LINGHUI KONG
Preclinical Pharmacokinetic Studies of 3'-Azido-2',3'-Dideoxyuridine and Its Novel
    Prodrugs
(Under the direction of F. DOUGLAS BOUDINOT)

3'-Azido-2',3'-dideoxyuridine (AZDU, AzddU, CS-87, Uravidine) is a nucleoside analog with a similar chemical structure to 3'-azido-3'-deoxythymidine (AZT, zidovudine), the most frequently used drug in the treatment of HIV-infected patients. AZDU has been found to have potent anti-HIV activity in human peripheral blood mononuclear cells with significantly reduced human bone marrow toxicity, 30-fold less than AZT. Nevertheless, the potential of AZDU as a promising anti-HIV agent has been limited by its relatively short half-life, relatively low bioavailabilities as illustrated in various animal models as well as its extensive glucuronidation in HIV-infected patients.

As means of improving its pharmacokinetic profile, several novel compounds were synthesized as prodrugs of AZDU in order to obtain prolonged half-lives and good bioavailabilities.

Oral bioavailabilities of AZDU were determined in rats. A rapid, sensitive, reproducible high performance liquid chromatography (HPLC) method using gradient elution was developed to simultaneously quantitate AZDU and its prodrugs in rat plasma. Preclinical pharmacokinetic studies on one of the prodrugs, 3'-azido-2',3'-dideoxyuridine-5'-$O$-valinate hydrochloride, showed improved bioavailability of AZDU compared with that obtained after oral administration of the parent drug. Therefore, AZDU-VAL is a promising prodrug of AZDU.

2'-Amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) was recently synthesized as a prodrug of  the anti-HIV nucleoside analog 2', 3'-dideoxydidehydroguanine (D4G). In the present dissertation, a

reliable, sensitive isocratic HPLC analytical method was developed to determine ADV in rat plasma and rat liver homogenates. This analytical method was employed in the bioconversion studies of ADV *in vitro*.

INDEX WORDS:    3'-Azido-2',3'-dideoxyuridine, AZDU, Bioavailability, HPLC, Prodrug, Pharmacokinetics, Cyclopropyl, Guanine, Bioconversion

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DIDEOXYURIDINE AND ITS NOVEL PRODRUGS

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B.S., Shenyang Pharmaceutical University, China, 1993

M.S., Shenyang Pharmaceutical University, China, 1996

A Dissertation Submitted to the Graduate Faculty

of The University of Georgia in Partial Fulfillment

of the

Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2001

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August  2001

**DEDICATION**

TO

MOM, DAD, BROTHER AND ANDY

WITH LOVE

## ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude and appreciation to my major advisor, Dr. F. D. Boudinot, for his guidance, encouragement and patience all through my graduate program at the College of Pharmacy at the University of Georgia.

I would like to thank my committee members, Drs. C. K. Chu, Robert Lu, Bill McCormick and James Price for reading my dissertation and for their invaluable comments and suggestions. Also, my sincere gratitude goes to all of my colleagues and friends at the University who have helped me in many ways making my life wonderful in Athens.

Lastly, I would like to express my appreciation to my loving mom, dad, brother and Andy for their unconditional love, understanding and support throughout my academic career.

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## CHAPTER I:

## Introduction and Literature Review

**AIDS-- acquired immunodeficiency syndrome**

Since the first case of AIDS occurred in 1981 with report of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma in homosexual men in the United States (1), AIDS has become a major worldwide epidemic. Today an estimated 30.6 million people are living with HIV infection or AIDS (2) and as many as 900,000 Americans may be infected with HIV. AIDS is caused by the human immunodeficiency virus (HIV). People diagnosed with AIDS may get life-threatening diseases called opportunistic infections, which are caused by microbes such as viruses or bacteria that usually do not make healthy people sick. Infections with HIV-1 initiates progressive destruction of CD4+ T lymphocytes, the rate of decline determining the rate of immunodeficiency and the subsequent development of HIV-related opportunistic infections (3).

**Life Cycle of HIV Infection**

HIV begins its infection of a susceptible host cell by binding to the CD4 receptor on the host cell. Following fusion of the virus with the host cell, HIV enters the cell. The virus RNA is released and undergoes reverse transcription into DNA by an enzyme in HIV called reverse transcriptase. Once the genetic material of HIV has been changed into DNA, this viral DNA enters the host cell nucleus where it can be integrated into the genetic material of the cell. Once the viral DNA is integrated into the genetic material of the host, it is possible that HIV may persist in a latent state for many years.

Activation of the host cells results in the transcription of viral DNA into messenger RNA (mRNA), which is then translated into viral proteins. The new viral RNA forms the genetic material of the next generation of viruses. The viral RNA and viral proteins assemble at the cell membrane into a new virus. Following assembly at the cell surface, the virus then buds forth from the cell and is released to infect another cell.

**Targets for antiretroviral agents**

Theoretically, the multiple steps in the replication of HIV could be targets for antiviral action (4). However, currently agents that affect only steps, reverse transcription and protein processing, have been approved by Food and Drug Administration as therapies in the treatment of HIV infections (5).

**Reverse Transcriptase Inhibitors (RT)**

<u>Nucleoside Analogs</u>

Zidovudine/Retrovir (AZT, ZDV)

Didanosine/Videx (ddI)

Zalcitabine/HIVID (ddC)

Stavudine/Zerit (d4T)

Lamivudine/Epivir (3TC)

Abacavir/Ziagen (ABC)

<u>Non-Nucleoside Reverse Transcriptase Inhibitors</u>

Nevirapine/Viramune

Delavirdine/Rescriptor

Efavirenz/Sustiva (DMP-266)

<u>Nucleotide Analog</u>

Adefovir

Dipivoxil/Preveon

**Protease Inhibitors**

Indinavir/Crixivan

Ritonavir/Norvir

Saquinavir/Invirase

Fortovase

Nelfinavir/Viracept

Amprenavir/Agenerase

**Nucleoside Analogs are the cornerstones of antiretroviral therapy**

The natural history of HIV-1 infection has been dramatically modified by the use of multidrug highly active antiretroviral therapy (HAART) regimens (6). The recommended HAART regimens are all built around a backbone of 2 nucleoside analogs with either protease inhibitors or non-nucleoside reverse transcriptase inhibitors. Combinations involving 3 nucleoside analogs are also under evaluation. Current HAART regimens are unable to eradicate HIV-1 infection (7, 8), and thus the current therapeutic paradigm involves continuing therapy for an indefinite period (9).

The first drug to be approved for use in HIV infection was AZT (zidovudine), an inhibitor of reverse transcriptase developed over 30 years ago (4). AZT is a dideoxynucleoside, a structure that lacks a hydroxyl group on the 3' position in the ribose ring. AZT, along with other nucleoside drugs in this class, continues to represent a major chemotherapeutic approach towards the management of HIV-1 infections (10-15). These nucleoside analogs enter cells by nonfacilitated passive diffusion (16). Following

intracellular conversion to their 5'-triphosphate derivatives (nucleotide), they compete with natural nucleotides for binding to RT and, subsequently, cause chain termination through incorporation into the nascent DNA strand. Chain termination is caused by the lack of a hydroxyl motif at the 3' carbon of the pentose ring that is necessary to form a 3'-5' phosphodiester bond with the next nucleoside substrate in the elongating DNA strand (17-21). The efficacy of a nucleoside analog is dependent on several factors, including its oral bioavailability, cellular uptake, the intracellular anabolism to its triphosphate derivative, the ability to compete with natural nucleosides as a substrate for RT, and the degree of drug resistance developed by the virus (22, 23).

Currently, drug resistance to HIV viruses is an inevitable consequence of long term exposure of antiretroviral therapy due to a high turnover of HIV-1 in patients (24, 25) and a low fidelity of the viral RT (26). Recent studies provided evidence that replication competent virus can be readily recovered from peripheral blood mononuclear cells, as well as from semen, in subjects who had undetectable plasma HIV RNA for up to two years (7, 8, 27, 28). Other studies showed that the number of latently infected CD4+ T cells decline only very slowly in patients on HAART (29).

The clinical pharmacokinetics of nucleoside analogs suggests that significant drug-drug interactions are unlikely. These drugs have a short elimination half-life and are excreted predominantly by the renal mechanism (30, 31, 32, 33).

Nucleoside analogs represent the cornerstones of antiretroviral regimens. A range of drug- or tissue-specific toxicities, such as peripheral neuropathy (34), myopathy, pancreatitis and lactic acidosis with hepatic steatosis, has been documented with these agents. The fat atrophy seen on long-term antiretroviral therapy may also be related to

nucleoside analogues (9). Long term administration of AZT in patients with AIDS has led to bone marrow suppression and other cytotoxic effects (35).

Therefore, it is of utmost importance to identify new agents that are active against these drug resistant strains of HIV-1 and are well tolerated by individuals living with HIV-1. It is evident from the volume of literature that many approaches are being used to develop new anti-HIV nucleosides. If there is any pattern to the research efforts, it might be in the emphasis on prodrug formulations of existing nucleosides.

**Prodrugs**

A prodrug is an agent that undergoes chemical or enzymatic thansformation in vivo to yield the active parent drug (36). The prodrug approach could be used to optimize the physicochemical properties thereby, improving the pharmacological and toxicological profiles of a given drug. Prodrugs can also be utilized to change the target organ specificity and tissue distribution. The objective of this approach can be to deliver the nucleoside to a site of action (e.g. the brain) or to decrease delivery to a site of toxicity (e.g. the kidney) (37). Technically, all nucleosides whose active species is a triphosphate (TP) anabolite are prodrugs. However, here 'prodrug' is used to describe one or more of the vast array of strategies employed to enhance the activity *in vivo*, or reduce the toxicity, of a nucleoside before it is converted to its TP form. The rationale for the selection of a particular prodrug strategy lies in the particular deficiency displayed by the parent nucleoside, such as poor oral bioavailability, CNS penetration, cellular uptake or the rate-limiting enzymatic step in the conversion to the TP species (38).

**AZDU and its novel prodrugs**

In the search for new and less toxic agents, a great deal of interest has been focused on 2', 3'-dideoxynucleosides.

3'-Azido-2', 3'-dideoxyuridine (AZDU, CS-87) was the first nucleoside analogue with a uracil base which has showed potent inhibition of human immunodeficiency virus replication *in vitro* (39, 40). In peripheral blood mononuclear cells, the major metabolite of AZDU is AZDU-5' monophosphate. It is believed that this monophosphate becomes di- and triphosphorylated by cellular enzymes. Thiphosphorylated AZDU interferes with the RNA-dependent DNA polymerase (reverse transcriptase) of several retroviruses, including HIV, and thus inhibits HIV replication preventing cDNA synthesis. Similar to AZT, it inhibits cellular polymerase alpha; however, concentrations of AZDU that inhibit cellular DNA polymerase are 3000- to 5000-fold greater than those required to inhibit HIV reverse transcriptase. In various cell-virus systems, the activity of AZDU varied from system to system: the anti-viral activity of AZDU in peripheral blood mononuclear (PBM) and MT-4 cell cultures was 100 times less than that of AZT, regardless of the type of retrovirus used; in Hela-T4 cell cultures, AZT was only 3.5 times more active than AZDU; in ATH8 cells, AZDU and AZT inhibited HIV-induced CPE equally well; in human monocytes and macrophages, AZDU was 10-20 times more active than in PBM cell cultures. According to one *in vitro* study, AZDU is 30 times less toxic to human bone marrow derived granulocyte-macrophage and erythroid precursor cells than AZT (41, 42, 43, 44). No overt toxicity was observed in animal studies using rats and dogs (given, respectively, 2500 and 1000 mg/kg, orally for 28 days). In human studies, maximum tolerated dose has not been reached. AZDU has been well tolerated with mild headache

being the only major subjective side effect to date. No patient has developed anemia, but one patient in a phase I trial developed grade 3 neutropenia that resolved with a 50% dose decrease (46, 47).

The pharmacokinetics of AZDU has been characterized in different animal models, such as rats, mice and monkeys in comparison with AZT. AZDU has a similar chemical structure to that of AZT, thus, it is not surprising to see comparable disposition of these two compounds. AZDU and AZT exhibit linear pharmacokinetics up to 100 mg/kg in rats. However, dose-dependent kinetics was observed with dose increase, as elucidated by decrease in total clearance and renal clearance. The deceased renal clearance, and hence total clearance, of AZDU results from saturation of active renal tubular secretion of the nucleoside (48). The pharmacokinetics of AZDU and AZT were also characterized following iv doses of 50 mg/kg and 250 mg/kg in uninfected mice to compare these two compounds and to estimate the extent of brain uptake of the drug. The pharmacokinetic parameters of half-life, total clearance and volume of distribution were similar for both compounds. Brain/serum concentration ratios for AZDU tended to be greater than those obtained for AZT and were significantly different at the 50 mg/kg dose (49). Preclinical pharmacokinetics of AZDU was examined using the rhesus monkey as an animal model. Similar half-life, total clearance and steady-state volume of distribution were observed for AZDU and AZT in this study. Oral absorption of AZDU and AZT was virtually complete after 60 mg/kg. However, bioavailability of both nucleosides was markedly lower after 200 mg/kg, possibly indicating the involvement of a saturable absorption mechanism. AZDU and AZT penetrated the cerebrospinal fluid (CSF) with

concentration ratios in CSF:serum ranging from 0.05 to 0.25 one hour after drug administration (50).

It has been shown that human immunodeficiency virus (HIV) infected cells preferentially locate in lymphoid tissue early in the course of infection (51, 52, 53, 54). Thus, it is of great importance to deliver the anti-HIV agents to lymphatic system. The lymphatic distribution of AZDU and AZT was studied in mice after single dose intravenous, oral, and intraperitoneal administrations of 50 mg/kg of both compounds. Patterns of regional distribution in lymph nodes were similar for both drugs; however, the accumulation of AZDU in the various lymph nodes, according to AUC values, was 3-76% greater than that for AZT. The relative exposure for both nucleosides exhibited a dependence on route of administration. Intravenous and oral administration resulted in a greater distribution of nucleoside into axillary lymph nodes, compared with neck and mesenteric lymph nodes. Following intraperitoneal administration, however, distribution was similar in all three regions (55).

Another important pharmacokinetic consideration for any antiviral agent is its distribution in the brain. HIV produces devastating effects on the central nervous system (CNS). Thus, antiviral agents also need to gain access to the CNS. For example, concentrations of AZT in cerebrospinal fluid are lower than systemic concentrations. Consequently, a novel system for CNS delivery of this drug has been developed by attaching AZT to a molecule that facilitates its entry (56). In an animal study, this approach showed some promise (57).

As a nucleoside analog of AZT, the potential of AZDU has been limited by its relative short half-life as illustrated in various animal models, extensive glucuronidation in patients as well as its ineffective delivery in the brain and lymphatic system.

Several prodrugs of AZDU have been designed and synthesized in an attempt to improve its distribution in the brain, lymphatic system and anti-HIV efficacy. Various methods have been utilized to improve brain targetability of drugs. Bodor and co-workers' strategy appears to be very attractive. The approach uses dihydropyridine-pyridinium salt redox system (58). Thus, a dihydropyridine derivative of AZDU (AZDU-DHP) was synthesized as a prodrug in an effort to increase brain delivery of AZDU. AZDU-DHP showed the greatest *in vitro* stability in human serum, followed by mouse serum and homogenate, with half-lives of 4.33, 0.56, 0.17 h, respectively. The extended half-life of AZDU-DHP in human serum would allow sufficient time to cross the human blood-brain barrier (BBB), which is the main barrier for penetration of drugs. *In vivo* studies in mice showed a significant increase in brain exposure of AZDU (area under the brain concentration-time curve of 11.4 μg h/mL) following intravenous administration of AZDU-DHP in comparison with the parent compound (2.1μg h/mL). In addition, the brain-elimination half-life of AZDU derived from the prodrug was much greater than that of the parent drug (4.34 h *vs* 0.84 h), indicating a prolonged retention of AZDU in the brain (59).

Esterification of the 5'-OH function of pyrimidine nucleoside analogs with various aliphatic acids to improve lipophilicity of the anti-HIV agents has been exploited by many researchers (60, 61, 62). Ether prodrugs of AZDU were prepared by substitution of benzyl or glucose at the 5' position to serve as viable nucleoside drug delivery system

to the brain. *In vitro* stability and pharmacokinetic studies of these prodrugs in mice indicated that they were stable in phosphate buffer (pH 7.4), human serum and mouse serum. In mouse brain homogenate, the degradation half-lives for benzyl AZDU (BzlAZDU) and glucose AZDU (GAZDU) were 1.66 and 0.98 h, respectively. However, neither of these two prodrugs produced the parent drug, AZDU, in mouse brain (63).

As means of improving the delivery of AZDU to lymphatic tissues, Chu *et al.* (64) synthesized the dipalmitoyl-phosphatidyl derivative of AZDU (AZDU-DPP). Maximum concentrations of AZDU derived from AZDU-DPP were lower than those obtained when parent compound was administered. However, AZDU concentration maintained longer after prodrug administration due to sustained release as indicated by enhanced half-lives in serum, neck, axillary and mesenteric lymph nodes. The thioether (1-S-alkyl) lipids linked by a pyrophosphate diester bond was synthesized to improve AZDU therapeutic efficacy (65). Racemic 1-S-octadecyl-2-*O*-palmitoyl-1-thioglycerol of AZDU diphosphate was found to protect 80% of HIV-infected CEM cells at concentrations as low as 0.58 μM, cause cytotoxicity at 180 μM. An 8-fold prolonged half-life after intravenous administration was observed.

**Objectives of this study**

Although pharmacokinetics of AZDU have been studied extensively in various animal models, such as rats, mice and monkeys, the bioavailability of this compound in rats has not been reported in the literature. Thus, bioavailability studies were conducted to gain this information in order to fully evaluate the novel compounds synthesized as prodrugs of AZDU. An HPLC method for simultaneous determination of AZDU and its prodrugs in rat plasma was developed. The *in vitro* stability and pharmacokinetics of a

prodrug of AZDU, 3'-Azido-2', 3'-dideoxyuridine-5'-*O*-valinate hydrochloride, were evaluated using rats as an animal model.

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**CHAPTER II:**

**Effect of Dose on the Bioavailability of 3´-Azido-2´, 3´-Dideoxyuridine (AZDU) in**

**Rats[1]**

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**ABSTRACT**

**Purpose.** The purpose of this study was to determine the oral bioavailability of 3′-azido-2′, 3′-dideoxyuridine (AZDU), a nucleoside analogue and promising anti-HIV agent in rats. **Methods.** The bioavailability of AZDU was determined in eight adult male Sprague-Dawley rats after intravenous and oral administration at two doses, 25 mg/kg and 100 mg/kg. For each dose, a crossover study design was employed. Blood samples were taken and AZDU plasma concentrations were determined by high performance liquid chromatography. The pharmacokinetic parameters were estimated by area-moment analysis. **Results.** Plasma concentrations of AZDU following intravenous administration declined in a biexponential manner. The areas under the plasma concentration-time curves (AUC) were $19.43 \pm 1.24$ mg·h/L and $155.82 \pm 6.97$ mg·h/L, following intravenous administration of 25 mg/kg and 100 mg/kg, respectively. Following oral administration $T_{max}$, $C_{max}$ and AUC were $0.38 \pm 0.13$ h, $10.24 \pm 2.55$ μg/ml and $11.72 \pm 0.54$ mg·h/L at a dose of 25 mg/kg, and at dose of 100 mg/kg they were $0.63 \pm 0.27$ h, $41.60 \pm 14.90$ μg/ml and $70.35 \pm 13.07$ mg·h/L, respectively. Bioavailability values of AZDU averaged $0.60 \pm 0.03$ and $0.46 \pm 0.10$ following oral administration of 25 and 100 mg/kg AZDU, respectively and were not statistically significantly different. **Conclusions**. The bioavailability of AZDU after oral administration to rats was approximately 53 % at both doses studied.

**INTRODUCTION**

Since the first case of acquired immunodeficiency syndrome (AIDS) was diagnosed in the United States in 1981 (1, 2, 3), major advances have been made in the understanding of the pathogenesis of human immunodeficiency virus (HIV) infection and modes of intervention at various stages of virus infection and replication (4, 5, 6, 7). Many compounds with *in vitro* anti-HIV activity have been proposed for clinical use and 14 anti-HIV agents have been approved by United States Food and Drug Administration (FDA) for the treatment of AIDS, including reverse transcriptase (RT) inhibitors and protease inhibitors. Based on structure and mechanism of action, the reverse transcriptase inhibitors can be classified further into two major groups, nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). NRTIs are usually 3'-deoxy or 2', 3'-dideoxy derivatives of natural nucleosides of DNA polymerases acting as DNA chain terminators and thus inhibiting viral reverse transcriptase (4, 8).

In 1987, AZT was first introduced for clinical use as a nucleoside analog reverse transcriptase inhibitor, and this compound still is the most frequently used drug for clinical treatment of HIV-infected patients. However, serious side effects associated with AZT therapy such as myelosuppression (9) have been reported, and cross-resistance with other anti-HIV drugs in combination therapy limit its long-term use. Recently it was found that HIV-1 can become multi-drug resistant under combination therapy (10, 11), making it necessary to develop new agents as alternative combination therapy. An intensive search for agents that inhibit RT has been undertaken and many small molecules with similar chemical structures as AZT have been evaluated for antiviral activity.

3'-Azido-2', 3'-dideoxyuridine (AZDU, AzddU, uravudine, CS-87 was first reported as a new 3'-azido nucleoside analog by Tai-Shun Lin et al. in 1983 (12). The chemical structure of AZDU, compared to AZT, is illustrated in Figure 1. The *in vitro* anti-HIV activity of AZDU was first reported in 1987 (13), and in 1988 Chu *et al* reported the synthesis of AZT and AZDU from mannitol (14). AZDU has been reported to inhibit HIV-1 replication in human peripheral blood mononuclear cells (PBMC) infected with HIV-1 in the 0.18 to 0.46 $\mu$M range (15). In the human T-cell lines MT-4 and ATH8, the median effective AZDU concentration for inhibition of HIV-mediated cytopathic effects was 0.4 $\mu$M. The 50% inhibition of cell growth occurred at concentrations of 200 $\mu$M or greater (16). Compared to AZT, AZDU demonstrated significantly less *in vitro* toxicity (30-fold) to human bone marrow cells (BMC) (17). Metabolic studies showed that AZDU is sequentially phosphorylated to its mono-, di-, and triphosphate metabolites by cellular kinases. The 5'- triphosphate of AZDU (AZDU-TP) is known to be the active form of the compound inhibiting HIV-1 and simian immunodeficiency virus (SIV) reverse transcriptase at concentrations of 5.9 and 4.1 mM**,** respectively (18). In rat hepatocytes AZDU has been shown to be metabolized to 3'-amino-2', 3'-dideoxyuridine (AMDU), through an azido reduction pathway similar to other 3'-azido-2', 3'-dideoxynucleosides, and to AZDU-glucuronide (GAZDU) (19).

Due to its anti-HIV activity and significantly reduced bone marrow toxicity, AZDU appears to be a good prospect for AIDS therapy. While much work has been reported on the pharmacokinetics and metabolism of AZDU, the evaluation of its pharmacokinetics and bioavailability following oral administration to rats has not been

reported. It is of importance to have this information for the development of AZDU and novel prodrugs of the nucleoside analog.

Therefore, the purpose of this study was to determine the oral bioavailability of AZDU in rats. A crossover oral and intravenous administration experimental design was used to assess the effects of dose on the bioavailability of AZDU.

## MATERIALS AND METHODS

### Chemicals

3'-Azido-2', 3'-dideoxyuridine (AZDU) and 3'-azido-3'-deoxythymidine (AZT, zidovudine), used as the internal standard for drug analysis, were synthesized as previously described (14). The chemical purity of each compound was greater than 99% as assessed by HPLC and spectral analysis. Acetonitrile, HPLC grade, and all other chemicals, analytical grade, were purchased from J.T. Baker (Phillipsburg, NJ). AZDU was dissolved in 0.1 N sodium hydroxide in physiological saline for intravenous and oral administration.

### Experimental Design

Eight adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing $311 \pm 22$ g (mean $\pm$ SD) were used for the study. Animals were housed in a 12-h light/12-h dark, constant temperature (22°C) environment with free access to standard laboratory chow and water in the University of Georgia College of Pharmacy Animal Care Facility, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Rats were acclimatized to this environment for at least one week before the study. Animal studies were approved by the

University of Georgia Animal Care and Use Committee and conducted in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

External jugular vein cannulas were implanted under (ketamine:acepromazine: xylazine; 50:3.3:3.3 mg/kg) anesthesia the day before the experiment. Animals were fasted overnight, however, water was allowed *ad libitum*. The eight rats were randomly divided into two groups of four each. For each group of rats, a randomized oral/intravenous crossover study design with two dosing periods and a 4-day washout period between doses was used. One group of animal received a dose of 25 mg/kg AZDU and the other was administered 100 mg/kg of the compound. AZDU was administered intravenously by bolus injection via the jugular vein cannula or orally by gastric gavage. The animals were housed in metabolism cages following drug administration. Blood samples (0.3 ml) were collected from the cannulas into heparinized tubes prior to and at 0.083, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12 h after intravenous administration and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 h following oral administration. Blood volume was replaced with normal saline. Blood samples were immediately centrifuged and plasma was frozen at -20°C until analysis. Urine samples were collected for 24 h, urine volume was measured, and samples were frozen at -20°C until analysis.

**Analytical Methodology**

Concentrations of AZDU in plasma and urine were determined by reverse-phase high-performance liquid chromatography using an octadecyl silane ($C_{18}$) column (4.6

mm ID × 15 cm, 5μm particle size; Alltech Associates, Deerfield, IL) as previously described (20).

**Pharmacokinetic Analysis**

Pharmacokinetic parameters for AZDU were determined by area-moment analysis. The area under the plasma concentration versus time curve (AUC) and the first moment (AUMC) were calculated by Lagrange polynomial interpolation from time zero to the last measured sample time, with extrapolation to time infinity by using the terminal slope ($\lambda z$) (21, 22). Total clearance ($CL_T$) was calculated from Dose/AUC. Mean residence time (MRT) was calculated from AUMC/AUC, and steady-state volume of distribution ($V_{ss}$) was calculated from $CL_T \times MRT$. Half-life ($t_{1/2}$) was calculated from $0.693/\lambda z$. Absolute bioavailability was calculated from $AUC_{po}/AUC_{iv}$. The maximum concentration achieved ($C_{max}$) and the time to $C_{max}$ ($t_{max}$) are reported as observed values. The fraction of the administered dose excreted in the urine ($f_e$) was calculated from $A_u$/dose, where $A_u$ is the total amount of AZDU excreted in the urine. Renal clearance ($CL_R$) was calculated from $f_e \times CL_T$ and non-renal clearance ($CL_{NR}$) from $CL_T - CL_R$.

Statistical analysis was performed using a two-way analysis of variance comparing the pharmacokinetic parameters. A probability level of less than 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

The purpose of this investigation was to characterize the pharmacokinetics and to determine the oral bioavailability of AZDU following intravenous and oral administration at two doses in the rat animal model. Representative plasma AZDU concentration versus

time profiles following intravenous administration of 25 and 100 mg/kg to rats are illustrated in Figure 2. Plasma concentrations of AZDU declined rapidly in a biexponential fashion at both doses. The AUC increased disproportionally with dose. The dose-normalized AUC after 100 mg/kg (1.56 ± 0.070; mean ± SD) was approximately 2-fold greater than that after the lower dose of 25 mg/kg (0.78 ± 0.05). This difference was statistically significant. Previous studies in rats demonstrated a dose-dependent disposition of AZDU following intravenous administration at doses of 10, 50, 100 and 250 mg/kg. The AUC increased proportionally with dose up to 100 mg/kg; however, the dose-normalized AUC after 250 mg/kg was 1.5-fold greater than that after the lower doses, indicating a linear kinetics over the dose range of 10 - 100 mg/kg (22). The reason that nonlinear pharmacokinetics were observed at dose 100 mg/kg in this study is likely due to the difference in the manner in which the animals were dosed. In the previous study, AZDU was administered over a 15-min infusion, whereas in this study the compound was administered as a bolus dose. As a result, a high initial drug concentration following administration of 100 mg/kg AZDU in the present study was comparable to that found in the previous study at 250 mg/kg (22).

A significant 2-fold decrease in $CL_T$ of AZDU as the dose increased, from 1.38 L/h/kg at 25 mg/kg to 0.70 L/h/kg at 100 mg/kg, was observed (Table 1). Consistent with previous studies (22), saturation of the active renal tubular secretion, as evidenced by a reduced renal clearance at the higher dose, was the primary cause of the observed dose dependent pharmacokinetics. Following a dose of 25 mg/kg, approximately 69% of the nucleoside was excreted unchanged in urine whereas 44% was excreted unchanged following administration of 100 mg/kg AZDU. Renal clearance of AZDU decreased

from 0.88 L/h/kg following administration of 25 mg/kg AZDU to 0.39 L/h/kg at a dose of 100 mg/kg. There was no significant difference in the $CL_{NR}$ of AZDU between the lower and higher doses, indicating that metabolism or other non-renal routes of elimination were not saturated.

The steady-state volume of distribution of AZDU also decreased significantly as the administered dose increased (Table 1). Due to comparable changes in clearance and volume of distribution, however, there were no statistically significant differences found between values of the terminal half-life.

Figure 3 depicts plasma concentrations of AZDU following oral administration of the compound. The $C_{max}$ values were 10.24 ± 2.55 µg/ml following 25 mg/kg dosing and 41.60 ± 14.90 µg/ml following oral administration of 100 mg/kg AZDU. The $t_{max}$ values were 0.38 ± 0.13 h and 0.63 ± 0.27 h following oral administration of 25 and 100 mg/kg AZDU, respectively. As dose increased, dose-normalized AUC values showed an increase similar to that observed after intravenous administration. Following oral administration of 25 mg/kg and 100 mg/kg AUC values increased from 11.72 to 70.35 mg·h/L. The nonlinearity in AUC is likely to due to nonlinear clearance as observed with the intravenous dosing. Generally an increase in AUC following oral dosing which is greater than dose proportional is more likely to be attributable to a decrease in elimination than to an increase in absorption (24). The half-life was approximately 1 h for both doses.

The oral bioavailability estimates of AZDU from the AUC values obtained after the crossover studies at doses of 25 and 100 mg/kg were 60% and 46%, respectively. These values were not statistically significantly different. The overall average oral

bioavailability of AZDU was 53%, which was lower than the 76% oral bioavailability reported in mice (23).   In rhesus monkeys, the oral bioavailability of AZDU was dose dependent, with F values ranging from greater than 90% following administration of 33 mg/kg to less than 50% following 200 mg/kg (25).

In summary, the disposition of AZDU in rats was dose-dependent over the range of 25 to 100 mg/kg following intravenous bolus injection of the nucleoside analog. Bioavailability of AZDU following oral administration, however, was independent of the dose.

**ACKNOWLEDGEMENTS**

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**Table 1.** Mean (SD) pharmacokinetic parameters of AZDU following intravenous and oral administration of 25 mg/kg and 100 mg/kg AZDU to rats.

| Route | Dose (mg/kg) | AUC (mgh/ml) | $CL_T$ (L/h/kg) | $CL_R$ (L/h/kg) | $CL_{NR}$ (L/h/kg) | $V_{ss}$ (L/kg) | $t_{1/2}$ (h) | F |
|---|---|---|---|---|---|---|---|---|
| iv | 25 | 19.43[a] (1.24) | 1.38[a] (0.20) | 0.90[a] (0.27) | 0.48[a] (0.15) | 0.78[a] (0.26) | 0.53[a] (0.04) | -- |
| | 100 | 155.80[b] (6.97) | 0.70[b] (0.09) | 0.43[b] (0.12) | 0.27[a] (0.05) | 0.34[b] (0.11) | 0.60[a] (0.05) | -- |
| po | 25 | 11.72[a] (0.54) | -- | -- | -- | -- | 0.99[a] (0.26) | 0.60[a] |
| | 100 | 70.35[b] (13.07) | -- | -- | -- | -- | 1.10[a] (0.38) | 0.46[a] |

Dissimilar letter superscripts indicate statistically significant difference ($p < 0.05$).
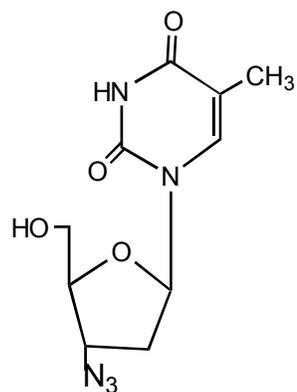
**FIGURE LEGENDS**

**Figure 1.** Chemical structures of AZDU and AZT

**Figure 2.** Mean plasma concentrations as a function of time of AZDU following

intravenous administration of AZDU at dose 25 mg/kg (○) and 100 mg/kg (●) to rats.

**Figure 3.** Mean plasma concentrations of AZDU following oral administration of AZDU

at dose 25 mg/kg (◊) and 100 mg/kg (♦) to rats.
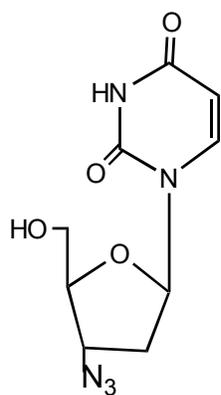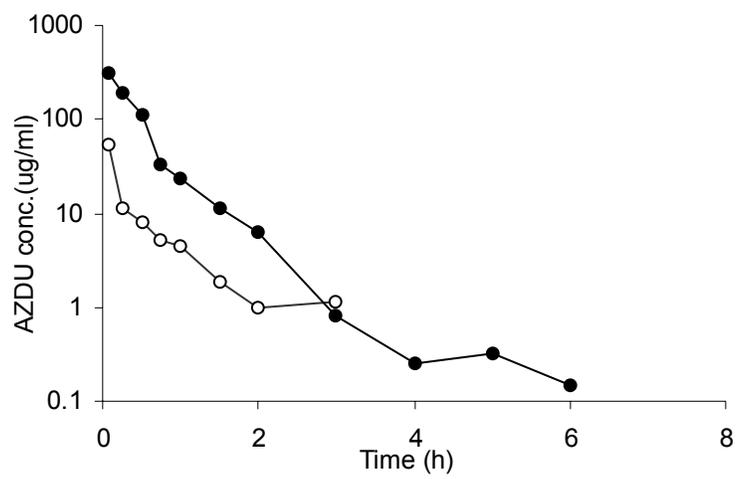
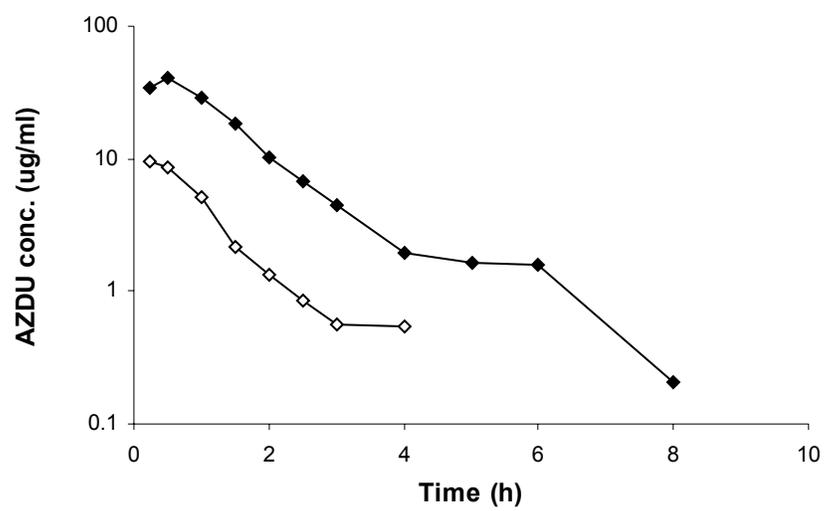AZDU                    AZT

Figure 1

Figure 2

Figure 3

**CHAPTER III:**

**Simultaneous Determination of 3'-Azido-2', 3'-Dideoxyuridine and Its Novel Prodrugs in Rat Plasma by High Performance Liquid Chromatography[1]**

**ABSTRACT**

**Purpose.** To develop a high performance liquid chromatographic (HPLC) method to quantitatively determine 3'-azido-2',3'-dideoxyuridine (AZDU) and prodrugs, N3-pivaloyloxymethyl-3'-azido-2',3'-dideoxyuridine (I), 5'-pivaloyloxy-methyl-3'-azido-2',3'-dideoxyuridine (II), 5'-*O*-valinyl-3'-azido-2',3'-dideoxyuridine hydrochloride (III) and 5'-*O*-phenylalaninyl-3'-azido-2',3'-dideoxyuridine hydrochloride (IV), in rat plasma simultaneously. **Methods.** A reversed-phase gradient elution HPLC method was developed to simultaneously quantitate AZDU and its prodrugs. AZDU and its prodrugs were analyzed using an octadecyl silane (C-18) column (4.6 mm ID X 15 cm, 5 μm). The mobile phase consisted of 0.04 mM sodium acetate buffer (pH 5.0) and acetonitrile, running in a segmented gradient manner at a flow-rate of 2 ml/min. Acetonitrile was increased from 10% to 50% during the first 8 min by 5% per min, followed by a 10% per min increase to 90% acetonitrile. 3'-Azido-2', 3'-dideoxy-5-ethyluridine (CS-85) at a concentration of 25 μg/ml was used as an internal standard. All compounds were detected by UV absorption at 261 nm. **Results.** Extraction recoveries for all compounds were greater than 80%. Retention times of AZDU, CS-85, prodrugs I, II, III and IV were 3.3, 5.2, 9.1, 8.8, 6.3 and 7.3 min, respectively. Calibration plots were linear over the range of 0.25 μg/ml to 100 μg/ml for compounds AZDU, II, III, and IV and 0.5 μg/ml to 100 μg/ml for prodrug I. The limit of quantitation was 0.25 μg/ml for prodrugs II, III and IV and 0.5 μg/ml for prodrug I. The intra- and inter-day variations were less than 10% and accuracies were greater than 90%. **Conclusions.** The HPLC method developed is rapid, sensitive and reproducible for the determination of AZDU and its prodrugs in rat plasma.

**Keyword:**   Reversed phase, high performance liquid chromatographic (HPLC), AZDU, prodrug

**INTRODUCTION**

Acquired immundeficiency syndrome (AIDS), first diagnosed in 1981 (1, 2, 3), has become a global epidemic. While major advances have been made in the understanding of the disease and clinical treatments, there currently is no cure for AIDS. Therefore, the search for highly effective therapeutics agents with low toxicity continues.

3'-Azido-2', 3'-dideoxyuridine (AZDU, CS-87) is a synthetic nucleoside analog structurally similar to zidovudine (AZT) with proven activity against human immunodeficiency virus (HIV) and lower bone marrow toxicity as compared to AZT (4, 5, 6, 7). However, the clinical application of AZDU is limited by its relatively short half-life, extensive glucuronidation in patients and insufficient delivery to the brain and lymphatic system. Thus, research has focused on the discovery of prodrugs of AZDU with improved pharmacokinetic properties such as higher oral bioavailability, longer half-life and enhanced brain and lymphatic delivery. Recently four novel compounds, N3-pivaloyloxymethyl-3'-azido-2',3'-dideoxyuridine (I), 5'-pivaloyloxymethyl-3'-azido-2',3'-dideoxyuridine (II), 5'-O-valinyl-3'-azido-2',3'-dideoxyuridine hydrochloride (III) and 5'-O-phenylalaninyl-3'-azido-2',3'-dideoxyuridine hydrochloride (IV), were synthesized as potential prodrugs of AZDU (8). The chemical structures of these potential prodrugs are shown in Figure 4.

To investigate the preclinical distribution and metabolism of these prodrugs, a method for the simultaneous quantitation of the prodrugs and parent compound, AZDU, is required. High-performance liquid chromatographic (HPLC) techniques have been reported for the determination of AZDU and other prodrugs (9, 10, 11, 12, 13, 14). The objective of the present study was to develop a rapid, accurate and sensitive HPLC

method for the quantitation of AZDU and its potential prodrugs, N3-pivaloyloxymethyl-3'-azido-2', 3'-dideoxyuridine (I), 5'-pivaloyloxymethyl-3'-azido-2', 3'-dideoxyuridine (II), 5'-O-valinyl-3'-azido-2', 3'-dideoxyuridine hydrochloride (III) and 5'-O-phenylalaninyl-3'-azido-2', 3'-dideoxyuridine hydrochloride (IV), suitable for preclinical pharmacokinetic studies in rats.

## EXPERIMENTAL SECTION

### Chemicals

3'-Azido-2', 3'-dideoxyuridine (AZDU), internal standard, 3'-azido-2', 3'-dideoxy-5-ethyluridine (CS-85), and prodrugs I, II, III and IV were synthesized as previously described (15, 16). The chemical purity of each compound, as assessed by HPLC and spectral analysis, was greater than 99%. Acetonitrile, HPLC grade, and other chemicals, analytical grade, were purchased from J. T. Baker (Phillipsburg, NJ, USA).

### Preparation of standards

Standard solutions of 100, 10 and 1.0 μg/ml of the compounds were prepared in distilled water. Standard solutions were added to rat plasma to obtain calibration concentrations of 0.1, 0.25, 0.5, 1, 5, 10, 20, 50, 80, 100 μg/ml.

### Extraction procedure

The extraction of the compounds from plasma was performed by slight modification of a liquid-liquid extraction method described previously (10, 14, 17). Standard solutions were added to 1.5 ml polypropylene centrifuge tubes containing 100 μl rat plasma, followed by 50 μl of internal standard, CS-85 (25 μg/ml). Ice-cold acetonitrile (0.9 ml) was added to precipitate plasma proteins. The tubes were vigorously

mixed for 1 min and centrifuged for 10 min at 9000 g. The supernatant was transferred to a polypropylene centrifuge tube, and excess crystalline magnesium sulfate was added. After mixing for 3 min, the tubes were centrifuged for 10 min at 9000 g. The supernatant was transferred to a clean polypropylene tube and evaporated to dryness under a stream of nitrogen gas at room temperature. The residual film was reconstituted in 200 μl of water and transferred to a disposable 300 μl polypropylene injection tube. Fifty microliters (50 μl) was injected onto HPLC.

**Chromatography**

The HPLC system used for analysis of rat plasma samples consisted of a Shimadzu LC-10A solvent delivery system, a Shimadzu SCL-10A system controller, a Shimadzu SPD-10A UV-VIS detector, SIL-10A auto injector and CR-501 chromatopac integrator. Concentrations of AZDU and its prodrugs in plasma were determined by reversed-phase high-performance liquid chromatography using an octadecyl silane ($C_{18}$) column (4.6 mm ID × 15 cm, 5 μm particle size; Alltech Associates, Deerfield, IL) protected by a Nova-Pak $C_{18}$ guard column with 4 μm particle size. The mobile phase was consisted of solvent A, 0.04 M sodium acetate buffer (pH 5.0) and solvent B, acetonitrile. A segmented gradient elution was used to separate compounds at a flow-rate of 2 ml/min. During the first 8 min, the concentration of acetonitrile was increased from 10% to 50% by 5% per min and then by 10% per min until the mobile phase consisted of 10% buffer (A) and 90% acetonitrile (B). After each analysis, the column was equilibrated for 10 min to initial conditions. The chromatography was performed at room temperature.

**Quantitation**

Concentrations of AZDU and prodrugs in unknown samples were determined from the slope of calibration plots of the peak-area ratio of AZDU or prodrug/internal standard versus the calibration standard AZDU or prodrug concentrations. Slopes were determined using linear regression analysis with a weighting factor of 1/concentration$^2$ (18). Use of this weighting factor generated a normal distribution of weighted residuals around the standard curve over the entire range of drug concentrations.

**Assay specifications**

The extraction recoveries of AZDU and prodrugs I, II, III and IV were assessed at plasma concentrations of 0.5, 5.0 and 50 μg/ml. The recovery of internal standard was assessed at 25 μg/ml at which it was used for the assay. Three plasma samples (100 μl) containing drug and internal standard were extracted and injected. Three injections of the same amount of compound in water were directly injected. The peak areas of the compounds were measured and the percentage recovery was calculated from 100 × (peak area, $_{extraction}$/mean peak area, $_{direct\ injection}$).

The intra- and inter-day precision and accuracy of the analytical method were determined by analysis of six plasma samples containing 0.5, 5.0, 50 μg/ml concentrations for five compounds. Assay precision was determined by calculating relative standard deviations (RSD) for each drug concentration. Accuracy was calculated by comparing measured concentrations to the known values.

**RESULTS AND DISCUSSION**

The purpose of this study was to develop an HPLC analytical method for the simultaneous determination of AZDU and its prodrugs, I, II, III, IV, in rat plasma. The

method employed a liquid-liquid extraction procedure with slight modifications (10, 14, 17). AZDU and its prodrugs have a wide retention range (19). Under isocratic conditions, AZDU tends to be an early elute while prodrugs, especially prodrug I and II, are late elutes. Thus, to measure the parent drug, AZDU, and its prodrugs simultaneously, a gradient elution method was developed. In this method, a binary-solvent mobile phase consisted of 40 mM sodium acetate buffer (pH 5.0) and acetonitrile was used throughout the separation, with the concentration of acetonitrile (B) increasing from 10% to 90% in a segmented fashion during each run. This method provided appropriate retention times for both AZDU and all its prodrugs, while good separation was achieved.

Figure 5 illustrates chromatograms corresponding to the extracts of blank rat plasma and rat plamsa with AZDU, prodrug I, prodrug II, prodrug III, prodrug IV at a concentration of 50 μg/ml and internal standard. Each compound eluted with a sharp peak and distinct separation at baseline. The retention times of AZDU, CS-85, prodrugs I, II, III and IV were 3.3, 5.2, 9.1, 8.8, 6.3 and 7.3 min, respectively. Blank plasma sample was free of interferences at the retention times corresponding to the compounds of interest.

The limit of quantitation was 0.25 μg/ml for prodrugs II, III and IV and 0.5 μg/ml for prodrug I when a signal-to-noise ratio of 3 was used as a criterion for a significant response. Calibration plots were linear over the range of 0.25 μg/ml to 100 μg/ml for compounds AZDU, II, III, IV and CS-85, and 0.5 μg/ml to 100 μg/ml for prodrug I. The assay specifications including extraction recovery, intra- and inter-day precision and accuracy for AZDU and its prodrugs at 0.5, 5.0 and 50 μg/ml concentrations are

presented in Table 2. The extraction recoveries for AZDU, all prodrugs and internal standard were greater than 80%. The precision of the assay was acceptable with relative standard deviations less than 10%. The accuracy of the method was greater than 90% for all the compounds at low, medium, and high concentrations.

In summary, the determination of AZDU and its prodrugs I, II, III and IV in rat plasma by the HPLC method described is simple, rapid, sensitive and reproducible. The limit of quantitation of this method is sufficiently sensitive to characterize the pharmacokinetics of the prodrugs and their biotransformation to AZDU. With slight modifications, this method can also be applied to studies on the pharmacokinetics of AZDU and its prodrugs in other animal models.

**ACKNOWLEDGEMENT**

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**FIGURE LEGENDS**

**Figure 4.** Chemical structures of AZDU, internal standard CS-85, and prodrugs N3-pivaloyloxymethyl-3'-azido-2', 3'-dideoxyuridine (I), 5'-pivaloyloxymethyl-3'-azido-2', 3'-dideoxyuridine (II), 5'-O-valinyl-3'-azido-2',3'-dideoxyuridine hydrochloride (III) and 5'-O-phenylalaninyl-3'-azido-2',3'-dideoxyuridine hydrochloride (IV).

**Figure 5.** Representative chromatograms for (A) blank plasma and (B) rat plasma with (1) AZDU, (2) internal standard, CS-85, (3) prodrug III, (4) prodrug IV, (5) prodrug II and (6) prodrug I added.

**Table 2.** Assay specifications for the simultaneous determination of AZDU and prodrugs N3-pivaloyloxymethyl-3'-azido-2', 3'-dideoxyuridine (I), 5'-pivaloyloxymethyl-3'-azido-2',3'-dideoxyuridine (II), 5'-O-valinyl-3'-azido-2',3'-dideoxyuridine hydrochloride (III) and 5'-O-phenylalaninyl-3'-azido-2',3'-dideoxyuridine hydrochloride (IV).

| Compound | Concentration (µg/ml) | Recovery (%) | Precision (%) | | Accuracy (%) | |
|---|---|---|---|---|---|---|
| | | | Intra-day | Inter-day | Intra-day | Inter-day |
| AZDU | 0.5 | 95.2 ± 1.5 | 2.22 | 1.10 | 92.10 | 95.98 |
| | 5.0 | 94.6 ± 2.8 | 1.02 | 7.34 | 97.55 | 100.00 |
| | 50.0 | 90.4 ± 1.0 | 5.65 | 1.20 | 92.33 | 98.77 |
| I | 0.5 | 93.1 ± 5.2 | 7.98 | 9.49 | 96.59 | 90.03 |
| | 5.0 | 94.4 ± 3.7 | 9.19 | 8.23 | 95.56 | 92.48 |
| | 50.0 | 94.8 ± 5.7 | 6.75 | 8.07 | 99.30 | 97.79 |
| II | 0.5 | 82.2 ± 8.9 | 1.98 | 7.11 | 97.69 | 94.85 |
| | 5.0 | 91.1 ± 5.2 | 0.48 | 6.72 | 94.70 | 93.93 |
| | 50.0 | 97.1 ± 1.5 | 3.01 | 6.38 | 99.00 | 93.11 |
| III | 0.5 | 81.2 ± 6.8 | 7.31 | 3.37 | 95.43 | 92.78 |
| | 5.0 | 92.2 ± 1.1 | 0.68 | 2.85 | 98.90 | 97.87 |
| | 50.0 | 92.5 ± 4.2 | 3.51 | 6.93 | 99.52 | 92.81 |
| IV | 0.5 | 84.7 ± 6.3 | 3.70 | 7.05 | 92.96 | 93.73 |
| | 5.0 | 91.1 ± 2.9 | 2.63 | 7.21 | 97.66 | 96.10 |
| | 50.0 | 92.4 ± 1.8 | 1.13 | 3.12 | 92.61 | 96.21 |

AZDU

CS-85

Prodrug I
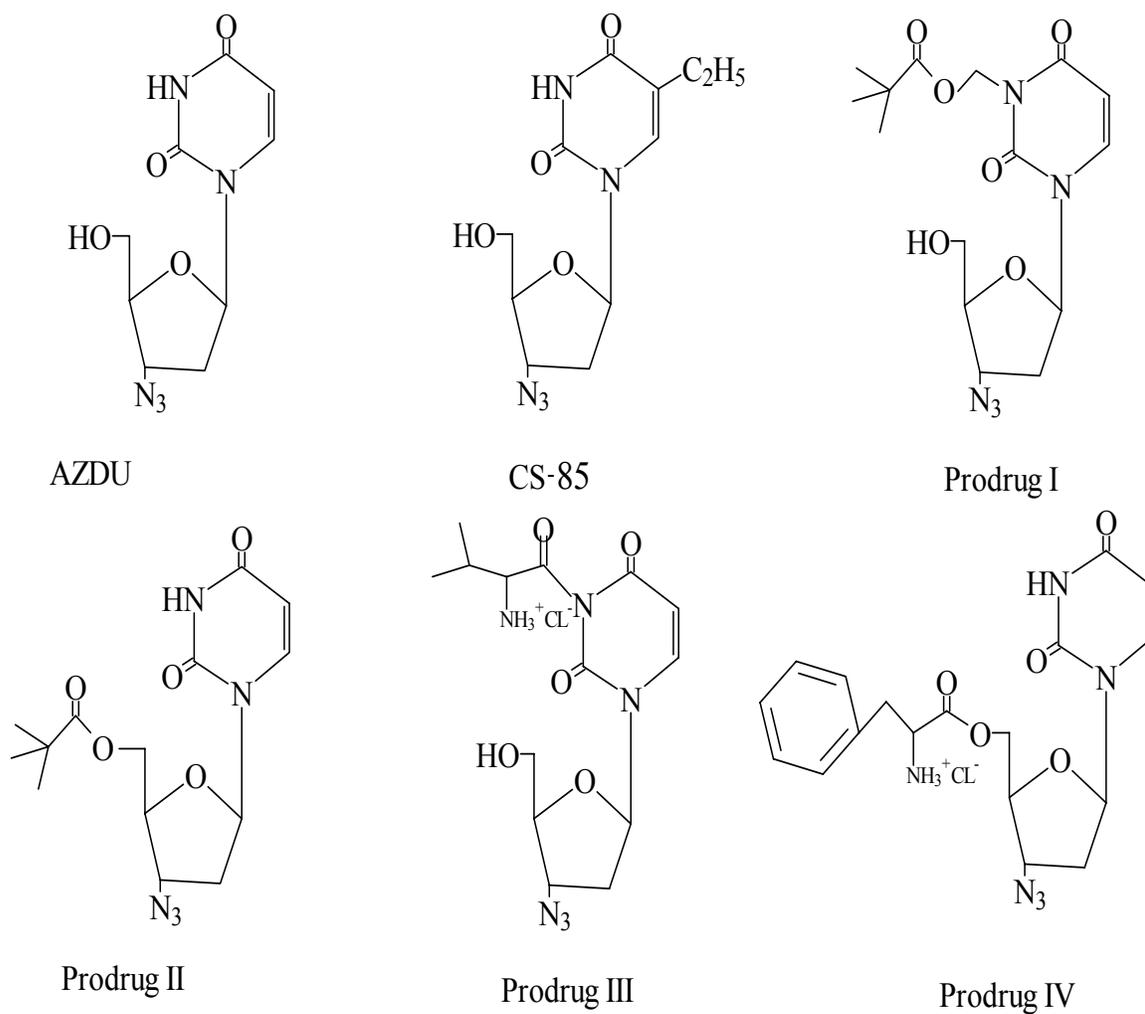
Prodrug II
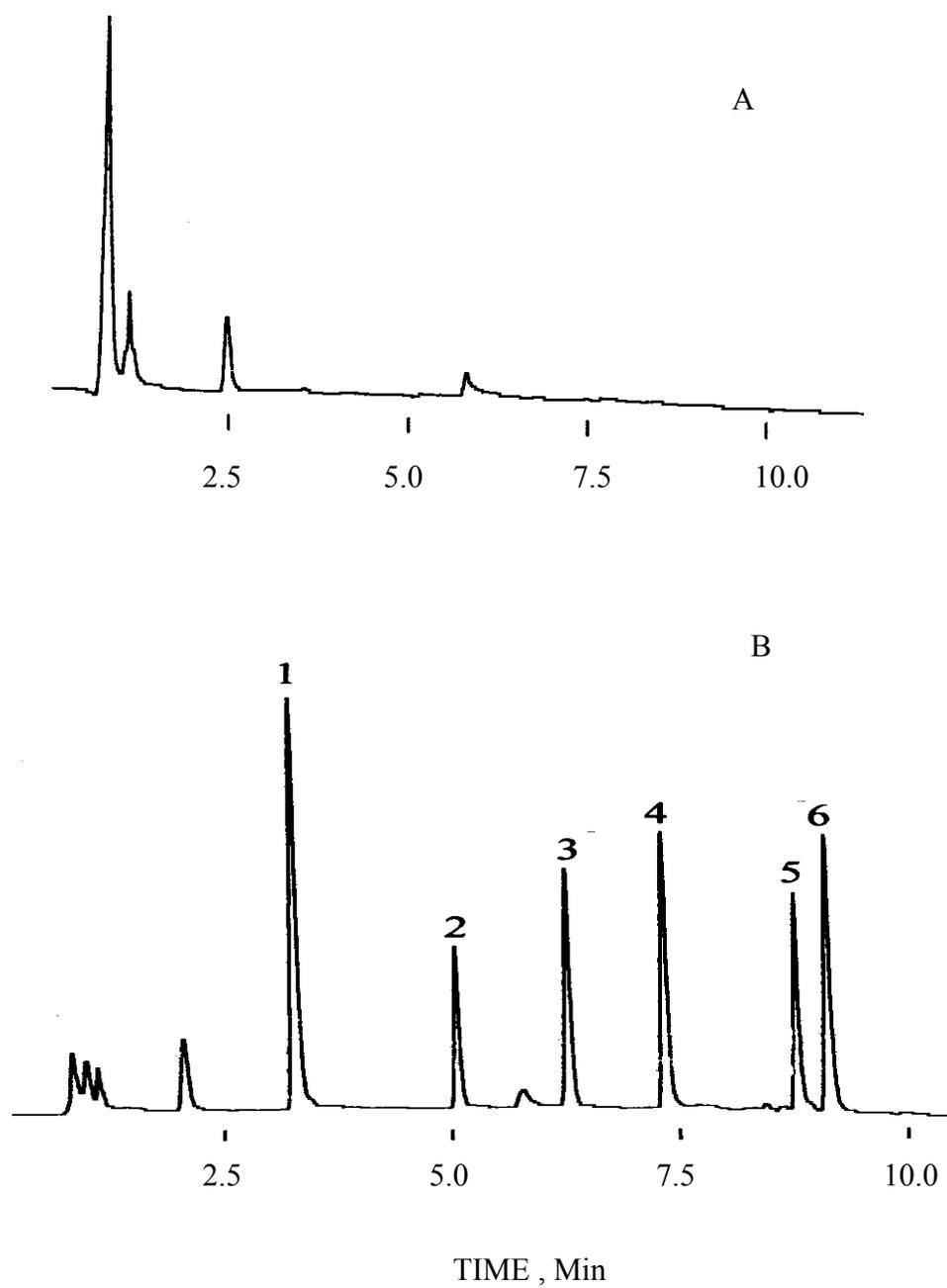
Prodrug III

Prodrug IV

Figure 4

A

B

TIME , Min

Figure 5

# CHAPTER IV:

## *In Vitro* and *in Vivo* Evaluation of 3'-Azido-2', 3'-Dideoxyuridine-5'-*O*-Valinate-Hydrochloride as a Prodrug of the Anti-HIV Nucleoside, 3'-Azido-2', 3'-Dideoxyuridine[1]

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[1] Kong, L., Huang, C., Cooperwood, J. S., Boudinot, F. D., Chu, C. K. To be submitted to Antimicrobial Agents and Chemotherapy.

**ABSTRACT**

**Purpose.** 3'-Azido-2', 3'-dideoxyuridine (AZDU, AzddU, CS-87) has been shown to have potent anti-HIV activity *in vitro*. In an effort to increase it's relatively short half-life and incomplete bioavailability, prodrug 3'-azido-2',3'-dideoxyuridine-5'-*O*-valinate hydrochloride (AZDU-VAL) was synthesized by the esterification of 5'-OH function in AZDU. The objective of this study was to investigate the *in vitro* stability of the potential prodrug AZDU-VAL in phosphate buffer and *in vivo* biotransformation and pharmacokinetics of AZDU-VAL along with its antiviral parent compound AZDU following intravenous and oral administration to rats. **Methods.** The partition coefficients of AZDU and AZDU-VAL were determined using shake-flask method in octanol/water. *In vitro* stability of AZDU-VAL was investigated in phosphate buffer at pH values of 2.6, 5 and 7.4. Adult male Sprague-Dawley rats were administered 75 mg/kg and 100 mg/kg of AZDU-VAL intravenously and orally, respectively. Concentrations of AZDU-VAL and AZDU were determined by HPLC. Pharmacokinetic parameters were generated by area-moment analysis. **Results.** The partition coefficients of AZDU and AZDU-VAL were 0.43 and 0.01, respectively, indicating the prodrug is markedly water soluble. *In vitro* stability studies in buffer solutions demonstrated favorable stability of AZDU-VAL at pH values of 2.6, 5 and 7.4. *In vivo* the prodrug was rapidly and efficiently bioconverted to yield AZDU following intravenous and oral administration. Maximum plasma concentrations ($C_{max}$) of AZDU varied from 15.1 to 32.4 µg/ml and were achieved within $0.35 \pm 0.14$ h after oral administration of AZDU-VAL. The terminal phase half-life of AZDU formed from AZDU-VAL was $8.80 \pm 4.36$ h. The dose-normalized areas under the concentration-time curves (AUC) were 0.55 and

0.42 µg·h/ml/mg/kg following intravenous and oral administration, respectively. The apparent availability of AZDU following oral administration of prodrug AZDU-VAL ranged from 50.9% to 140.1 with an average value of 101%. The bioavailability of AZDU following intravenous administration of prodrug AZDU-VAL averaged 106%.

**Conclusions.** The results suggest that AZDU-VAL appears to be a promising prodrug for the delivery of AZDU.

**Keywords:** 3'-Azido-2',3'-dideoxyuridine, AZDU, AZDU-VAL, prodrug, pharmacokinetics

**INTRODUCTION**

3'-Azido-2',3'-dideoxyuridine (AZDU, AzddU, CS-87, Uravidine) is a nucleoside analog similar in structure to 3'-azido-3'-deoxythymidine (AZT, zidovudine), the most frequently used drug in the treatment of human immunodeficiency virus (HIV) infection. AZDU has been shown to have significant anti-HIV activity *in vitro* in human peripheral blood mononuclear cells (1, 2) with significantly reduced human bone marrow toxicity as compared to AZT (4). Preclinical studies demonstrated that the pharmacokinetic characteristics of AZDU and AZT were similar in mice, rats and rhesus monkeys (5, 6, 7, 8). Thus, the selective anti-HIV-1 activity of AZDU makes it a potential drug for AIDS therapy. However, preclinical studies also showed that AZDU had a relatively short half-life and incomplete bioavailability in some animal models (5, 6, 7, 8).

To improve its pharmacokinetic properties, a prodrug, 3'-azido-2',3'-dideoxyuridine-5'-*O*-valinate hydrochloride (AZDU-VAL) was synthesized (3) to obtain a prolonged half-life and increased bioavailability of the parent compound. Figure 1 shows the chemical structures of AZDU and potential prodrug AZDU-VAL. This prodrug was designed based on the fact that 5'-L-valyl ester of acyclovir (L-Val-ACV) enhance the uptake of ACV 10 times more than the parent drug and its D-isomer D-Val-ACV. *In vitro* studies using thansfected Chinese Hamster Ovary (CHO) cells over-expressing the hPEPT1 transporter, L-Val-ACV showed strong affinity for the hPEPT1 transporter with an IC50 of 1.1 mM (inhibition of gly-sar uptake) in comparison with cephradine (IC50 = 15 mM) and enalapril (IC50 = 4.5 mM). Furthermore, *in vivo* L-Val-ACV was well absorbed and rapidly converted to ACV in humans (3). The purpose of

this study was to characterize the preclinical pharmacokinetics of AZDU-VAL following intravenous and oral administration of the prodrug to rats.

## MATERIALS AND METHODS

### Chemicals

3'-Azido-2',3'-dideoxyuridine (AZDU) and AZDU-VAL were synthesized as previously described (2, 3). Internal standard, D4T, was obtained from Sigma Chemical Co. (St. Louis, MO). The chemical purity of the compounds, confirmed by spectral and high-performance liquid chromatography (HPLC) analysis, was greater than 98%. For intravenous and oral administration, AZDU and AZDU-VAL were dissolved in phosphate-buffered saline, pH 7.4. Acetonitrile (HPLC grade) and all other chemicals (reagent grade) were obtained from J. T. Baker (Phillipsburg, NJ).

### Synthesis of 3'-azido-2',3'-dideoxyuridine- 5'-*O*-valinate hydrochloride

Melting points were determined on a Mel-temp II and are uncorrected. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker 400 AMX spectrometer for 400 MHz with Me$_4$Si as internal standard. Chemical shifts ($\delta$) are reported in parts per million (ppm), and signals are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or br s (broad singlet). Optical rotations were performed on a Jasco DIP- 370 Digital Polarimeter. TLC were performed on Uniplates (silica gel) purchased from Analtech Co.. Column chromatography was performed using either silica gel 60 (220- 440 mesh) for flash chromatography or silica gel G (TLC grade > 440 mesh) for vacuum flash column chromagraphy. UV spectra were obtained on a Bechman DU- 650 Spectrophotometer. Elemental analysis was performed by Atlantic Microlab Inc., Norcross, GA.

DMAP (86.5 mg, 0.7 mmol), N-t-Boc valine (1.5 mg, 7 mmol) and DCC (2 g, 9.7 mmol) was added to a solution of AZDU (1.25 g, 4.95 mmol) in DMF (25 ml). After completion (ca. 48 h), the reaction mixture was filtered, and the filtrate concentrated to dryness in vacuo. The residue was dissolved in $CH_2Cl_2$, washed with water (2 x 50 ml), sat. $NaHCO_3$ solution (2 x 50 ml) and dried over $MgSO_4$ then purified by silica gel chromatography using 0-2 % MeOH/$CH_2Cl_2$ to give 5'-$O$-N-t-Boc valinyl-3'-azido-2',3'-dideoxyuridine (I) as a white solid (2 g, 89%): $[\alpha]^{25}_D$ 34.2° (c 0.6, MeOH); UV (MeOH) $\lambda_{max}$ 260 nm (log ε 3.8) $^1$H NMR (400 MHz, CDCl$_3$) δ 8.4 (s, 1H), 7.50 (d, J = 7.9 Hz, 1H), 6.11 (t, J = 6.0 Hz, 1H), 5.84 (d, J = 7.8 Hz, 1H), 4.97 (d, J = 8.8 Hz, 1H), 4.29 (m, 1H), 4.22 – 4.11 (m, 4H), 2.52 (m, 1H), 2.34 (m, 1H), 1.44 (s, 9H), 0.99 (d, J = 6.8 Hz, 3H), 0.93 (d, J = 6.8 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 172.2,162.5,155.6, 149.7, 139.5, 103.0, 85.7, 81.7, 80.3, 63.6, 60.4, 58.9, 37.7, 30.9, 28.3, 19.1, 17.8; FABMS m/z 368 (M + H)$^+$. Anal. Calcd for $C_{19}H_{27}O_7N_6$ 0.85 EtOAc: C, 51.14; H, 6.49; N, 15.97. Found: C, 51.49; H, 6.48; N, 16.36.

Compound I was dissolved in 15% HCl/THF (50 ml) cooled to 10°C, then the mixture was allowed to warm to ambient temperature and stirred for 12 h. After completion, the reaction mixture was diluted with diethyl ether (500 ml) resulting in the precipitation of 3'-azido-2',3'-dideoxyuridine-5'-$O$-valinate hydrochloride (AZDU-VAL) as an off-white amorphous solid: $[\alpha]^{25}_D$ 38.3° (c 0.5, $H_2O$); UV (MeOH) $\lambda_{max}$ 259 nm (log ε 3.4); $^1$H NMR (400 MHz, DMSO-d$_6$) δ 11.17(s, 1H), 8.27 (br s, 2H),7.44 (d, J = 8.1 Hz, 1H), 5.87 (t, J = 6.2 Hz, 1H), 5.43 (d, J = 8.1 Hz, 1H), 4.2 – 4.1 (m, 4H), 3.8 (m, 1H), 3.68 (d, J = 4.6 Hz, 1H), 2.13 (m, 1H), 1.95 (m, 1H), 0.74 (d, J = 6.8 Hz, 3H), 0.70 (d, J = 6.8 Hz, 3H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 167.3,161.6,148.9, 139.7,

100.7, 83.9, 79.0, 63.6, 58.6, 55.8, 34.0, 27.9, 16.7, 16.1; FABMS m/z 353 $(M + H - Cl)^+$.

Anal. Calcd for $C_{14}H_{16}O_5N_5$ 0.1 EtOAc: C, 49.97; H, 5.77; N, 20.23. Found: C, 50.07; H, 5.98; N, 19.76.

**Partition Coefficients**

The partition coefficients of AZDU-VAL and AZDU were determined by the shake flask method (9) using mutually saturated octanol and water at 37°C. AZDU (2 mg) or AZDU-VAL (2 mg) were dissolved in 4 ml water and shaken for 24 h with 4 ml octanol on a horizontal shaker at 37°C. The concentrations of the compounds in the aqueous phase were determined by HPLC and the partition coefficients (PC) were calculated according the equation $PC = (C_0 - Ce)/Ce$, where $C_0$ represents the initial concentration of compounds in aqueous phase and Ce is the aqueous concentration at equilibrium.

***In vitro* stability studies**

The *in vitro* stability of AZDU-VAL was assessed in buffer solutions at pH values of 2.6, 5.0 and 7.4. AZDU-VAL was dissolved in the buffers at a concentration 100 μg/ml. Buffer solutions were incubated at 37°C with gentle agitation in a shaking water bath. Aliquots (200 μl) of buffers were collected at specified times after initiation of the reaction into tubes placed on ice. Samples were immediately analyzed for concentrations of AZDU-VAL by high performance liquid chromatography (HPLC). First-order degradation rate constants (*k*) and half-lives ($t_{1/2} = 0.693/k$) for the *in vitro* studies in buffer were determined by linear least-squares regression of the natural logarithm of drug concentration versus time values.

**Pharmacokinetic studies**

Adult male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing $392 \pm 10$g (mean $\pm$ SD) were used for the study. Animals were housed in a 12-h light/12-h dark, constant temperature (22°C) environment with free access to standard laboratory chow and water in the University of Georgia, college of Pharmacy Animal Care Facility, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Rats were acclimatized to this environment for at least one week before the study. Animal studies were approved by the University of Georgia Animal Care and Use Committee and conducted in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (10).

External jugular vein cannulas were implanted under (ketamine:acepromazine:xylazine; 50:3.3:3.4 mg/kg) anesthesia the day before the experiment. Rats were fasted overnight, however, water was allowed *ad libitum*. A randomized oral/intravenous crossover study design with two dosing periods and a 4 day washout period between doses was used. Each animal received a 75 mg/kg dose of AZDU-VAL orally and a 100 mg/kg dose intravenously. Following dosing, the animals were housed in metabolism cages. Blood samples (0.3 ml) were collected prior to and at 0.083, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8 h after drug administration from the cannulas into tubes that contained 1.8 ml 2 M ice-cold acetonitrile.

**Drug analysis**

Concentrations of AZDU-VAL and AZDU in plasma were determined by reverse phase high performance liquid chromatography (HPLC). Plasma samples were added to

tubes containing 1.8 ml 2 M ice-cold acetonitrile, followed by 50 μl internal standard (D4T, 20 μg/ml). Tubes were vigorously mixed for 30 seconds and centrifuged at 4500 g for 30 min. The supernatant was transferred to a clean tube and dried by nitrogen stream. The residual film was reconstituted with 75 μl HPLC water, and 50 μl was injected onto the HPLC.

Separation was achieved using a Hypersil ODS ($C_{18}$) column (150 mm × 4.6 mm, 5 μm particle size, Alltech Associates, Deerfield, IL) and a mobile phase consiting of 5% acetonitrile in 40 mM sodium phosphate monobasic, pH 5.0 (v/v) at a flow rate of 2 ml/min. The compounds were quantitated at an UV wavelength of 261nm. The retention times for AZDU-VAL, AZDU and internal standard were 46, 17.5 and 9.6 min, respectively.

Standard curves ranging from 0.5 to 25 μg/ml for AZDU-VAL and 0.25 to 50 μg/ml for AZDU were prepared in blank rat plasma. AZDU-VAL and AZDU concentrations in the samples were calculated from the slope of calibration plots of the peak area ratio of drug/internal standard versus standard drug concentrations. A weighing factor of 1/concentration was used for standard curve regression analysis. Standard curves were linear in the range of 0.5 to 25 μg/ml for AZDU-VAL and 0.25 to 50 μg/ml for AZDU with the limits of quantitation 0.5 and 0.25 μg/ml, respectively. The intra- and inter-day coefficients of variation over the range of the standard concentrations were less than 10%. Extraction recoveries of AZDU-VAL, AZDU and internal standard D4T were greater than 85%.

**Pharmacokinetic analysis**

Pharmacokinetic parameters were determined by area-moment analysis. The maximum

plasma concentrations ($C_{max}$) and the time to achieve maximum concentrations ($t_{max}$)

were determined from observed data. The area under the plasma concentration versus

time curve (AUC) and the first moment (AUMC) were calculated by Lagrange

polynomial interpolation from time zero to the last measured sample time, with

extrapolation to time infinity by using the terminal slope ($\lambda z$) (11, 12). Total clearance

($CL_T$) was calculated from Dose/AUC. Mean residence time (MRT) was calculated from

AUMC/AUC, and steady-state volume of distribution ($V_{ss}$) was calculated from $CL_T \times$

MRT. Half-life ($t_{1/2}$) was calculated from $0.693/\lambda_z$. Bioavailability (F) of AZDU

following intravenous prodrug administration was calculated from ($AZDU_{iv}^{pro} \times D_{iv}^{par}/$

$AZDU_{iv}^{par} \times D_{iv}^{pro}$) $\times$ 100%. Oral bioavailability (F) of AZDU following oral

administration was calculated from ($AZDU_{po}^{pro} \times D_{iv}^{pro}/ AZDU_{iv}^{pro} \times D_{po}^{pro}$) $\times$ 100%.

Statistical analysis was performed using a one-way analysis of variance

comparing the pharmacokinetic parameters. A probability level of less than 0.05 was

considered statistically significant.


**RESULTS AND DISCUSSION**

3'-Azido-2', 3'-dideoxyuridine (AZDU) is a nucleoside analog demonstrating

potent anti-HIV activity in cell culture (1, 2). However, studies in animal models such as

rats, mice and rhesus monkeys showed that AZDU has a relatively short half-lives as well

as incomplete oral bioavailability (5, 6, 7, 8). As a continuation of the development of

prodrug strategies to improve the pharmacokinetic profile of AZDU 3'-azido-2',3'-

dideoxyuridine-5'-*O*-valinate hydrochloride (AZDU-VAL) was recently synthesized as a novel prodrug of AZDU by esterifying the 5'-OH function of the parent compound. Previous studies have reported that prodrugs of AZDU through esterification of the 5'-OH function of pyrimidine nucleoside analogs with various aliphatic acids showed improved pharmacokinetic distribution such as enhanced brain and lymphatic delivery and extended half-lives compared to that of parent drug AZDU (13).

The purpose of this study was to evaluate the *in vitro* chemical stability of AZDU-VAL and to characterize its pharmacokinetics in rats. AZDU-VAL is a salt form of parent nucleoside (Figure 6), therefore it is markedly more water soluble. The octanol/water partition coefficients, an indicator of lipid solubility, were 0.01 for AZDU-VAL and 0.43 for AZDU, suggesting that AZDU-VAL is much more hydrophilic than parent drug AZDU.

The *in vitro* chemical stability of AZDU-VAL was assessed in buffer solutions at pH 2.6, 5, and 7.4. As shown in Figure 7, AZDU-VAL did not demonstrate any significant degradation over the 204 h incubation period ($t_{1/2}$ = 477 days) at pH 2.6. At pH 5 and pH 7.4, AZDU-VAL degraded slowly with half-life values of 30 days and 25 days, respectively, indicating that the prodrug is highly stable at physiological pH values.

Mean plasma concentrations of AZDU-VAL as a function of time following intravenous and oral administration of 75 mg/kg and 100 mg/kg of the prodrug, respectively, are illustrated in Figure 8. Concentrations of AZDU-VAL in plasma after intravenous and oral administration declined rapidly such that the prodrug became undetectable shortly after dosing. AZDU-VAL plasma concentrations were detectable for 5 minute after intravenous administration and for 45 minutes after oral administration.

These *in vivo* results suggested AZDU-VAL was subject to rapid enzymatic metabolism. Since AZDU-VAL is an ester of AZDU through esterification of the 5'-OH function, it is likely to be hydrolyzed by the carboxylesterase existing in the rat blood and liver. It has been reported that esters with lower carbon chain length from $C_2$ to $C_5$ were immediately hydrolyzed to the parent compound in human serum (13). Due to the rapid elimination of AZDU-VAL after both intravenous and oral administration, it was not possible to calculate pharmacokinetic parameters accurately for the prodrug.

Pharmacokinetic profiles of AZDU derived from AZDU-VAL in plasma after intravenous and oral administration of AZDU-VAL are also shown in Figure 8. Maximum concentrations of AZDU, ranging from 0.53 to 0.67 µg/ml/mg/kg, were achieved 5 min after intravenous administration. AZDU plasma concentrations declined in a biexponential manner with terminal phase half-life of $8.8 \pm 4.36$ h. Compared with that of AZDU given as the parent drug (13), the elimination half-life was extended significantly by nearly 6-fold. However, the extended half-life was evident only at low AZDU concentrations, suggesting that the prodrug may distribute to a deep tissue compartment and release AZDU slowly. Extended half-lives have also been observed in previous reports for other nucleoside analogs such as (-)-β-D-dioxolane guanine (DXG), (-)-β-D-2, 6-diaminopurine dioxolane (DAPD), 5-fluorouracil (5FU) and prodrugs following intravenous administration (14, 15, 16). Intravenous administration of AZDU-VAL produced an AUC value for AZDU (0.55 µg·h/ml/mg/kg) similar to that seen when AZDU itself was administered (0.52 µg·h/ml/mg/kg).

Following oral administration of 100 mg/kg AZDU-VAL, AZDU rapidly achieved a maximum concentration ($C_{max}$) of $0.24 \pm 0.07$ µg/ml/mg/kg at $T_{max}$ 0.35 h $\pm$

0.14 (Table 3). The area under the AZDU plasma concentration versus time curve following oral administration was 0.42 ± 0.18 μgh/ml/mg/kg. Thus, the apparent bioavailability ($F_{AZDU}$) of the metabolite, AZDU, following oral administration averaged 101%. $F_{AZDU}$ was nearly 2-fold greater than that obtained by directly administering AZDU in rats (53%) (3, 4). The bioavailability of AZDU following intravenous administration of AZDU-VAL averaged 106%. The results following oral administration also suggested that the prodrug, AZDU-VAL, underwent extensive first-pass metabolism following administration by this route.

In summary, the results of this study demonstrated that prodrug, AZDU-VAL, was rapidly and efficiently bioconverted to parent nucleoside drug AZDU after intravenous and oral administration to rats. AZDU plasma concentrations were above the $EC_{50}$ value (0.046-0.12 μg/ml) for up to at least 8 h and 10 h, following intravenous and oral administration of AZDU-VAL of 75 and 100 mg/kg, respectively. The significant increases in oral bioavailability of AZDU make AZDU-VAL a promising prodrug of AZDU.

**ACKNOWLEDGMENTS**

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**Table 3.** Mean ± SD pharmacokinetic parameters of AZDU derived from the prodrug AZDU-VAL (AZDU [pro]) in comparison to those obtained when AZDU was administered (AZDU [par]) intravenously (14).

| Parameter | AZDU [pro] | | AZDU [par] |
|---|---|---|---|
| | iv | po | iv |
| Dose (mg/kg) | 75 | 100 | 100 |
| $T_{max}$ (h) | $0.08 \pm 0.00$ | $0.35 \pm 0.14$ | $0.08 \pm 0.00$ |
| $C_{max}$ (µg/ml) | $0.69 \pm 0.07$ | $0.24 \pm 0.07$ | - |
| AUC (µg·h/ml/mg/kg) | $0.55 \pm 0.51$ | $0.42 \pm 0.18$ | $0.52 \pm 0.08$ |
| $t_{1/2}$ (h) | $7.65 \pm 2.75$ | $8.8 \pm 4.36$ | $1.51 \pm 0.79$ |
| F, % | 106 | 101 | 100 |

**FIGURE LEGENDS**

**Figure 6.** Chemical structures of AZDU and AZDU-VAL.

**Figure 7.** AZDU-VAL hydrolysis profile at pH 2.6, 5.0 and 7.4.

**Figure 8.** Mean plasma concentrations of AZDU-VAL (□) and AZDU (■) following intravenous administration of 75 mg/kg AZDU-VAL, AZDU-VAL (○), AZDU (●) following oral administration of 100 mg/kg AZDU-VAL, and AZDU (▲)following intravenous administration of 100 mg/kg AZDU itself (14).
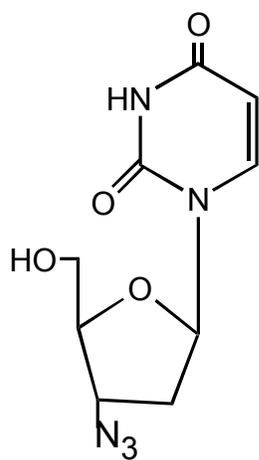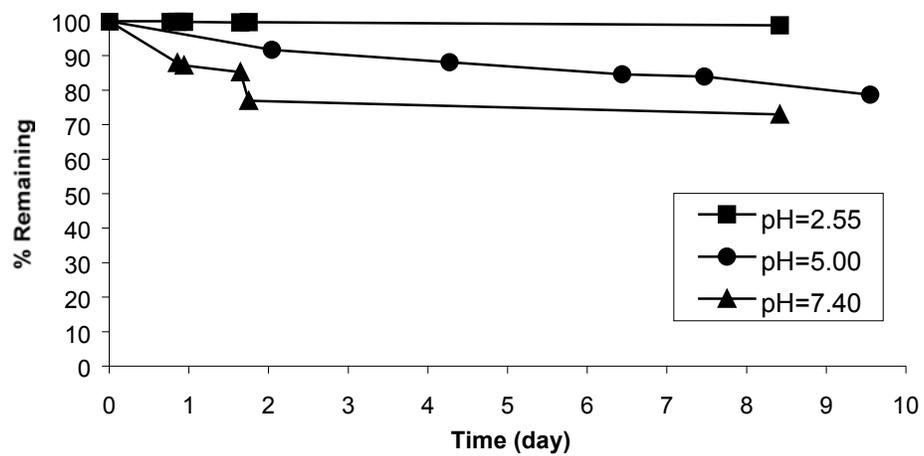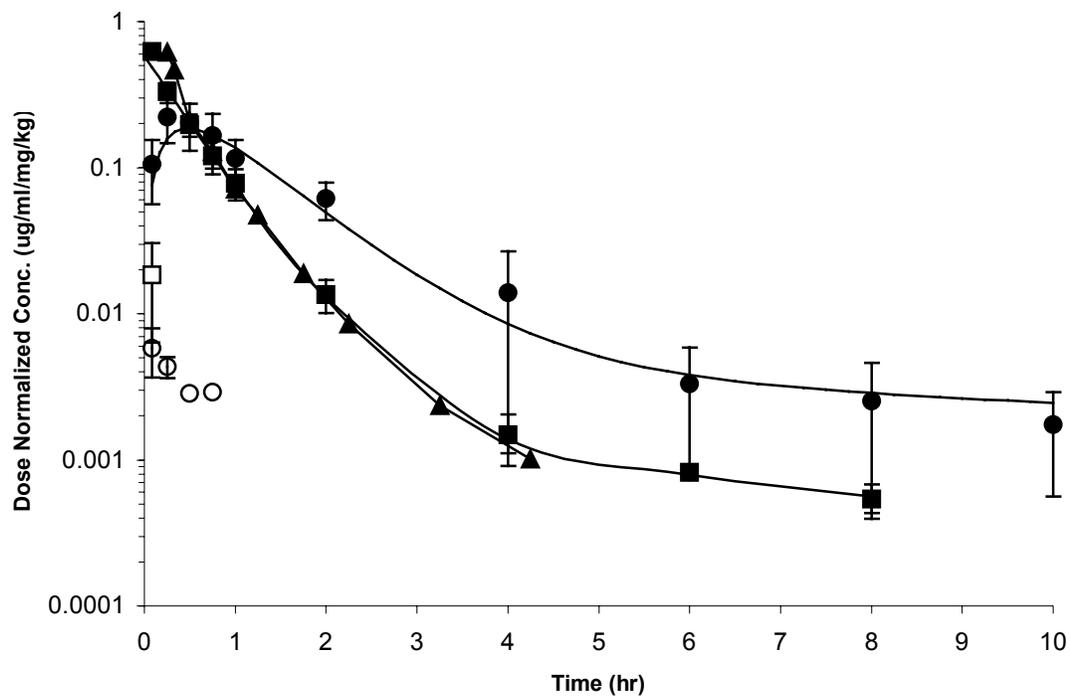
AZDU
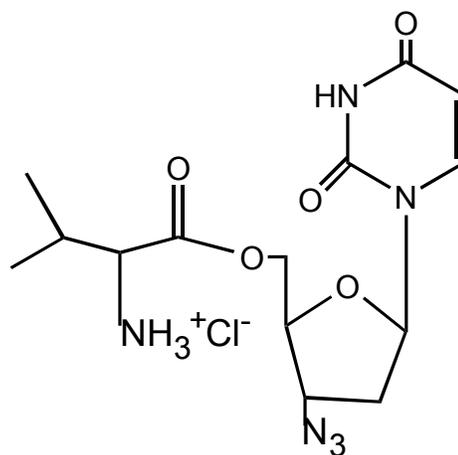
AZDU-VAL

Figure 6

Figure 7

Figure 8

**CHAPTER V:**

**High Performance Liquid Chromatography**

**Determination of 2'-Amino-6-Cyclopropylamino-9-(2', 3'-Dideoxy-β-D-Glycero-**

**Pent-2-Enofuranosyl) Purine, a Novel Anti –HIV Prodrug of 2', 3'-**

**Dideoxydidehydroguanine: Applications to *InVitro* Biotransformation Studies[1]**

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**ABSTRACT**

**Purpose.** To develop and validate a reversed-phase high performance liquid chromatographic (HPLC) method to quantitatively determine 2'-amino-6-cyclopropylamino-9-(2', 3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) in rat plasma and liver homogenate. **Methods.** A reversed-phase isocratic HPLC method was developed to quantitate ADV in rat plasma and liver homogenates. ADV was analyzed using an octadecyl silane (C-18) column (4.6 mm ID X 15 cm, 5 μm). The mobile phase consisted of 0.025 M phosphate buffer (pH 7.0) and 7% acetonitrile at a flow-rate of 1.0 ml/min. 3'-Azido-2', 3'-dideoxythymidine (AZT) was used as an internal standard (100 μg/ml). ADV and AZT were detected by UV absorption at 283 nm. **Results.** Extraction recoveries for ADV in rat plasma and rat liver homogenates were greater than 85%. Retention times of ADV and AZT were 10.3 min and 16.6 min, respectively. Calibration plots were linear over the range of 0.5 μg/ml to 80 μg/ml for ADV in rat plasma as well as in liver homogenates. The limit of quantitation was 0.5 μg/ml for ADV. The intra- and inter-day variations were less than 15% and accuracies were greater than 90%. **Conclusions.** This method is rapid, sensitive and reproducible for the determination of ADV in rat plasma and liver homogenates.

**Keyword:** Reversed phase, high performance liquid chromatographic (HPLC), cyclopropyl, purine

**INTRODUCTION**

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) (1, 2).  Efforts have been made to search for new chemotherapeutic agents that possess greater anti-HIV potency as well as less toxicity than currently available treatments for HIV infection and AIDS (3).  Theoretically, there are several steps in the HIV life cycle that could be targets for antiviral action (4).  However, currently agents that have been approved by Food and Drug Administration (FDA) as therapies for the treatment of HIV infection (5) affect only reverse transcription and protein processing.  Among the enzymes involved in the replication of HIV, reverse transcriptase (RT) is of the most practical for developing anti-HIV agents.  Reverse transcriptase, an essential enzyme for conversion of retroviral RNA to proviral DNA, catalyzes the incorporation of deoxyribonucleotides using both RNA and DNA templates (6, 7).  Currently, drug resistance to HIV viruses is an inevitable consequence of long-term exposure of antiretroviral therapy due to a high turnover of HIV-1 in patients (8, 9) and a low fidelity of the viral RT (10).  Recent studies provided evidence that replication competent virus can be readily recovered from peripheral blood mononuclear cells, as well as from semen, in subjects who had undetectable plasma HIV RNA for up to two years (11, 12, 14, 15).  Other studies showed that the number of latently infected CD4+ T cells decline only very slowly in patients on highly active antiretroviral therapy (HAART) (13). Therefore, it is of utmost importance to identify new agents that are active against these drug resistant strains of HIV and are well tolerated by individuals living with HIV.

Nucleoside analogs, particularly pirimidine and purine derived compounds, have been evaluated extensively *in vitro* and *in vivo* for anti-HIV activity (16, 17, 18). It has been reported that the β-D analogs of purine bases containing a dioxolane sugar derivative such as (-)-β-D-2,6-diaminopurine dioxolane (DAPD) and (-)-β-D-1',3'-dioxolane guanosine (DXG) have potent anti-HIV-1 activity (19, 20, 21, 22, 23, 24, 25). In addition, a variety of acyclic nucleosides were designed and tested for their antiviral and antitproliferative activity (16, 17, 18, 26). In particular, the acyclic nucleoside phosphates containing a cyclopropyl substituent at the N6 position of the 2,6-diaminopurine ring, which are structurally similar to the FDA approved anti-HIV drug abacavir (1592U89), represent a novel class of nucleoside analogs due to a highly stable carbon-phosphorus bond between the acyclic nucleoside and the phosphate moiety (16). It has been recognized that abacavir has a unique intracellular activation involving phosphoralation first to the monophosphate and then deamination by a novel cytosolic enzyme to the guanine analog, carbovir monophosphate (27).

Therefore, as part of efforts in the continuous search for new acyclic nucleosides with potential anti-HIV activity, 2'-amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) was recently developed as a prodrug of the guanine nucleoside analog 2',3'-dideoxydidehydroguanine (D4G) (Figure 9). D4G, an analog of the anti-HIV nucleoside DXG, has potent anti-HIV activity. As a potential drug candidate, *in vitro* studies have showed that ADV was a selective inhibitor of HIV reverse transcriptase. Due to the structural similarities between ADV and abacavir, it is possible that ADV may be bioconverted by the same enzyme involved in the activation of abacavir. To help understand the pharmacokinetic characteristics of this newly

synthesized nucleoside analog and its metabolism it is essential to develop a sensitive, reproducible analytical method to quantitate drug concentrations in biological samples. Thus, the purpose of this study was to develop an high performance liquid chromatographic (HPLC) analytical method for the determination of ADV in rat plasma and liver homogenate and to apply the method to investigate the biotransformation of ADV *in vitro*.

## EXPERIMENTAL SECTION

### Chemicals

2'-Amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) was synthesized in our laboratories (unpublished results). 3'-Azido-2', 3'-dideoxythymidine (AZT), used as an internal standard, was synthesized as previously described (28).  The chemical purity of each compound, as assessed by high performance liquid chromatography (HPLC) and spectral analysis, was greater than 99%. Acetonitrile, HPLC grade, and other chemicals, analytical grade, were purchased from J. T. Baker (Phillipsburg, NJ, USA).

### Preparation of standards

Standard solutions of 100, 10 and 1.0 μg/ml of ADV were prepared in distilled water.  Calibration curves for ADV in rat plasma were prepared by adding standard solutions to blank rat plasma yielding concentrations of 0.5, 1, 5, 10, 20, 50, 80 μg/ml for ADV.  Standard curves for the analysis of ADV in rat liver homogenates were prepared in blank liver homogenate at concentration 0.5, 1, 5, 10, 20, 50 and 80 μg/ml with a final volume of 1 ml.

**Extraction procedure**

The extraction of the nucleoside was performed by slight modification of a previously described method (29, 30, 31). Standard solutions were added to 1.5 ml polypropylene centrifuge tubes containing 100 μl rat plasma or liver homogenate, followed by 20μl internal standard, AZT (100 μg/ml). Ice-cold acetonitrile (3 ml) was added to precipitate proteins. Tubes were vigorously mixed for 1 min and centrifuged for 15 min at 5000 g. The supernatant was transferred to a polypropylene centrifuge tube, and excess crystalline magnesium sulfate was added. After mixing for 3 min, the tubes were centrifuged for 15 min at 5000 g. The supernatant was transferred to a clean polypropylene tube and evaporated to dryness under a stream of nitrogen gas at room temperature. The residual film was reconstituted in 500 μl of water and transferred to a disposable 1 ml polypropylene injection tube. Twenty microliters (20 μl) was injected onto the HPLC.

**Chromatography**

The HPLC system used for analysis of ADV in rat plasma and liver homogenate samples consisted of a Shimadzu LC-10A solvent delivery system, a Shimadzu SCL-10A system controller, a Shimadzu SPD-10A UV-VIS detector, SIL-10A auto injector and CR-501 chromatopac integrator. Concentrations of ADV in biological samples were determined by reversed-phase high-performance liquid chromatography using an octadecyl silane ($C_{18}$) column (4.6 mm ID × 15 cm, 5 μm particle size; Alltech Associates, Deerfield, IL). The column was protected by a Nova-Pak C18 guard column with particle size 4 μm. The mobile phase consisted of 0.025 M phosphate buffer (pH

7.0) and 7% acetonitrile.  The flow-rate was 1.0 ml/min.  ADV and AZT were detected by UV absorption at 283 nm.   The chromatography was performed at room temperature.

**Quantitation**

Concentrations of ADV in unknown samples were determined from the slope of calibration plots of the peak-area ratio of ADV/internal standard versus the calibration standard ADV concentrations.  Slopes were determined using linear regression analysis with a weighting factor of $1/x^2$ (32).  Use of this weighting factor generated a normal distribution of weighted residuals around the standard curve over the entire range of drug concentrations.

**Assay specifications**

The extraction recoveries of ADV were assessed at concentrations of 1, 10 and 80 μg/ml in rat plasma and liver homogenates. The recovery of internal standard was assessed at 100 μg/ml at which it was used for the assay.   Five plasma or liver homogenate samples (100 μl) containing drug and internal standard were extracted and injected.   Five injections of the same amount of compound in water were directly injected.  The peak areas of the compounds were measured and the percentage recovery was calculated from 100 × (peak area, extraction/mean peak area, direct injection).

The intra- and inter-day precision and accuracy of the analytical method were determined by analysis of six plasma samples containing 1, 10, 80 μg/ml concentrations for ADV. Assay precision was determined by calculating relative standard deviations (RSD) for each drug concentration.  Accuracy was calculated by comparing measured concentrations to the known values.

### *In vitro* biotransformation studies

The *in vitro* biotransformation of ADV was assessed in rat plasma and rat liver homogenate. Rat plasma was purchased from Harlan Laboratories, Indianapolis, IN. Rat liver was obtained from rats under anesthetized with diethyl ether. Livers were blotted dry, rinsed with isotonic potassium chloride (1.15%), and homogenized in 0.9% sodium chloride in a 1:2 ratio.

For *in vitro* bioconversion studies, ADV was dissolved in rat plasma or liver homogenate at concentrations of 1, 10, 80 μg/ml. Samples were incubated at 37°C with gentle agitation in a shaking water bath. Aliquots (100 μl) of samples were collected at specified time after initiation of the reaction into tubes placed on ice. Samples were immediately analyzed for concentrations of ADV by high performance liquid chromatography (HPLC). First-order degradation rate constants ($k$) and half-lives ($t_{1/2} = 0.693/k$) for the *in vitro* studies in rat plasma and liver homogenate were determined by linear least-squares regression of the natural logarithm of drug concentration versus time values.

## RESULTS AND DISCUSSION

2'-Amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) is a newly synthesized nucleoside analog being developed as a prodrug of the anti-HIV agent 2',3'-dideoxydidehydroguanine (D4G) (Figure 9). The purpose of this study was to develop a reliable, sensitive, reproducible HPLC analytical method for the determination of ADV in rat plasma and liver homogenate. The method was used to investigate the bioconversion of ADV *in vitro*.

Chromatograms of (A) blank rat plasma, (B) rat plasma spiked with ADV and the internal standard AZT, (C) blank rat liver homogenate, and (D) rat liver homogenate spiked with ADV and AZT are shown in Figure 10. For ADV and AZT, baseline separation was achieved with this method, and there was no interference from endogenous substances. The retention times of ADV and AZT were 16.6 min and 10.8 min, respectively, in rat plasma. In rat liver homogenate ADV and AZT were eluted at 18.8 min and 11.9 min, respectively.

The range of linearity for ADV was 0.5 - 80 μg/ml in both rat plasma [y = 0.0038 + 0.4997X, r = 0.9999] and liver homogenate [y = 0.0057 + 0.3985X, r = 0.9999]. The limit of quantitation for the method was 0.5 μg/ml. The assay specifications including extraction recovery, assay precision and accuracy are presented in Table 4. The extraction recoveries of ADV were greater than 90% in rat plasma and greater than 85% in rat liver homogenate. The intra- and inter- day precision of ADV at low, medium and high concentrations were less than 15% in both rat plasma and liver homogenate. The intra- and inter-day accuracies of ADV were also satisfactory being greater than 90%.

The *in vitro* biotranformation studies of ADV using the present HPLC analytical method revealed that ADV was highly stable in rat plasma with half-lives of 1136, 613 and 247 h at concentrations of 1, 10, 80 μg/ml. There was no significant metabolic degradation in plasma, indicating that ADV does not undergo enzymatic bioconversion in rat plasma (Figure 11). Structurally ADV is similar to abacavir (1592U89), a synthetic carbocyclic nucleoside analog with inhibitory activity against HIV. Both compounds have a cyclopropylamino substituent at the N6 position of the 2, 6-diaminopurine ring. Cyclopropyl bonds are intermediate in character between sigma (σ) and pi (π) bonds.

Cyclopropane mimics a C=C bond in its ability to conjugate with an adjacent olefinic bond, but unlike a C=C bond, it does not transmit electronic effects (33). This character, at least in part, may be contributed to the good stability of this class of compounds. However, in rat liver homogenate, ADV appeared to undergo a slow metabolic degradation with half-life values of 12.4, 4.9 and 16.8 h at concentrations of 1, 10, 80 μg/ml (Figure 12). No D4G was detected after the initiation of the incubation of ADV in rat plasma and rat liver homogenate, suggesting ADV might undergo a different metabolism rather than directly bioconvert to D4G. It has been reported that abacavir, similar to ADV in chemical structure, is not significantly metabolized by cytochrome P450 enzymes in humans. Abacavir is intracellularly converted by cellular enzymes to the active metabolite carbovir triphosphate (34). However, it has been recognized that abacavir has a unique intracellular activation involving phosphoralation first to the monophosphate and then deamination by a novel cytosolic enzyme to the guanine analog, carbovir monophosphate (27). Therefore, it is possible that the same enzyme may activate ADV.

In summary, the determination of ADV in rat plasma and rat liver homogenate by the HPLC method developed in the present study is simple, rapid, sensitive and reproducible. The limit of quantitation of this method is sufficiently sensitive to characterize the pharmacokinetics and biotransformation of ADV in rats.

**ACKNOWLEDGEMENT**

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**Table 4.** Assay specifications for the determination of 2'-amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) in rat plasma and rat liver homogenate

|  | Concentration (μg/ml) | Recovery[a] (%) | Precision (%) | | Accuracy (%) | |
|---|---|---|---|---|---|---|
|  |  |  | Intra-day | Inter-day | Intra-day | Inter-day |
|  | 1 | 90.8 ± 0.6 | 3.0 | 7.8 | 90.7 | 102.4 |
| Rat plasma | 10 | 97.6 ± 1.0 | 5.6 | 6.5 | 97.6 | 95.8 |
|  | 80 | 104.8 ± 1.9 | 12.6 | 10.5 | 97.0 | 109.2 |
| Liver | 1 | 105.0±5.6 | 1.8 | 5.9 | 91.2 | 90.6 |
| homogenate | 10 | 85.9±1.3 | 1.5 | 6.1 | 98.9 | 96.4 |
|  | 80 | 107.0±1.7 | 1.1 | 5.9 | 104.8 | 103.4 |

[a]Mean ± SD (n = 5)

**FIGURE LEGENDS**

**Figure 9.** Chemical structures of prodrug 2'-Amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) and 2',3'-dideoxydidehydroguanine (D4G).

**Figure 10.** Representative chromatograms for (A) blank plasma, (B) rat plamsa spiked with ADV and internal standard, AZT, (C) blank rat liver homogenate, (D) liver homogenate spiked with ADV and AZT.

**Figure 11.** Biocoversion study of 2'-Amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) in rat plasma at concentrations of 1 μg/ml (♦), 10 μg/ml (■) and 80 μg/ml (▲).

**Figure 12.** Biocoversion study of 2'-Amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) in rat liver homogenate at concentrations of 1 μg/ml (♦), 10 μg/ml (■) and 80 μg/ml (▲).
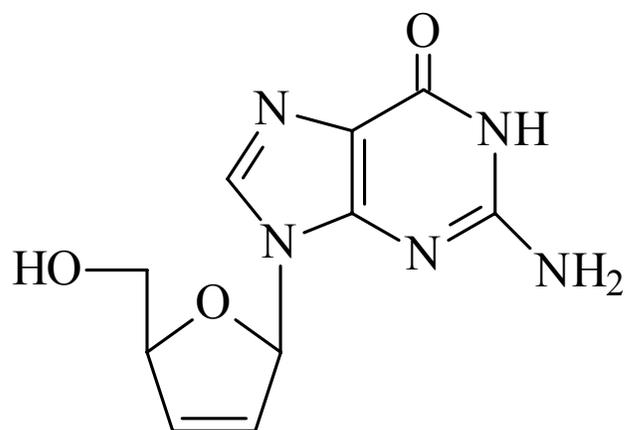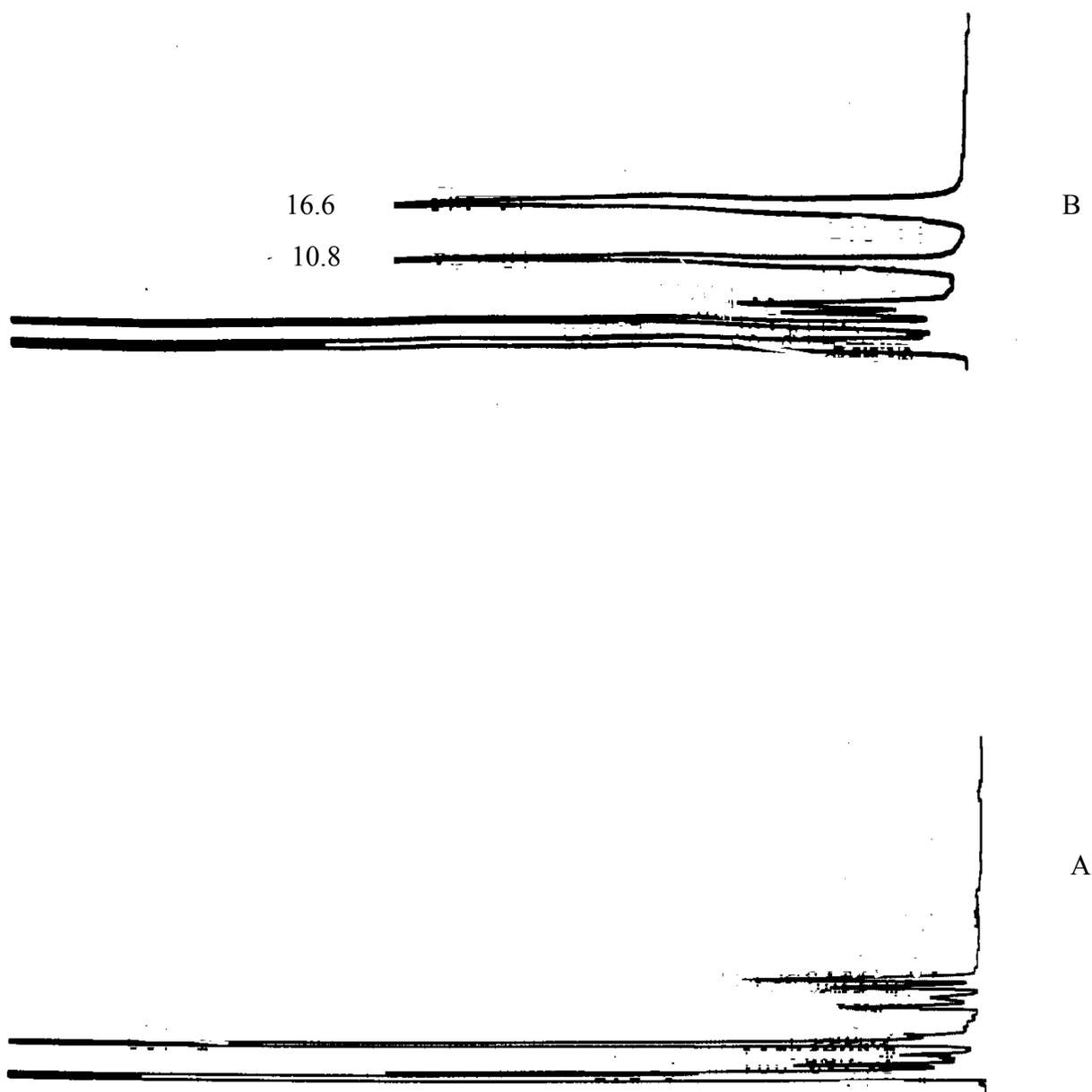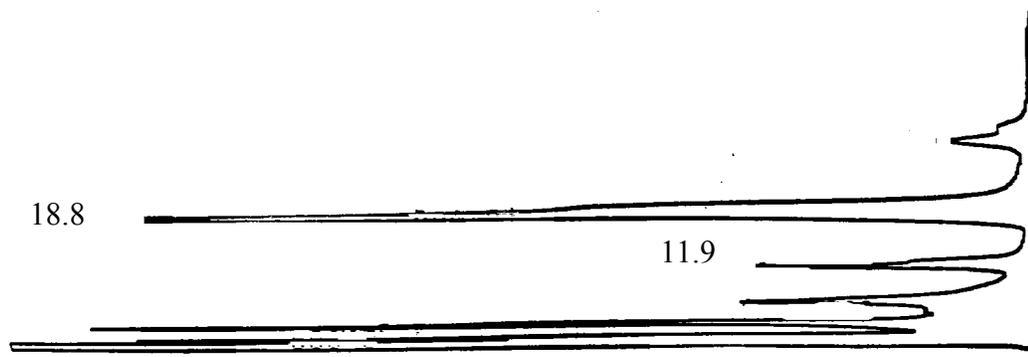
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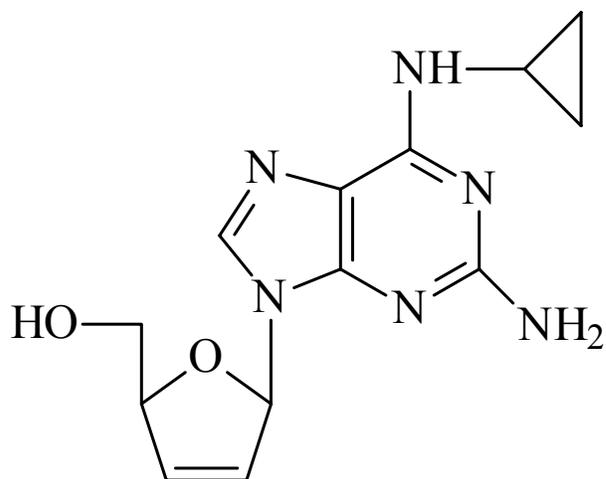


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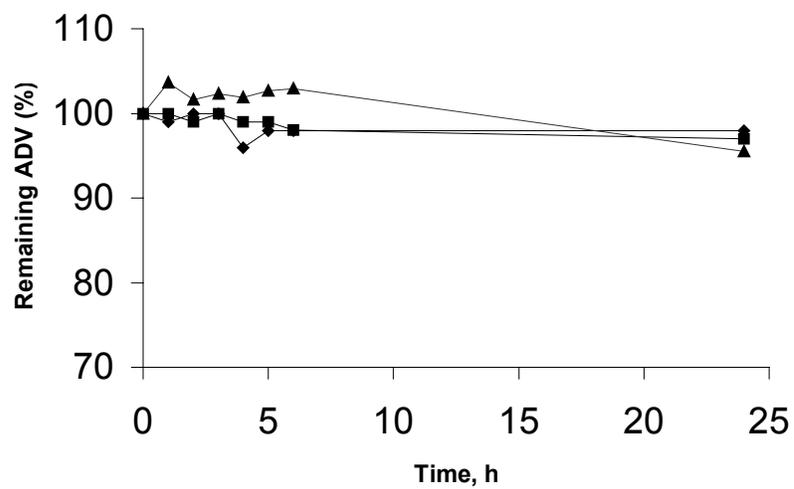
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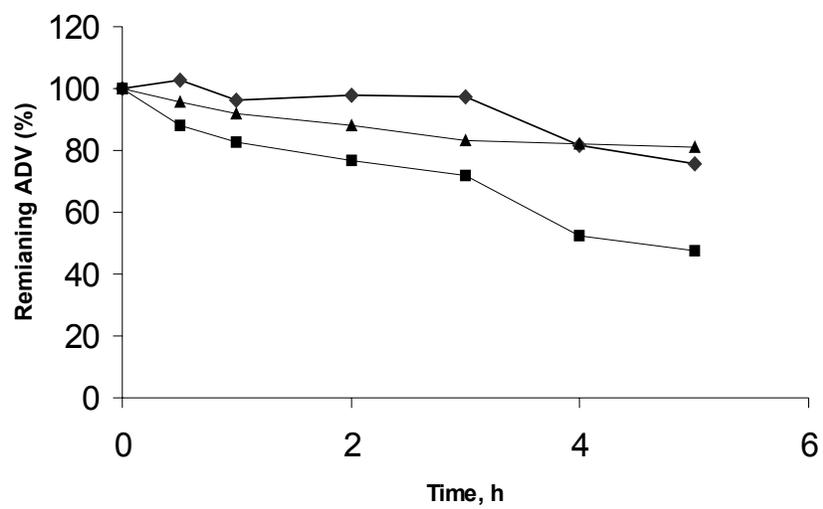
Figure 10

18.8

11.9

C

Figure 10 (Cont)

Figure 11

Figure 12

## CHAPTER VI:

## CONCLUSIONS

Since the discovery of human immunodeficiency virus (HIV) as a pathogenic retrovirus linked to AIDS, a number of potentially useful strategies for antiretroviral therapy of AIDS and its related diseases have emerged. One such strategy involves use of the broad family of 2', 3'-dideoxynucleoside, to which 3'-azido-2', 3'-dideoxythymidine (AZT) belongs. The natural history HIV-1 infection has been dramatically modified by the use of multi-drug highly active antiretroviral therapy (HAART) regimens. The nucleoside-based compounds are the major drugs employed in today's chemotherapy for HIV-infected patients.

3'-Azido-2', 3'-dideoxyuridine (AZDU, CS-87) is a nucleoside analog that has been found to inhibit the replication of human immunodeficiency virus (HIV) *in vitro*. The preclinical pharmacokinetics of AZDU has been investigated in various animal models. Preclinical and clinical phaseI/II results showed AZDU is a promising anti-HIV agent; however, due to the short half-life, variable oral bioavailability and limited delivery into central nervous and lymphatic systems, continuous efforts have been made in the search for prodrugs of AZDU. Four new chemical entities have been synthesized in our lab as prodrugs of AZDU.

In the present dissertation, oral bioavailability of AZDU was determined using rats as an animal model. An HPLC analytical method was developed to simultaneously determine AZDU and its novel prodrugs in rat plasma. 3'-Azido-2', 3'-dideoxyuridine

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5'-*O*-valinate hydrochloride, a newly synthesized prodrug of AZDU, was evaluated *in vitro* and *in vivo.Effect of Dose on the Bioavailability of 3'-Azido-2', 3'-Dideoxyuridine (AZDU) Following Oral Administration to Rats*

The effect of dose on the bioavailability of AZDU was investigated in eight adult male Sprague-Dawley rats after intravenous and oral administration at two doses, 25 mg/kg and 100 mg/kg. For each dose, a crossover study design was employed. Blood samples were taken and AZDU plasma concentrations were determined by high performance liquid chromatography. The pharmacokinetic parameters were estimated by area-moment analysis.

The oral bioavailability estimates of AZDU from the area under the curve (AUC) values obtained after the crossover studies following intravenous and oral administrations of AZDU at doses 25, 100 mg/kg were 60% and 46%, respectively, with no statistically significant difference at a probability level of 0.05 ($\alpha = 0.05$).

*Simultaneous Determination of AZDU and its Prodrugs in Rat Plasma by HPLC*

A reversed-phase gradient elution HPLC method was developed to simultaneously quantitate AZDU and its prodrugs, N3-pivaloyloxymethyl-3'-azido-2', 3'-dideoxyuridine (I), 5'- pivaloyloxymethyl-3'-azido-2', 3'-dideoxyuridine (II), 5'-*O*-valinyl-3'-azido-2', 3'-dideoxyuridine hydrochloride (III) and 5'-*O*-phenylalaninyl-3'-azido-2', 3'-dideoxyuridine hydrochloride (IV), in rat plasma.  AZDU and its prodrugs were analyzed using a C-18 column. The mobile phase consisted of 0.04 μM sodium acetate buffer (pH 5.0) and acetonitrile, running in a segmented gradient manner at a flow-rate of 2 ml/min. All compounds were detected by UV absorption at 261 nm.

Extraction recoveries for all compounds were greater than 80%. Retention times of AZDU, CS-85, prodrugs I, II, III and IV were 3.3, 5.2, 9.1, 8.8, 6.3 and 7.3 min, respectively. Calibration plots were linear over the range of 0.25 μg/ml to 100 μg/ml for compounds AZDU, II, III, and IV and 0.5 μg/ml to 100 μg/ml for prodrug I. The limit of quantitation was 0.25 μg/ml for prodrugs II, III and IV and 0.5 μg/ml for prodrug I. The intra- and inter-day variations were less than 10% and accuracies were greater than 90%. This method is rapid, sensitive and reproducible for the determination of AZDU and its prodrugs in rat plasma.

*In Vitro and in Vivo Evaluation of AZDU-VAL, a Prodrug of AZDU*

*In vitro* stability studies revealed that AZDU-VAL was highly stable in phosphate buffer at various pH values, indicating that there is no detectable chemical degradation of this compound in this media. Pharmacokinetics of AZDU-VAL were characterized in male Sprague-Dawley rats following intravenous and oral administration of AZDU-VAL of 75 and 100 mg/kg, respectively. Concentrations of AZDU-VAL in plasma after intravenous and oral administrations declined rapidly that the drug became undetectable shortly after dosing, suggesting AZDU-VAL was possibly subject to enzymatic metabolism in the rat blood and liver. Since AZDU-VAL is an ester of AZDU through esterification of the 5'-OH function, it is very likely to be hydrolyzed by the carboxylesterase existing in the rat blood and liver. The oral results also suggested that the prodrug, AZDU-VAL, underwent extensive first-pass metabolism after oral administration. Following intravenous administration, AZDU-VAL converted to AZDU completely. The oral bioavailability of AZDU derived from AZDU-VAL was 76%,

which was 20% higher than that obtained by directly administration of AZDU in rats (53%). In summary, AZDU-VAL was efficiently converted to parent nucleoside drug AZDU after intravenous and oral administration to rats. The significant improved bioavailabilities make AZDU-VAL a promising prodrug of AZDU.

*HPLC Determination of 2'-Amino-6-Cyclopropylamino-9-(2', 3'-Dideoxy-β-D-Glycero-Pent-2-Enofuranosyl) Purine, A Novel Anti-HIV Prodrug of 2',3'-Dideoxydidehydroguanine: Applications to In Vitro Biotransformation Studies*

A reversed-phase isocratic elution HPLC method was developed to quantitate 2'-amino-6-cyclopropylamino-9-(2', 3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) in rat plasma and liver homogenates. Extraction recoveries for ADV in rat plasma and rat liver homogenates were greater than 85%. Retention times of ADV and AZT were 10.3 min and 16.6 min, respectively. Calibration plots were linear over the range of 0.5 μg/ml to 80 μg/ml for ADV in rat plasma as well as rat liver homogenates. The limit of quantitation was 0.5 μg/ml for ADV. The intra- and inter-day variations were less than 15% and accuracies were greater than 90%. Biotransformation of ADV in rat plasma and liver homogenate was investigated using this analytical method. The results revealed that ADV was highly stable in rat plasma with half-lives ($t_{1/2}$) of 1136, 613 and 247 h at concentrations of 1, 10, 80 μg/ml. There was no significant metabolic degradation in plasma. However, in rat liver plasma, ADV appeared to undergo a slow chemical degradation with half-lives ($t_{1/2}$) of 12.4, 4.87 and 16.8 h at low, medium and high concentrations of 1, 10, 80 μg/ml. No parent drug D4G was detected after the initiation of

the incubation of ADV in rat plasma and rat liver homogenate, suggesting ADV might undergo a different metabolism rather than directly bioconvert to D4G.

*Summary*

AIDS – acquired immunodeficiency syndrome – was first reported in the United States in 1981 and has become a major worldwide epidemic. AIDS is caused by the human immunodeficiency virus (HIV). By killing or damaging cells of the body's immune system, HIV progressively destroys the body's ability to fight infections and certain cancers. More than 700,000 cases of AIDS have been reported in the United States since 1981, and as many as 900,000 Americans may be infected with HIV. Over the past 10 years, researchers have developed drugs to fight both HIV infection and its associated infections and cancers.

3'-Azido-2', 3'-dideoxyuridine (AZDU, AzddU, CS-87, Uravidine) is a synthetic nucleoside analog structurally related to 3'-azido-3'-deoxythymidine (AZT, zidovudine), the most frequently used drug in the treatment of HIV-infected patients. AZDU has been proven to have potent anti-HIV activity in human peripheral blood mononuclear cells with significantly reduced human bone marrow toxicity, 30-fold less than AZT. Nevertheless, the potential of AZDU as a promising anti-HIV agent has been limited by its relatively short half-life, relatively low bioavailabilities as illustrated in various animal models as well as its extensive glucuronidation in HIV-infected patients.

As a continuation of the development of prodrug strategies in improving its pharmacokinetic profile by utilizing the *in vivo* biotransformation systems, several novel

ompounds were synthesized as prodrugs of AZDU in order to obtain prolonged half-lives and good bioavailabilities.

Oral bioavailabilities of AZDU were determined in rats, averaging 53%. A rapid, sensitive, reproducible HPLC method using gradient elution was developed to simultaneously quantitate AZDU and its prodrugs in rat plasma. Preclinical pharmacokinetic studies on one of the prodrugs, 3'-Azido-2', 3'-dideoxyuridine-5'-*O*-valinate hydrochloride, showed improved bioavailability of AZDU compared with that obtained after oral administration of the parent drug. Thus, AZDU-VAL, as the novel prodrug of AZDU, appears to be a promising anti-HIV agent.

2'-Amino-6-cyclopropylamino-9-(2',3'-dideoxy-β-D-glycero-pent-2-enofuranosyl) purine (ADV) was recently synthesized as a prodrug of  the anti-HIV nucleoside analog 2', 3'-dideoxydidehydroguanine (D4G). In the present dissertation, a reliable, sensitive isocratic HPLC analytical method was developed to determine ADV in rat plasma and rat liver homogenates. This analytical method was employed in the bioconversion studies of ADV *in vitro*.