen et al. 1976). Because of the overexpression of P-gp, CH8C5 cells has become one of the useful models for examining the role of P-gp in drug transport (Van der Bliek et al. 1986).
CHAPTER 3

CHARACTERIZATION OF LAMIVUDINE (3TC) RENAL TRANSPORT PROPERTIES
BY LLC-PK₁ MONOLAYER CELLS

Part of this chapter is in preparation for publication.

3.1. ABSTRACT

3TC, a cytidine nucleoside analog, is commonly used in combination with other antiretroviral drugs in the treatment of HIV-1 infection. In humans, 3TC is highly (~70-80%) excreted unchanged in urine via glomerular filtration and tubular secretion. We hypothesized that similar to other nucleoside analog drugs (i.e., zidovudine and zalcitabine), the renal tubular transport of 3TC may involve multiple membrane carrier systems (i.e., organic cation, anion, and/or nucleoside). The purpose of this study was to characterize the transport properties of 3TC by a continuous renal epithelial cell line (i.e., LLC-PK₁) grown as a monolayer on an impermeable support or a permeable membrane filter. 3TC (5 μM) uptake by LLC-PK₁ monolayer cells was saturable (Km = 4.6 ± 0.7 mM and Vmax = 2042 ± 172 pmol/mg protein/min), sodium independent and not significantly altered in the presence of various purine and pyrimidine nucleoside and nucleobase substrates (i.e., adenosine, cytidine, thymidine, guanosine, 2’-deoxyadenosine, adenine, and acyclovir) and nucleoside transport inhibitors (i.e., NBMPR, dipyridamole, and dilazep), this suggesting a nucleoside transporter is not involved in 3TC uptake by renal epithelial cells. However, a number of endogenous and exogenous organic cation probes and inhibitors (i.e., choline, guanidine, thiamine, amantadine, cimetidine,
mepiperphenidol, NMN, quinidine, quinine, spermine, TEA, TMP, and verapamil) significantly reduced 3TC uptake while high concentrations of 3TC inhibited the uptake of the typical organic cation probe TEA. In addition, an outwardly directed proton gradient generated by a standard NH4Cl protocol significantly increased 3TC uptake. Altogether, these results suggest that the renal transport of 3TC may involve an organic cation/proton exchanger.

3.2. INTRODUCTION

Lamivudine (3TC), a human immunodeficiency virus-1’ (HIV-1) reverse transcriptase inhibitor is commonly used in combination with other antiretroviral drugs in the treatment of HIV-1 infection (Eron et al. 1995; Larder et al. 1995; van Leeuwen et al. 1995; van Leeuwen et al. 1992). 3TC is a cytidine nucleoside analog [(-)-2’4’-olty-3’-thiacytidine] (Figure 3.1) that has been shown to be effective in inhibiting the replication of HIV-1 and HIV-2 in vitro (Coates et al. 1992; Schinazi et al. 1992). In addition, 3TC is a potent inhibitor of ZDV resistant HIV-1 isolates (Rooke et al. 1991; Soudeyns et al. 1991) and hepatitis B virus in vitro and in humans (Dienstag et al. 1995; Chang et al. 1992a and 1992b; Doong et al. 1991).

In asymptomatic HIV-1 infected male patients, 3TC has been reported to be primarily excreted unchanged in the urine (approximately 70-80%) through glomerular filtration and tubular secretion (van Leeuwen et al. 1992). 3TC unbound renal clearance (i.e., 210-384 ml/min) exceeds the glomerular filtration rate (around 100 ml/min), which implies that a secretory process is involved in 3TC renal clearance (van Leeuwen et al. 1992). Although very little information is available on the renal transport mechanisms of 3TC, its renal disposition has
Figure 3.1. Chemical Structures of 3TC, cytidine, thymidine and ZDV
been investigated in the isolated perfused rat kidney (Sweeney et al. 1995). In this study, the renal clearance of 3TC was found to be nonlinear between 500 ng/ml and 5000 ng/ml and the excretion ratio decreased from 3.67 to 2.49 consistent with a decrease in 3TC secretion. In this system, trimethoprim (TMP) induced a reduction of 3TC renal clearance and excretion ratio by approximately 60% while zidovudine (ZDV), didanosine (ddI), and zalcitabine (ddC) did not exert any significant effect (Sweeney et al. 1995). In humans, trimethoprim-sulfamethoxazole (TMP/SMX) has also been found to alter the pharmacokinetic properties of oral 3TC (Moore et al. 1996). A 43% increase in area under the curve and a 35% decrease in renal clearance were observed when 3TC was coadministered with TMP/SMX compared with 3TC alone.

It has been reported that the renal transport of dideoxynucleoside analog drugs (i.e., ZDV and ddC) involves multiple membrane transporters (i.e., organic cation, organic anion and nucleoside systems) (Yao et al. 1996; Aiba et al. 1995; Bendayan et al. 1995; Griffiths et al. 1992 and 1991). From the results of a number of in vitro studies, it has been proposed that organic cations permeate the renal BLM via a carrier mediated process driven by the intracellular negative electrical potential (Besseghir et al. 1990; Sokol and McKinney 1990; Jung et al. 1989). At the BBM, the extrusion of organic cations into the tubular lumen is believed to be driven by an electroneutral organic cation/H⁺ exchanger (Bendayan et al. 1994; Dantzler et al. 1989; Jung et al. 1989; Hsyu and Giacomini 1987; Inui et al. 1985). This organic cation/H⁺ exchange process appears to be functionally regulated by both the luminal Na⁺/H⁺-antiporter and the H⁺-ATPase (Bendayan et al. 1994; McKinney and Hosford 1993; Hsyu and Giacomini 1987).

Equilibrative and concentrative (Na⁺-dependent) nucleoside transport (NT) mechanisms have been characterized in various mammalian tissues and cells, particularly in the renal proximal tubule (Cass 1995). The NT equilibrative systems are divided into sensitive (es; Kd ~
0.1-1.0 nM) and insensitive (e.g.; Kd > 10 μM) to nitrobenzylthioinosine (NBMPR) and can facilitate the membrane transport of a diverse group of nucleosides in a bi-directional manner (Cass 1995). At least five concentrative NT systems have been identified and are classified according to their substrate selectivity (i.e., purine or pyrimidine). These concentrative NT systems transport nucleosides into the cells primarily by a Na⁺-cotransport process (Wang et al. 1997; Cass 1995). In the kidney, two Na⁺-dependent nucleoside transporters have been identified to play an important role in the nucleoside salvage process (Wang et al. 1997; Cass 1995; Brett et al. 1993; Griffith et al. 1992). These Na⁺-dependent NT systems are labelled N1 (cif) for purine and N2 (cit) for pyrimidine nucleosides respectively. Uridine, adenosine, and deoxyadenosine appear to be substrates for both transport systems (Wang et al. 1997; Cass 1995). Both systems display a 1:1 Na⁺:nucleoside coupling ratio. A K⁺-dependent uridine nucleoside carrier (with a 2 K⁺:3 uridine stoichiometry ratio) has also been identified in BBM vesicles isolated from rat kidney cortex (Lee et al. 1990 and 1988). An N3 (cib) nucleoside transporter, primarily found in tumor cells, has a 2 Na⁺:1 nucleoside stoichiometry and is permeant to both purines and pyrimidines (Wang et al. 1997; Cass 1995). In human kidney, the transport properties of the N4 nucleoside transporter were similar to the N2 nucleoside transporter (Wang et al. 1997; Cass 1995).

Although it is well established that 3TC undergoes renal tubular secretion in humans and mammals (Sweeney et al. 1995; van Leeuwen et al. 1992), little information is available on the mechanisms by which this occurs. Since 3TC is a nucleoside analog and a zwitterion (pKa 4.3), we hypothesized that its renal transport like for other nucleoside analog drugs may involve multiple renal membrane transporters. In addition, TMP has been reported to inhibit the renal elimination of 3TC (Moore et al. 1996; Sweeney et al. 1995). Since TMP appears to be secreted
by an organic cation transporter (Cacini 1987; Cacini and Myre 1985), drug-drug interactions between 3TC and TMP may occur at the renal proximal tubular site through competition for a common membrane carrier protein (i.e., an organic cation transporter). The purpose of this study was to characterize the renal tubular uptake properties of 3TC using a continuous renal epithelial cell line, LLC-PK₁, grown as a monolayer on an impermeable surface. LLC-PK₁, a continuous renal epithelial cell line, has been widely used as an in vitro model to investigate the renal transport processes of numerous compounds including organic cations (i.e., gentamicin, cimetidine, and TEA), and nucleosides (i.e., ZDV and uridine) (Bendayan et al. 1995; Bendayan et al. 1994; Griffiths et al. 1992; Inui et al. 1985; Handler 1983).

3.3. RESULTS

3.3.1. Time Course and Metabolism of 3TC Uptake by LLC-PK₁ Monolayer Cells

The chemical structure of 3TC is similar to the cytidine nucleoside with a sulfur atom substituted for the 3' carbon in the ribose ring (Figure 3.1). Although 3TC is considered as a weak organic anion (pKa 4.3), it is a zwitterion which possesses a hydroxyl group (acidic group) at the 5' carbon of the ribose ring and an amine group (basic group) at the 4' carbon of the cytosine base. ZDV, a thymidine nucleoside analog, is also a zwitterion with an azide group attached to the 3' carbon ribose ring.

The TLC data confirmed that no significant cell metabolism of 3TC occurred within the first minute of incubation. Following 1, 10, and 30 minute cell incubation with 3TC, 89.3%, 77.7%, 76.8% of the intracellular radioactivity was recovered in the 3TC fraction while the remaining fractions were associated with cytidine and nucleotide derivatives (Figure 3.2).
Figure 3.2. Cellular Metabolism of 3TC by LLC-PK₁ Monolayer Cells
Uptake of 5 μM of 3TC was measured at 1, 10, and 30 minutes at 37 °C in EBSS followed by metabolism studies using TLC method as described in Methods and Materials (Chapter 2). Results are expressed as mean ± standard deviation of a typical representative experiment.
The time course of 5 μM 3TC uptake by LLC-PK₁ monolayer cells was measured in standard incubation medium over two hours at 37 °C (Figure 3.3). 3TC uptake was linear up to 10 minutes (28.5 ± 2.8 pmole/mg protein/ml) and began to equilibrate by 30 minutes (39.0 ± 2.5 pmole/mg protein/ml) (Figure 3.3). Therefore, 1-minute and 10-minute uptakes were used as a representative of the net influx of 3TC into LLC-PK₁ monolayer cells. A 3TC concentration of 5 μM was chosen because it is within the therapeutic range of 3TC serum concentrations observed in humans (Johnson et al. 1999).

3.3.2. Effect of Temperature on 3TC Uptake

The uptake of 3TC (5 μM) was measured over one hour at 37 °C and 4 °C. At 4 °C, 3TC uptake was significantly reduced (Figure 3.4), suggesting the involvement of a temperature dependent uptake process by the monolayer cells.
Figure 3.3. Time Course of 3TC Uptake by LLC-PK₁ Monolayer Cells.
Uptake time profile of 5 μM of 3TC was measured over 2 hr at 37 °C in EBSS. Results are expressed as mean ± standard deviation of five separate experiments. Linear regression analysis was applied for the linear portion of the graph ($r^2 = 0.99$).
Figure 3.4. Effect of Temperature on 3TC Uptake by LLC-PK₁ Monolayer Cells. Uptake of 5 μM of 3TC was measured over 1 hr at 37 °C (●) and 4 °C (○) in EBSS. Results are expressed as mean ± standard deviation of three separate experiments. *Significant difference from control at p < 0.05, as determined by one way ANOVA followed by unpaired Student’s t test.
3.3.3. Kinetics of 3TC Uptake by LLC-PK₁ Monolayer Cells

Ten-minute 3TC uptake by LLC-PK₁ monolayer cells was measured as a function of 3TC concentrations (0.05 to 5 mM) at 37 °C (Figure 3.5). Nonlinear least-squares regression analysis was applied with the use of a computer program (Enzfitter, Elsevier Biosoft). The total rate of 3TC uptake involves a saturable and a nonsaturable component (i.e., diffusion) and can be described by the equation:

\[ V = \frac{V_{\text{max}} \times [3\text{TC}]}{K_m + [3\text{TC}]} + D \times [3\text{TC}], \]

where \( V \) is the total rate of 3TC uptake, \( V_{\text{max}} \) is the maximum rate of carrier mediated transport, \( K_m \) is the affinity constant of the carrier, and \( D \) is the coefficient for the diffusional entry (i.e., non-specific) of 3TC into the monolayer cells. 3TC specific rate (i.e., saturable component) was obtained by subtracting the non-specific (i.e., diffusion component) from the total rate. The estimated kinetic parameters for 3TC were: \( K_m = 4.6 \pm 0.7 \) mM, \( V_{\text{max}} = 2042 \pm 172 \) pmol/mg protein/min, and \( D = 0.07 \pm 0.1 \) pmol/mg protein/min/mM.

3.3.4. Effect of Sodium, Nucleosides, Nucleobases, and NT inhibitors on 3TC Uptake

Since 3TC is a cytidine nucleoside analog and both concentrative and equilibrative NT systems have been identified in the kidney (Wang et al. 1997; Cass 1995; Brett et al. 1993; Griffith et al. 1992), we have investigated the possible involvement of these NT systems on the renal transport of 3TC by LLC-PK₁ monolayer cells. The uptake of \( [3\text{H}]3\text{TC} \) was measured in the presence and absence of sodium. In these experiments, we replaced sodium with N-methyl-D-glucamine. As shown in Figure 3.6, no significant difference was found between the two
Figure 3.5. Concentration Dependency Study of 3TC by LLC-PK₁ Monolayer Cells.
3TC uptake rate was measured at various 3TC concentrations (0.05 to 5 mM) over 10 min at 37 °C in EBSS. The kinetic constants ($K_m$ and $V_{max}$) and the diffusion coefficient ($D$) were determined by a nonlinear least-squares fit of the Michaelis Menten equation with a diffusion component to the data. Specific rate (i.e., saturable component) was calculated by subtracting the non-specific (i.e., diffusion component) from the total rate. A nonlinear least-squares regression analysis was applied with the use of computer software (EnzFitter, Elsevier Biosoft). The linear transformation of data, Lineweaver-Burk Plot, is shown in the inset (Results are the mean of six separate experiments).
Figure 3.6. Effect of Sodium on 3TC Uptake by LLC-PK₁ Monolayer Cells. Uptake of 5 μM 3TC was measured over 1 hr at 37 ºC in EBSS with (●) or without (○) 138 mM Na⁺ (Na⁺ was replaced by equimolar concentrations of N-methyl-D-glucamine). Results are expressed as mean ± standard deviation of two separate experiments. *Significant difference from control at p < 0.05, as determined by one way ANOVA followed by unpaired Student’s t test.
treatments suggesting that 3TC uptake is not Na⁺-dependent. Furthermore, high concentrations of NT inhibitors (i.e., 1 μM NBMPR, 50 μM dipyridamole, and 50 μM dilazep), purine and pyrimidine nucleosides (i.e., 100 μM adenosine, cytidine, thymidine, guanosine, and 2'-deoxyadenosine), and purine nucleobases (i.e., 100 μM adenine and 1 mM acyclovir) had no significant effect on ten minutes 3TC uptake by the monolayer cells (Table 3.1). To further examine the possible involvement of the equilibrative nucleoside carrier in 3TC uptake, we investigated the effect of high concentrations of two NT inhibitors (i.e., 1 μM NBMPR and dipyridamole) in the absence of Na⁺ (i.e., Na⁺ was replaced by equimolar concentration of N-methyl-D-glucamine in the uptake medium) on the uptake of 3TC by LLC-PK₁ monolayer cells. We had also observed no significant changes on 3TC uptake under these conditions (Table 3.2). These data suggest that 3TC uptake by the renal epithelial cells does not involve NT systems.

3.3.5. Effect of Organic Cations on 3TC Uptake

In humans and animals, TMP, an organic cation has been shown to significantly reduce the renal clearance of 3TC (Moore et al. 1996; Sweeney et al. 1995). Therefore we have investigated the effect of various organic cations on 3TC uptake by LLC-PK₁ monolayer cells. Non-specific 3TC uptake was determined by measuring the uptake of [H3]3TC in the presence of an excess concentration (5 mM) of the most potent inhibitor we identified (i.e., quinine). 3TC (5 μM) specific uptake was significantly inhibited by 1 mM of various endogenous and exogenous organic cations (i.e., guanidine, mepiperphenidol, NMN, nicotine, quinine, spermine, TEA, TMP, thiamine, and verapamil) and 250 μM of imipramine (Figure 3.7). Quinine, TMP, and TEA inhibited 3TC uptake in a dose dependent manner with IC₅₀ values of 0.6 mM, 0.63 mM, and 1.9 mM respectively (Figure 3.8). These data suggest that an organic cation carrier
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% 3TC Uptake mean ± SD</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td>Control (5 μM 3TC)</td>
<td>100 ± 0.6</td>
<td></td>
</tr>
<tr>
<td><strong>Dideoxynucleoside Analog Drugs</strong></td>
<td></td>
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<tr>
<td>Zidovudine 1 mM</td>
<td>92.5 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>Didanosine 1 mM</td>
<td>95.5 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>Zalcitabine 1 mM</td>
<td>88.6 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>Abacavir 1 mM</td>
<td>99.0 ± 2.6</td>
<td>ns</td>
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<tr>
<td><strong>Purine Nucleoside Permeants</strong></td>
<td></td>
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</tr>
<tr>
<td>Adenosine 100 μM</td>
<td>100.34 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>2'-Deoxyadenosine 100 μM</td>
<td>105.9 ± 4.4</td>
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</tr>
<tr>
<td>Guanosine 100 μM</td>
<td>105.7 ± 7.8</td>
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<tr>
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<tr>
<td>Cytidine 100 μM</td>
<td>91.3 ± 1.4</td>
<td>ns</td>
</tr>
<tr>
<td>Thymidine 100 μM</td>
<td>96.8 ± 2.5</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Purine Nucleobase Permeants</strong></td>
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<td></td>
</tr>
<tr>
<td>Adenine 100 μM</td>
<td>94.0 ± 8.1</td>
<td>ns</td>
</tr>
<tr>
<td>Acyclovir 1 mM</td>
<td>99.8 ± 7.4</td>
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<td><strong>NT Inhibitors</strong></td>
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</tr>
<tr>
<td>NBMPR 1 μM</td>
<td>98.8 ± 8.2</td>
<td>ns</td>
</tr>
<tr>
<td>Dipyridamole 50 μM</td>
<td>96.6 ± 7.5</td>
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</tr>
<tr>
<td>Dilazep 50 μM</td>
<td>98.8 ± 11.1</td>
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Table 3.1. Effect of Various Dideoxynucleoside Analog Drugs, Nucleosides, Nucleobases, and NT Inhibitors on 3TC Uptake by LLC-PK₁ Monolayer Cells

Uptake of 5 μM 3TC was measured over 10 minutes in standard EBSS media at 37 °C in the presence of dideoxynucleoside analog drugs (i.e., zidovudine, didanosine, zalcitabine, and abacavir), purine and pyrimidine nucleosides (i.e., adenosine, 2'-deoxyadenosine, guanosine, cytidine, and thymidine), purine nucleobases (i.e., adenine and acyclovir), and NT inhibitors (i.e., NBMPR, dipyridamole, and dilazep). Results are expressed as mean ± standard deviation of two separate experiments. *Significant difference from control at $p < 0.05$ as determined by one way ANOVA followed by unpaired Student's $t$ test (ns = not significant)
<table>
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<th>Inhibitor</th>
<th>% 3TC Uptake mean ± SD</th>
<th>Significance</th>
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<tr>
<td>Control (5 μM 3TC)</td>
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<tr>
<td><strong>NT Inhibitors</strong></td>
<td></td>
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<tr>
<td>NBMPR 1 μM</td>
<td>111 ± 6.1</td>
<td>ns</td>
</tr>
<tr>
<td>Dipyridamole 50 μM</td>
<td>95.2 ± 8.9</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 3.2. Effect of NT Inhibitors in the absence of sodium on 3TC Uptake by LLC-PK1 Monolayer Cells

Uptake of 5 μM 3TC was measured over 10 minutes in standard EBSS media at 37 °C in the presence of NT inhibitors (i.e., 1 μM NBMPR and dipyridamole) without Na⁺ (Na⁺ was replaced by equimolar concentrations of N-methyl-D-glucamine). Results are expressed as mean ± standard deviation of one representative experiment.

* Significant difference from control at p < 0.05 as determined by one way ANOVA followed by unpaired Student's t test (ns = not significant)
Figure 3.7. Effect of Various Organic Cations on 3TC Specific Uptake by LLC-PK₁ Monolayer Cells.

Uptake of 5 μM 3TC was measured over 10 minutes at 37 °C in EBSS in the presence of 1 mM of various endogenous and exogenous organic cations (imipramine concentration was 250 μM). Specific uptake of 3TC was determined by subtracting the total 3TC uptake from the non-specific uptake of 3TC obtained in the presence of an excess concentration (5 mM) of the most potent inhibitor we identified (i.e., quinine). Results are expressed as mean ± standard deviation of four separate experiments. *Significant difference from control at \( p < 0.05 \), as determined by one way ANOVA followed by unpaired Student’s \( t \) test.
Figure 3.8. Dose Response Effect of Quinine, TMP, and TEA on 3TC Specific Uptake by LLC-PK₁ Monolayer Cells.
Ten-minute 3TC (5 µM) uptake was measured at 37 °C in EBSS in the absence (control) and presence of the indicated concentrations of quinine (♦), TMP (■), and TEA (▲). Results are presented as percentage of control uptake vs log dose curves and are the mean ± standard deviation of three separate experiments. Nonlinear regression analysis was applied to determine the IC₅₀ values.
may be involved in the renal transport of 3TC. To further examine the possible involvement of an organic cation transporter in 3TC transport by LLC-PK₁ monolayer cells, we measured the uptake of the organic cation probe, [¹⁴C]TEA (40 μM), in the presence of various concentrations of 3TC. 3TC inhibited TEA uptake in a dose dependent manner (IC₅₀ = 0.44 mM) (Figure 3.9).

3.3.6. Effect of an Outwardly Directed Proton Gradient on 3TC Uptake

Since a number of in vitro studies have shown that the transport of organic cations at the luminal membrane of the renal proximal tubule can be stimulated by a proton gradient (Bendayan et al. 1994; Dantzler et al. 1989; Hsyu and Giacomini 1987; Inui et al. 1985), we further examined whether 3TC uptake into LLC-PK₁ monolayer cells involved the counter-transport of protons. The uptake of 5 μM 3TC was measured under control pH conditions (pH 7.4) and after intracellular acidification (pH 6.0-6.5) generated by a standard NH₄Cl protocol (Bendayan et al. 1994; Jans et al. 1987). We have previously shown in our laboratory using the fluorescent probe biscarboxyethylcarboxyfluorescein that LLC-PK₁ cells are acidified by this NH₄Cl protocol (Bendayan et al. 1994). Under acidification conditions, 3TC uptake was significantly enhanced over a fifteen-minute time interval (Figure 3.10) suggesting that a proton exchanger may be involved in the renal transport of 3TC.

To verify that the enhanced uptake of 3TC in the presence of an outwardly directed proton gradient was not a result of membrane potential effects, we measured the uptake of 3TC in the presence of 138 mM KCl with or without valinomycin (1 μM), a potassium ionophore. Figure 3.11 illustrates that 3TC uptake was significantly enhanced when Na⁺ was replaced by K⁺ in the incubation media. It has been shown that in the absence of sodium, the activity of Na⁺/H⁺-antiporter is eliminated and results in a drop in the intracellular pH (Jans et al. 1987). If 3TC
Figure 3.9. Dose Response Effect of 3TC on TEA Uptake by LLC-PK1 Monolayer Cells. Five-minute TEA (40 μM) uptake was measured at 37 °C in EBSS in the absence (control) and presence of the indicated concentrations of 3TC. Results are presented as percentage of control uptake vs log dose curves and are the mean ± standard deviation of three separate experiments. Non-linear regression analysis was applied to determine the IC$_{50}$ value.
Figure 3.10. Effect of an Outwardly Directed Proton Gradient on 3TC Specific Uptake by LLC-PK₁ Monolayer Cells.

Uptake of 5 μM 3TC was measured over 20 min at 37 °C at pH 7.4 (○) and acidification condition (●) in EBSS. An outwardly directed proton gradient was generated by a standard NH₄Cl protocol as described in the Methods and Materials. Results are expressed as mean ± standard deviation of four separate experiments. *Significant difference from control at p < 0.05 as determined by one way ANOVA followed by unpaired Student’s t test.
Figure 3.11. Effect of Potassium on 3TC Uptake by LLC-PK₁ Monolayer Cells.
Uptake of 5 μM 3TC was measured over 10 min at 37 °C in the presence of Na⁺ (●), K⁺ (▽), and K⁺ plus 1 μM valinomycin (□) in EBSS. Equimolar concentrations of potassium replaced sodium in both the incubation and uptake media. Results are expressed as mean ± standard deviation of three separate experiments. *Significant difference from control at $p < 0.05$ as determined by one way ANOVA followed by unpaired Student’s $t$ test.
transport across the apical membrane is coupled to $\text{H}^+$ exchange, replacing $\text{Na}^+$ with $\text{K}^+$ should produce a marked $[\text{H}^+]$ gradient $>$ media leading to an enhanced 3TC uptake (Figure 3.11). We then wanted to verify that the enhanced 3TC uptake in the presence of potassium was not a result of membrane potential effects (i.e., depolarization from the addition of KCl). We repeated these experiments in the presence of valinomycin, a potassium ionophore, and observed similar results implying that the increase in 3TC uptake observed in the absence of sodium was due to the maintenance of intracellular acidification resulting from the absence of $\text{Na}^+$/H$^+$-antiporter activity. These data suggest that like for a number of other organic cations, 3TC uptake by the renal epithelial cells is not dependent on membrane potential but rather coupled to a proton exchange process, i.e., organic cation/H$^+$ exchange.

3.4. DISCUSSION

The renal transport of dideoxynucleoside analog drugs (i.e., ZDV and ddC) can involve multiple membrane transporters (i.e., organic cation, organic anion, and nucleoside systems) (Yao et al. 1996; Aiba et al. 1995; Bendayan et al. 1995; Griffiths et al. 1992 and 1991). In purified membrane vesicles isolated from rat kidney cortex, ZDV inhibited the transport of the organic cation probe, $N1$-methylnicotinamide (NMN), ($IC_{50} = 2.5$ mM) at the brush border site while at the basolateral site, it inhibited the transport of the organic anion probe, p-aminohippuric acid (PAH), ($IC_{50} = 225$ $\mu$M) (Griffiths et al. 1992 and 1991). These results suggest that ZDV may be a substrate for the renal basolateral membrane (BLM) organic anion transporter and the renal brush border membrane (BBM) organic cation transporter. Recent results from in vivo and in vitro work undertaken in rat and pig kidney cell lines further support these findings (Bendayan et al. 1995; Aiba et al. 1995). Cimetidine, an organic cation, and probenecid, a standard organic
anion transport inhibitor, have been shown to significantly reduce the renal excretion of ZDV (Aiba et al. 1995). Furthermore, a number of organic cations (i.e., cimetidine, quinine, quinidine, and TMP) have also been shown to significantly inhibit the basolateral to brush border transcellular membrane transport of ZDV by a continuous renal epithelial cell line (LLC-PK1) grown as a monolayer on a permeable support (Bendayan et al. 1995). In addition, recombinant cDNA encoding a rat intestinal/kidney Na\(^+\)-dependent nucleoside transporter protein (rCNT) when functionally expressed in Xenopus oocytes has shown to mediate the Na\(^+\)-dependent influx of ZDV and ddC with apparent \(K_m\) values of 0.5 mM (Yao et al. 1996).

3TC is an important antiretroviral agent in the treatment of HIV-1 infection as well as hepatitis B infection (Dienstag et al. 1995; Chang et al. 1992a and 1992b; Doong et al. 1991; Johnson et al. 1999; Carpenter et al. 1998). Although in vivo and clinical studies have shown that approximately 70-80% of 3TC is primarily excreted unchanged in urine (Johnson et al. 1999; Moore et al. 1996; Sweeney et al. 1995; van Leeuwen et al. 1992), little information is available on the mechanisms of 3TC renal transport. Since TMP has been reported to interfere with the renal secretion of 3TC in humans (Moore et al. 1996; Sweeney et al. 1995), we hypothesized that this effect could occur through a common membrane transporter (i.e., an organic cation). The objective of our work was to characterize the renal transport properties of 3TC using a continuous renal epithelial cell line (i.e., LLC-PK1 monolayer cells).

Our data show that 3TC uptake by LLC-PK1 monolayer cells is saturable and specific. Kinetic analysis of 3TC uptake revealed a saturable membrane process with a \(K_m = 4.6\) mM and \(V_{max} = 2042 \pm 172\) pmol/mg protein/min, suggesting that a high capacity and low affinity transport system is involved in the renal transport of 3TC.
Although 3TC is a nucleoside analog, our data show that its transport into LLC-PK₁ monolayer cells is neither Na⁺-dependent nor affected by purine and pyrimidine nucleoside substrates (i.e., adenosine, cytidine, 2'-deoxyadenosine, guanosine, and thymidine), purine nucleobases (i.e., adenine and acyclovir) and NT inhibitors (i.e., NBMPR, dilazep, and dipyridamole). We had also observed no significant changes on 3TC uptake by LLC-PK₁ monolayer cells when 3TC uptake was measured in the presence of NBMPR and dipyridamole in the absence of sodium. These data suggest that nucleoside and nucleobase membrane transporters are not involved in the renal transport of 3TC.

We also investigated the effect of several organic cation substrates and inhibitors on 3TC uptake by LLC-PK₁ monolayer cells. Under normal physiological pH conditions, 3TC uptake into LLC-PK₁ monolayer cells was inhibited by a number of endogenous and exogenous organic cations (i.e., choline, guanidine, thiamine, imipramine, mepiperphenidol, NMN, nicotine, quinine, spermine, TEA, TMP, and verapamil). In addition, the organic cation probes (i.e., quinine, TMP, and TEA) inhibited 3TC uptake by LLC-PK₁ monolayer cells in a dose dependent manner. In turn, 3TC is a moderate inhibitor of the prototypical organic cation TEA ($IC_{50}$ 0.44 mM). These results suggest the possible involvement of an organic cation transport system in 3TC uptake. It has been reported that three minute TEA uptake by LLC-PK₁ monolayer cells grown on 24 well plastic plates has an apparent $K_m$ and $V_{max}$ values of 34.0 ± 3.4 μM and 1.7 ± 0.1 pmol/μg DNA, respectively (McKinney et al. 1988). In addition, in purified BBM vesicles isolated from LLC-PK₁ cells, the apparent $K_m$ and $V_{max}$ values of TEA were 1.9 mM and 1.7 nmol/mg protein/min (Inui et al. 1985). Compared to TEA uptake, 3TC has a lower affinity than TEA for the transport system. This lower affinity can explain the observation that the uptake of 40 μM TEA was not completely inhibited by high concentrations of 3TC ($IC_{50}$ = 0.44 mM),
whereas the same effect was observed with 5 μM 3TC specific uptake in the presence of different concentrations of TEA ($IC_{50} = 1.9$ mM). Although TEA is a typical organic cation transport substrate, it has been shown to be a weak inhibitor of the transport of a number of organic cations (i.e., cimetidine and gentamicin), including 3TC (Bendayan et al. 1994; Lui and Bendayan 1998). This may be explained by a different binding site on the same transporter or by the involvement of a different organic cation membrane transporter.

It has been reported that the uptake of TMP (10 μM) into renal cortical slices is significantly inhibited by typical organic cations (i.e., TEA, cyanine 863, and quinine), but not by organic anions (i.e., p-aminohippurate and probenecid), suggesting that TMP is selective for the organic cation system (Cacini and Myre 1985). This uptake process of TMP was found to be active (i.e., energy dependent), temperature sensitive and the slice-to-medium ratio of radiolabelled TMP decreased as the unlabelled TMP in the medium increased, suggesting a saturable component involved. TMP also inhibited TEA uptake but had no effect on PAH. These findings were subsequently confirmed with in vivo TMP excretion studies using the Sperber technique in chickens (Cacini 1987). The tubular excretion ratio of TMP was significantly reduced by quinine but not by probenecid (infusion rate: 1 and 15 μmol/min, respectively). These data led to the conclusion that TMP renal secretion is mediated by an organic cation transporter. Standard therapeutic doses of TMP in healthy humans produce steady state serum concentrations of about 10 μM. As previously mentioned, 3TC undergoes tubular secretion and its renal clearance is significantly reduced by TMP in isolated perfused rat kidney and in humans by 59% and 35% respectively (Moore et al. 1996; Sweeney et al. 1995). Our data shows that TMP reduces the uptake of 3TC in a dose dependent manner with an $IC_{50}$ of 0.63 mM which is 60-fold greater than the normal in vivo TMP levels, it is unlikely that recommended
doses of TMP will produce levels that will be higher than the \( IC_{50} \) value clinically. However, 3TC co-administered with high doses of TMP/SMX in the treatment of opportunistic infections in normal and particularly renal impaired patients could lead to drug interactions of clinical significance.

Findings from numerous *in vitro* studies undertaken in mammalian BBM vesicles and cell lines have suggested that an organic cation/H\(^{+}\) exchanger may be involved in the renal membrane transport of organic cations (i.e., cimetidine, guanidine, NMN, procainamide, and TEA) at the luminal site (Bendayan *et al.* 1994; Dantzler *et al.* 1989; Jung *et al.* 1989; Hsyu and Giacomini 1987; Inui *et al.* 1985). This process is primarily driven by an outwardly directed proton gradient, generated by both the Na\(^{+}/H^{+}\)-antiporter system and the H\(^{+}\)-ATPase pump (Bendayan *et al.* 1994; McKinney and Hosford 1993; Hsyu and Giacomini 1987). According to the results of our previous specificity experiments suggesting the involvement of an organic cation carrier in the renal transport of 3TC, we proceed to explore the effect of intracellular acidification on 3TC uptake by LLC-PK\(_1\) monolayer cells. In the past, several studies using \(^{31}\)P-NMR spectroscopy and fluorescence microscopy techniques have demonstrated that a standard NH\(_4\)Cl loading protocol generates a rapid intracellular acidification in LLC-PK\(_1\) cells (i.e., pH decreased by \(~0.65\) units) with a slow recovery to baseline over 10 to 15 minutes (Bendayan *et al.* 1994; Jans *et al.* 1987). The recovery of intracellular pH to baseline is regulated by the apical Na\(^{+}/H^{+}\)-antiporter (Bendayan *et al.* 1994). Numerous studies have used this intracellular acidification protocol to characterize the driving force for organic cation transport by cultured cells (Lui and Bendayan 1997; Bendayan *et al.* 1994).

Under intracellular acidification conditions, the early uptakes of 3TC were significantly enhanced suggesting a proton exchange process. Because 3TC an amphoteric molecule, having
both amine and hydroxyl groups, and are ionized as mostly zwitterion and anion ($pK_a = 43$), it is unlikely that the enhanced 3TC intracellular accumulation following intracellular acidification condition could be explained by nonionic diffusion effects. In addition, to further assess that the enhanced 3TC uptake by LLC-PK$_1$ cells was due to intracellular acidification and not to membrane potential changes, we investigated the uptake of 3TC in the presence of potassium with or without valinomycin. Similar results were observed with both treatments suggesting that changes in membrane potential cannot account for the observed enhanced 3TC uptake.

Because LLC-PK$_1$ cells do not express organic anion transporters, the possible involvement of these transporters in 3TC renal transport was not examined. However, this question could be further explored in OK cells which have shown to express a PAH transporter (Takano et al. 1994; Hori et al. 1993). P-gp, an energy dependent efflux pump, has been suggested to be involved in the emergence of HIV-1 resistance to DADs. To further investigate this controversial question, we have recently observed that P-gp does not play an important role in the renal transport of dideoxynucleoside analog drugs, including 3TC (Leung and Bendayan 1999 in press, see Chapter 4). In summary, our findings are a reflection of the renal BBM effects since in our model we did not have access to the BLM site of the renal epithelial cells. The renal transport of 3TC appears to be mediated by an organic cation/H$^+$ exchanger in a cultured epithelial system. Nucleoside transporters do not appear to play a significant role in the renal elimination of 3TC. Further investigation is needed to explore the possible involvement of an organic anion transport in 3TC renal tubular elimination.
CHAPTER 4

THE ROLE OF P-GLYCOPROTEIN IN THE RENAL TRANSPORT OF DIDEOXYNUCLEOSIDE ANALOG DRUGS

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4.1. ABSTRACT

P-glycoprotein (P-gp), the MDRI multidrug transporter, is known to be expressed in several human organs and tissues, including at the apical membrane of the renal proximal tubular cells. It has been reported that human immunodeficiency virus 1 (HIV-1) can trigger the expression of P-gp in cultured cells (i.e., H9, a T-lymphocyte cell line, and U937, a monocyte cell line) which may render the cells resistant to antiretrovirals. Since multiple membrane transport systems (i.e., organic cation, organic anion, and nucleoside systems) can be involved in the renal tubular transport of dideoxynucleoside analog drugs (DADs) (i.e., zidovudine and zalcitabine), we have questioned if P-gp is involved in the renal transport of DADs. Chinese hamster ovary colchicine resistant cells (CH₈CS), a cell line that is well known to highly express P-gp and continuous renal epithelial cell lines (LLC-PK₁ and OK) which have also been shown to express P-gp, were used. The accumulation of [³H]vinblastine (20 nM), an established P-gp substrate, by the monolayer cells was significantly enhanced in the presence of two P-gp inhibitors (i.e., verapamil and cyclosporin A) and nucleoside transport inhibitors (i.e., dipyridamole and dilazep). In contrast, DADs (i.e., zidovudine, lamivudine, didanosine, and
zalcitabine) did not significantly affect vinblastine accumulation by these cell lines. These data suggest that P-gp does not play a significant role in the renal tubular transport of DADs. Dipyridamole and dilazep, two nucleoside membrane transport inhibitors, appear to be P-gp inhibitors.

4.2. INTRODUCTION

The MDRI multidrug transporter, P-glycoprotein (P-gp), a 170- to 180-kDa energy dependent efflux membrane-bound glycoprotein, has been found to be expressed in a number of organs and tissues including the brain, liver, intestine, adrenal gland, placenta, and the kidney, especially at the apical membrane of the renal proximal tubular cells (Ernest et al. 1997; Chin et al. 1989; Cordon-Cardo et al. 1989; Lieberman et al. 1989; Fojo et al. 1987; Thiebaut et al. 1987). Although the physiological role of P-gp in normal tissues is unclear, its substrate specificity and distribution suggest that this protein may play a role in the tissue excretion of toxins, hormones and other endogenous compounds as well as a wide range of xenobiotics and their metabolites. Anticancer drugs (i.e., vinblastine, doxorubicin, vincristine, and daunomycin), immunosuppressants (i.e., cyclosporin A and FK506), cardiac glycoside (i.e., digoxin) and certain antiretroviral drugs (i.e., protease inhibitors) are among the therapeutic agents that have been identified as being P-gp substrates and/or inhibitors (Saeki et al. 1993; de Lannoy and Silverman 1992; Tanigawara et al. 1992; Ford and Hait 1990; Lee et al. 1988).

Dideoxynucleoside analog drugs (DADs), also known as HIV reverse transcriptase inhibitors (i.e., zidovudine [ZDV], didanosine [ddI], zalcitabine [ddC], lamivudine [3TC], and stavudine [d4T]), are part of the standard therapy for human immunodeficiency virus 1 (HIV-1) infection (Beach 1998). Failure to antiretroviral therapy in the management of HIV-1 infection
is highly associated with the development of HIV-1 resistant strains (Moyle 1997). One of the proposed mechanisms of cellular resistance to antiretrovirals is the limited accessibility of drugs to the targeting cells and tissues. For instance, HIV-1 protease inhibitors (*i.e.*, indinavir, nelfinavir, and saquinavir) have been reported to have a poor membrane permeability into the GI mucosa as well as the blood brain barrier, this apparently due to the involvement of the *MDRI* transporter (Kim *et al.* 1998; Lee *et al.* 1998).

Although it has been demonstrated that HIV-1 protease inhibitors are substrates of the human multidrug transporter (Lee *et al.* 1998), the involvement of P-gp in the development of drug resistance to DADs remains a controversial issue. It has been reported that HIV-1 could trigger the expression of P-gp in cultured cells (*i.e.*, H9, a T cell line, and U937, a monocyte cell line) (Gollapudi and Gupta 1990). These cell lines show a decrease in ZDV and daunorubicin accumulation when compared to uninfected cells (Gollapudi and Gupta 1990). Induction of the expression of *MDRI* and a decreased cytotoxic effect of vinblastine have also been demonstrated in human lymphoblastoid tumor cells by exposing the cells to ZDV for a short period (Signoretti *et al.* 1997). In addition, Yusa and colleagues have demonstrated that human multidrug-resistant cells (*i.e.*, K562/ADM, CEM/VLB 100 and AdrR MCF-7) show a decreased accumulation of ZDV as compared to the wild type parental cells (Yusa *et al.* 1990). It has also been reported that multidrug resistant lymphoblastoid T-cells (CEM/VBL100) expressing high levels of P-gp are less sensitive to ZDV (Antonelli *et al.* 1992). Moreover, trifluoperazine, a P-gp inhibitor, reverses the low sensitivity effect to ZDV in CEM/VBL100 cells (Antonelli *et al.* 1992). These results suggest that P-gp may be associated with the diminished sensitivity of HIV-1 infected cells to HIV-1 reverse transcriptase inhibitors (*i.e.*, ZDV and ddC) by actively pumping these drugs out from the target cells.
However, results from other \textit{in vitro} and clinical studies have suggested that cellular resistance to DADs (\textit{i.e.}, ZDV, ddI and ddC) does not relate to the expression of P-gp (Glynn and Yazdanian 1998; Andreana \textit{et al.} 1996; Peter and Gambertoglio 1996; Lucia \textit{et al.} 1995; Dianzani \textit{et al.} 1994). For example, using the wild type lymphoblastoid T-cells (\textit{i.e.}, CEM) cultured in the presence of increasing concentrations of ZDV, it was found that these cells become resistant to the antiviral activity of ZDV despite an undetectable cellular level of P-gp (Dianzani \textit{et al.} 1994). Also, studies undertaken on peripheral blood lymphocytes (\textit{i.e.}, CD4$^+$ and CD8$^+$) have demonstrated that DADs do not inhibit or increase the function and expression of P-gp \textit{in vitro} and \textit{in vivo} (Lucia \textit{et al.} 1995; Andreana \textit{et al.} 1996). Furthermore, P-gp expression and ZDV phosphate concentrations in peripheral blood mononuclear cells of HIV-1 infected patients who have received long-term (> 18 months) ZDV therapy were not found significantly different from a group of patients receiving short term (< 2 months) ZDV therapy (Peter and Gambertoglio 1996).

A number of DADs (\textit{i.e.}, ZDV, 3TC, and ddC) are eliminated moderately or highly unchanged by the kidney through glomerular filtration and renal tubular secretion (Beach 1998) and the involvement of multiple membrane transporters (\textit{i.e.}, organic cation, anion, and nucleoside) has been suggested in the renal tubular transport of DADs (Yao \textit{et al.} 1996; Aiba \textit{et al.} 1995; Bendayan \textit{et al.} 1995; Griffiths \textit{et al.} 1992; Griffiths \textit{et al.} 1991). Because of the controversy on the role of P-gp in HIV resistance to the DADs, especially ZDV, we questioned whether P-gp could play a role in the renal tubular elimination of DADs. The purpose of this study was to investigate \textit{in vitro} the possible involvement of P-gp in the renal tubular transport of DADs. Using the continuous Chinese hamster ovary cell line colchicine resistant (\textit{i.e.}, CH$^R$C5), a cell line well known to express high levels of P-gp (Doige and Sharom 1992), and continuous
renal epithelial cell lines (i.e., LLC-PK₁ and OK) also known to express P-gp (Horio et al. 1990), the accumulation of vinblastine, an established P-gp substrate, was determined by the three cell lines grown as a monolayer on an impermeable surface in the presence of various DADs and known P-gp inhibitors.

4.3. RESULTS

4.3.1. Accumulation of vinblastine by CH₂C₅ monolayer cells in the presence of P-gp inhibitors

To verify in our own hands that the CH₂C₅ monolayer cells cultured in our laboratory, exhibit functional expression of P-gp, we determined over time, the accumulation of vinblastine in the presence and absence of well known P-gp inhibitors (i.e., verapamil and cyclosporin A). As shown in Figure 4.1, the accumulation of vinblastine was significantly enhanced by more than 5 fold increase in the presence of these two P-gp inhibitors, this confirming previous observations (Hunter et al. 1993; Shoji et al. 1991; Horio et al. 1990).

4.3.2. Effect of DADs on vinblastine accumulation by CH₂C₅, LLC-PK₁ and OK monolayer cells

The effect of various DADs (i.e., ZDV, 3TC, ddI and ddC) on 1 hr vinblastine accumulation by CH₂C₅, LLC-PK₁ and OK monolayer cells was evaluated at 37 °C. The results from the experiments show that as expected vinblastine has a low accumulation in all the three cell lines, in particular CH₂C₅ cells. Furthermore, the accumulation of 20 nM of [³H]vinblastine by CH₂C₅ monolayer cells (Figure 4.2) was significantly enhanced by 50 µM of verapamil and
Figure 4.1. Effect of Verapamil and Cyclosporin A on Vinblastine Accumulation by CH₈C5 Monolayer Cells.
Accumulation of [³H]vinblastine (20nM) by CH₈C5 monolayer cells was measured in standard medium at 37 °C in the presence of verapamil (50 μM) and cyclosporin A (10 μM) for up to 2 hours. Results are expressed as mean ± standard deviation of at least two separate experiments. *Significant difference from control at p < 0.05 as determined by one way ANOVA followed by unpaired Student's t test.
Vinblastine Accumulation (pmol/mg protein/ml)

ANOVA followed by unpaired Student's t-test. Significant difference from control at $p < 0.05$ as determined by one way ANOVA. Results are expressed as mean ± standard deviation of two separate experiments. Vinblastine accumulation was measured in standard incubation medium at 37°C in the presence of indicated concentrations of various DADs and P-EP.

CH&G Mancosayer Cells

Figure 42. Effect of DADs and P-EP Inhibitors on Vinblastine Accumulation by CH&G Cells.
dipyridamole, two well-known P-gp inhibitors. While verapamil and dipyridamole led to an 8-fold and 15-fold increase on vinblastine accumulation respectively, a high concentration (i.e., 1 mM) of DADs (i.e., ZDV, ddI, ddC, and 3TC) did not have any significant effect, suggesting that DADs may not be inhibitors of P-gp (Figure 4.2). Interestingly, dilazep (50 μM), like dipyridamole, a standard nucleoside membrane transport inhibitor (Cass 1995), led to a significant enhancement of vinblastine accumulation (a 12 fold increase) by CH₈C5 monolayer cells (Figure 4.2). Similar results are obtained in the continuous renal epithelial cell lines (i.e., LLC-PK1 and OK), although the accumulation of vinblastine by the renal epithelial cells is higher than by the CH₈C5 cell line, this reflecting presumably the lower level of P-gp expression by these cell lines (Figures 4.3 and 4.4).

4.3.3. Effect of dilazep on vinblastine accumulation by CH₈C5 monolayer cells

Because our previous data suggested that dilazep may possibly be a P-gp inhibitor, we had examined the effect of various concentrations (1 μM to 50 μM) of dilazep on the accumulation of vinblastine. Dilazep significantly increased the accumulation of vinblastine by CH₈C5 monolayer cells in a concentration dependent manner (Figure 4.5). These results suggest that, in addition to dipyridamole, dilazep, a widely used equilibrative nucleoside transport inhibitor, may also be a P-gp inhibitor.
Figure 4.3. Effect of DADs and P-gp Inhibitors on Vinblastine Accumulation by LLC-PK₁ monolayer cells.

One hour [³H]vinblastine (20 nM) accumulation was measured in standard incubation medium at 37 °C in the presence of various DADs and P-gp inhibitors. Results are expressed as mean ± standard deviation of two separate experiments. *Significant difference from control at p < 0.05 as determined by one way ANOVA followed by unpaired Student’s t test.
Figure 4.4. Effect of DADs and P-gp Inhibitors on Vinblastine Accumulation by OK Monolayer Cells.

One-hour [3H]vinblastine (20 nM) accumulation was measured in standard incubation medium at 37 °C in the presence of various DADs and P-gp inhibitors. Results are expressed as mean ± standard deviation of three separate experiments. *Significant difference from control at $p < 0.05$ as determined by one way ANOVA followed by unpaired Student’s $t$ test.
Figure 4.5: Effect of Diaphragm on Vinblastine Accumulation by CHF Cells

Vinblastine Accumulation (pmol/mg protein/ml)

1 pm 10 pm 25 pm 50 pm

Diaphragm
Control
CHF Cells

Unpaired Student's t test

difference from control at 0.05 as determined by one-way ANOVA followed by significant as mean ± standard deviation of two separate experiments.

Results are expressed as mean ± standard deviation of two separate experiments. Significant difference from control at 0.05 as determined by one-way ANOVA.
4.4. DISCUSSION

Although it has recently been reported that P-gp may mediate the intestinal transport of protease inhibitors, a class of antiretroviral drugs commonly used in the treatment of HIV-1 infection, the involvement of P-gp in the membrane transport of DADs remains a controversial issue. Multiple membrane transporters (i.e., organic cation, organic anion, and nucleoside transporters) have been identified to mediate the renal tubular transport of DADs (i.e., ZDV and ddC) (Yao et al. 1996; Aiba et al. 1995; Bendayan et al. 1995; Griffiths et al. 1992; Griffiths et al. 1991). In purified brush border membrane vesicles isolated from rat kidney cortex, ZDV inhibited the transport of the organic cation probe N\textsuperscript{1}-methylnicotinamide (IC\textsubscript{50} = 2500 μM) while at the basolateral site, it inhibited the transport of the organic anion probe, para-aminohippuric acid (IC\textsubscript{50} = 225 μM) (Griffiths et al. 1992 and 1991). These results suggest that ZDV may be a substrate for the renal basolateral membrane organic anion transporter and the renal brush border membrane organic cation transporter respectively. Recent in vivo work undertaken in rat kidney further supports these findings. Cimetidine, an organic cation probe and inhibitor, and probenecid, a standard organic anion transport inhibitor, significantly inhibited the renal excretion of ZDV (Aiba et al. 1995). In addition, recombinant cDNA from rat jejunal epithelium encoding an intestinal/kidney Na\textsuperscript{+}-dependent nucleoside transporter protein (rCNT1) expressed in Xenopus oocytes mediated the Na\textsuperscript{+}-dependent influx of ZDV and ddC with apparent Km values of 0.5 mM (Yao et al. 1996).

With recent findings of P-gp involvement in the intestinal and brain transport of protease inhibitors (Lee et al. 1998; Kim et al. 1998) as well as the controversy on the possible involvement of P-gp in DADs cell resistance (Glynn and Yazdanian 1998; Signoretti et al. 1997; Andreana et al. 1996; Peter and Gambertoglio 1996; Lucia et al. 1995; Dianzani et al. 1994;
Antonelli et al. 1992; Gollapudi and Gupta 1990; Yusa et al. 1990), we questioned the possible involvement of P-gp in the renal tubular transport of DADs.

CH^R C5, a continuous colchicine resistant Chinese hamster ovary cell line, known to express high levels of P-gp, has previously been used as a suitable in vitro model for the investigation of possible P-gp substrates and inhibitors (i.e., vinblastine, digoxin, verapamil, and cyclosporin A) (de Lannoy and Silverman 1992; Doige and Sharom 1992). LLC-PK1 and OK cells are continuous renal epithelial cell lines that have shown to retain a number of physiological and morphological properties of the normal renal proximal tubule. In particular, these cells have served as a useful model for the characterization of the renal transport properties of a number of organic cations and anions (Bendayan et al. 1994; Hori et al. 1993; Saito et al. 1992). Although these cell lines have shown to express low levels of P-gp, they have also proven to be a useful model for the study of P-gp drug mediated renal tubular transport (Tanigawara et al. 1992; Horio et al. 1990). Net basolateral to apical transepithelial transport of vinblastine in renal epithelial cell lines LLC-PK1, MDCK, and OK has been found to be 13 fold, 5 fold and 2 fold higher than apical to basolateral transport respectively suggesting an order of potency for P-gp expression as LLC-PK1>MDCK>OK cells (Horio et al. 1990).

In CH^R C5, our data shows that high concentrations of a number of DADs (i.e., ZDV, ddI, ddC, and 3TC) do not significantly alter the accumulation of vinblastine, a specific P-gp substrate, while verapamil and cyclosporin A, two P-gp inhibitors, significantly enhance the accumulation of vinblastine by the monolayer cells. Similar results are observed in LLC-PK1 and OK monolayer cells suggesting that P-gp may not be involved in the renal transport of these antiretroviral drugs. Our data also shows that two nucleoside membrane transport inhibitors (i.e., dipyridamole and dilazep), are able to enhance significantly vinblastine accumulation by the
monolayer cell lines. While dipyridamole has previously been shown to be a potent P-gp inhibitor (Shalinsky et al. 1992), to the best of our knowledge, this is the first report suggesting that dilazep may also be a potent P-gp inhibitor. This finding is important since both compounds are used widely in studies characterizing the membrane transport of nucleosides.

In summary, DADs do not significantly alter the accumulation of vinblastine by CH~RC5, LLC-PK₁ or OK monolayer cells suggesting that P-gp may not play a significant role in the renal tubular transport of DADs. Further investigation is needed to evaluate the role of P-gp in the transport of DADs by HIV-1 infected cell lines.
CHAPTER 5

SUMMARY AND DISCUSSION

The use of multiple drug therapies in HIV-1 patients increases the incidence of drug interactions that may alter the pharmacokinetic and pharmacodynamic properties of a drug. As a result, this increases the risk of drug-related problems and may ultimately lead to adverse drug reactions and toxicity (Chow et al. 1993; Bjornson et al. 1989). In addition, it appears that a higher incidence of adverse reactions to drugs that are commonly used in the treatment of opportunistic infections (i.e., TMP/SMX) is observed among patients who have AIDS than among those who do not (Medina et al. 1990; Bowden et al. 1986; Gordin et al. 1984; Kovacs et al. 1986). Lamivudine (3TC), a nucleoside analog, is an effective agent in the treatment of HIV-1 and hepatitis B infections. This agent is highly excreted unchanged in urine through glomerular filtration and tubular secretion. Since multiple membrane transporters (i.e., organic cation, anion, and nucleoside) have been reported to be involved in the membrane transport of a number of dideoxynucleoside analog drugs (i.e., ZDV and ddC), we hypothesized that multiple membrane carriers could also be involved in 3TC renal tubular secretion. In the present work, we have investigated the in vitro renal transport properties of 3TC by a continuous renal epithelial cell line (i.e., LLC-PK1) and the Chinese hamster ovary cell line colchicine resistant (CHRCS).

The renal transport properties of 3TC were characterized in a continuous renal epithelial cell line (i.e., LLC-PK1) grown as a monolayer on an impermeable surface. A saturable, temperature sensitive, Na+-independent carrier mediated system for 3TC was identified. Despite its chemical nucleoside analog properties, 3TC uptake was not affected by various purine and
pyrimidine nucleoside substrates and nucleoside transport inhibitors, suggesting that nucleoside transporters are not involved in 3TC uptake by renal epithelial cells. These results corroborate previous work undertaken with ZDV and ddC in purified BBM vesicles isolated from human kidney cortex (Brett et al. 1993) which suggested that naturally occurring nucleosides (i.e., uridine, adenosine, cytidine, and thymidine) are substrates of a renal Na⁺-nucleoside transport system whereas synthetic nucleoside analog drugs (i.e., ZDV and ddC) are not (Brett et al. 1993). However in rat, an intestinal/renal Na⁺-dependent nucleoside transport protein (cNT1) expressed in Xenopus oocytes has been demonstrated to mediate the transport of ZDV and ddC (Huang et al. 1994, Yao et al. 1996).

Our studies show that 3TC uptake by LLC-PK₁ monolayer cells is inhibited by a number of organic cations and inhibitors suggesting an organic cation carrier may be involved. To further determine the driving force for this carrier-mediated process, 3TC uptake was examined in the presence of an outwardly directed proton gradient generated by a standard NH₄Cl loading and removal protocol (Lui and Bendayan 1997; Bendayan et al. 1994). An enhanced 3TC uptake was observed under intracellular acidification conditions suggesting that 3TC uptake may be coupled to a proton exchange, i.e., an organic cation/proton exchanger. To confirm the enhanced 3TC uptake was not due to membrane potential effects, we measured the uptake of 3TC by LLC-PK₁ monolayer cells under voltage-clamped conditions. No significant changes in 3TC uptake were observed suggesting that rather than membrane potential dependency, an organic cation/H⁺ exchanger is likely to be involved in the renal luminal transport of 3TC. Another dideoxynucleoside analog drug, (i.e., ZDV), has also been suggested to be a substrate for the basolateral organic anion and brush border organic cation transport systems in vitro (Griffiths et al. 1992 and 1991). Previously our lab has also demonstrated the involvement of the
organic cation transporter in ZDV basolateral to apical flux in a cultured renal epithelium (i.e., LLC-PK₁) (Bendayan et al. 1995).

The question of the possible involvement of P-gp in the renal transport of DADs, including 3TC, was explored. Several reports have suggested that P-gp may or may not be involved in the emergence of HIV-1 resistance to DADs. (Antonelli et al. 1992; Yusa et al. 1990). Using an indirect approach to examine the drug efflux properties of P-gp, the accumulation of vinblastine, a well-recognized P-gp substrate, by CH₅C₅, LLC-PK₁ and OK cells was examined in the presence of various DADs, including 3TC at different concentrations. The accumulation of vinblastine was not found to be significantly altered in the presence of DADs suggesting P-gp does not play an important role in the renal transport of 3TC.

Since our studies are undertaken on monolayer cells grown on an impermeable surface, our results reflect primarily uptake events occurring at the apical membrane of the polarized monolayer cells. To overcome this limitation, transport processes at the basolateral membrane could be better explored in either monolayer cells grown on a permeable surface (i.e., filter membrane) and/or in purified membrane vesicles isolated from animal kidney cortex.

The possible involvement of the organic anion transport in 3TC transport by LLC-PK₁ cells was not explored because these cells do not appear to express this transport system (Miller et al. 1992; Rabito et al. 1986). The OK cells, another continuous renal epithelial cell line, could be a useful model for the study of organic anion transport (i.e., Hori et al. 1993 and 1990). However, OK monolayer cells are very leaky when grown on a permeable support and do not appear to be the ideal system for transepithelial flux studies. Other approaches, such as purified BBM and BLM vesicles, could be considered.
In summary, the understanding, prediction and prevention of drug-drug interactions and adverse drug reactions are of great clinical importance in AIDS patients. This is particularly true in view of the rapid introduction of newer agents and the paucity of pharmacological information on their use, alone or in combination. The present work has established, for the first time, a model for the renal transport of 3TC in renal tissue cell culture systems. This model not only elucidates the mechanisms involved in the renal tubular secretion of 3TC but also warrants the investigation of drug-drug interactions at the renal proximal tubule. These interactions could become highly significant with 3TC in patients with renal insufficiency since this drug is mainly eliminated unchanged by the kidney. The study of these possible interactions will form the basis of parallel clinical studies in AIDS patients.
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