## PHARMACOKINETICS OF ANTI-HIV AGENTS IN RODENTS

by

## SHAWN K. BLUE

(Under the Direction of Catherine A. White)

#### ABSTRACT

The treatment of HIV/AIDS is one of, if not, the most challenging medical enigmas of the 20th and 21st centuries. At the time of its discovery, HIV patients were predominantly homosexual White males in America. The demographics have changed drastically over the past two decades. Now women make up nearly 50% of global HIV/AIDS patients. The current feminization of HIV demographics has led to another obstacle, mother to child transmission (MTCT). These statistics, coupled with the fact that vertical transmission is the largest factor in the number of newly diagnosed juvenile HIV/AIDS patients, create a need to optimize treatment of HIV positive pregnant women, to reduce vertical transmission of HIV. It has been shown that administration of anti-HIV medications during pregnancy, delivery and to the infant after birth greatly reduces the risk of vertical transmission. Understanding the pharmacokinetics of HIV/AIDS medications alone and in combination during pregnancy is necessary in the development of more effective methods of vertical transmission prophylaxis.

Using a pregnant rat model, we have developed analytical methods and investigated the pharmacokinetics and placental transport of anti-HIV drugs alone and in combination. These studies allowed us to determine and understand any possible interactions between drugs in

combination. Studies were performed on timed-pregnant Sprague-Dawley rats and pharmacokinetic analysis was performed using WinNonlin.

While current methods of treating HIV/AIDS patients have been highly successful, the chance of viral mutations and resistance to Anti-Retroviral Therapy often occurs. In order to combat the resistance of reverse transcriptase inhibitors (NRTI and NNRTI) and protease inhibitors, scientists have continually searched for additional targets on HIV. For instance, integrase inhibitors, block the action of integrase, a viral enzyme, which integrates HIV DNA into the target cells. We have determined the pharmacokinetic profile of an investigational integrase inhibitor. *In vivo* animal studies were being performed on male Sprague-Dawley rats and female PXR-KO and hPXR transgenic mice and pharmacokinetic analysis was performed using WinNonlin.

INDEX WORDS: HIV, Pharmacokinetics, Stavudine, Lamivudine, D4T, DDC, Placental Transport, NRTI, Nucleoside Reverse Transcriptase Inhibitors, HPLC, Antiviral Drugs, Integrase Inhibitors

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# DEDICATION

I would like to dedicate this dissertation to my loving and supportive mother, Mary L. Blue; I owe everything to you.

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## CHAPTER 1

#### INTRODUCTION

There has been a dramatic shift in the demographics of HIV since its discovery, in 1981. Primarily, it was seen almost exclusively in white homosexual males in America. However, since the early 1980's, HIV has crossed gender, race and sexuality lines. Women and children are the most affected by this change. Of the approximately 33 million people infected with HIV, nearly 50% are women and 2 million are children under 15. This included over 400,000 newly infected children in 2007 [1]. The primary mode of infection for children is vertical transmission which can occur *in utero*, at delivery or through breastfeeding. The risk of transmission for nonbreastfeeding populations is 15-30% without preventative measures while breastfeeding may increase risk to 45% [2]. Preventative measures include voluntary cesarean delivery, antiretroviral drug treatment and alternatives to breastfeeding. However, in areas where cesarean delivery and alternate feeding are not an option it is necessary to reduce transmission during labor by antiviral treatment [2].

Several studies have shown that antenatal, intrapartum and postpartum antiretroviral treatment can greatly reduce mother-to-child transmission (MTCT) [3-10]. In 1994, the Pediatric AIDS Clinical Trials Group (PACTG) protocol 076 established, the nucleoside reverse transcriptase inhibitor, zidovudine (AZT) as an effective method of reducing perinatal HIV transmission by 68% (9). Since then, AZT has become the standard prophylactic method to reduce vertical transmission. However, due to resistance, toxicity, preference or lack of resources, alternatives to zidovudine are necessary (4, 10, 27, 34, 35). The standard treatment of

HIV consists of combination therapy or highly active antiretroviral therapy (HAART); consisting of at least 2 nucleoside reverse transcriptase inhibitor (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (nNRTI) or protease inhibitor (PI). HAART has led to a significant improvement in the mortality and morbidity of HIV infection (11, 13, 27). Other studies have shown that prenatal, intrapartum and postpartum combination therapy may provide an even greater reduction in vertical transmission risk. Previous studies have shown that anti-HIV drugs cross the placenta via passive diffusion, reducing the chance of placental drug-drug interactions. However, additional studies have shown interactions between co-administered nucleosides. While combination therapy has been proven to reduce vertical transmission of HIV, drug-drug interactions may not allow maximum efficacy [6, 11-13]. Therefore, it is necessary to understand the pharmacokinetics and placental disposition of HIV drugs alone and in combination.

Placental and fetal drug distribution is difficult to study. This is due to the ethical exclusion of pregnant women in clinical trials coupled with difficulty in obtaining fetal concentration data. Anatomical similarities between rat and human placenta make the pregnant rat model ideal for the investigation of human placental transfer [14]. The large litter size allows serial sampling that can be used to create a viable concentration time profile for pharmacokinetic studies. The pregnant rat model has been utilized in maternal-fetal drug transfer studies of various compounds including nucleoside analogs [12-13, 15-19].

There have been significant advances in the treatment of HIV/AIDS since the introduction of the first nucleoside reverse transcriptase inhibitor (NRTI), zidovudine [11, 20-21]. The development of additional NRTIs, protease inhibitors and recently fusion inhibitors has resulted in potent antiretroviral therapy. The introduction of combination therapy has led to a

2

considerable improvement in the quality of life for HIV positive people. The United States and other developing countries have also shown a decrease in death rates [11, 20-21].

Currently, nearly all of the existing antiretroviral agents inhibit the activity of the two central HIV enzymes, reverse transcriptase and protease. While current methods of treating HIV/AIDS patients have been highly successful, viral mutations, resistance and adverse effects often occur. In order to combat mutations, toxicity and resistance of reverse transcriptase inhibitors (NRTI and NNRTI) and protease inhibitors, scientists have continually searched for additional targets on HIV. For instance, integrase inhibitors, block the action of integrase, a viral enzyme, that integrates HIV DNA into the target cells [11, 20-21]. Currently, there is only one integrase inhibitor available for treatment [22-23].  $\beta$ -diketo acids are a group of integrase inhibitors that are currently under investigation. While several compounds have exhibited inhibition of integrase,  $\beta$ -diketo acids are strong and aggressive inhibitors [24-28]. A cooperating lab has developed a  $\beta$ -diketo acid, 4-(1,3-dibenzyl-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid, which is a potent inhibitor of the strand transfer steps in HIV integrase [25, 29-30]. Animal studies are integral to the development of investigational drugs with pharmacokinetic analysis being central [31-34].

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## **CHAPTER 2**

#### LITERATURE REVIEW

#### HIV/AIDS

In 1981, an aggressive form of Kaposi's sarcoma and the rare lung infection, Pneumocystis carinii pneumonia, were found in young homosexual men in California and New York. The Centers for Disease Control (CDC) published a report about the occurrence, and further described an inexplicable immunodeficiency among the men [1, 11]. It was later discovered that the disease was not isolated to the gay community. Several cases among injecting drug users, hemophiliacs and women provided a better understanding of the transmission of the illness. The illness was properly named Acquired Immune Deficiency Syndrome (AIDS) and the causative virus, Human Immunodeficiency Virus (HIV), was isolated. Currently, there are approximately 33 million people living with HIV/AIDS worldwide. There were nearly 2.5 million new diagnoses in 2008 [1, 11]. Over 25 million have died as a result of AIDS related complications since 1981, including 2.5 million in 2008. Globally, more than 6800 people are newly infected with HIV and over 5700 people die from AIDS every day. Nearly 70% of all HIV positive adults and 90% of all HIV positive children live in Sub-Saharan Africa [1, 11]. Infection rates in Asia, the Caribbean, Central and South America have grown over the Globally, the HIV/AIDS pandemic is one of, if not, the greatest medical last few years. challenge today.

The origin of HIV is a much debated issue. It is well accepted in the scientific world that Simian Immunodeficiency Virus (SIV) passed to humans which then became HIV, is thought to have transpired as early as the beginning of the 20<sup>th</sup> century. It has been known for years that viruses can cross species. When this occurs between animals and humans, it is referred to as zoonosis. There have been several theories of how the zoonosis occurred. The most common theory is the "hunter theory", which states that SIVcpz was transferred to humans when chimpanzees were killed and eaten. SIV was normally fought off, however, on occasion it adapted to its new host by mutating into HIV-1. Recently there have been other theories, including the Oral Polio Vaccine theory, contaminated needles theory, colonialism theory and the ubiquitous conspiracy theory [35-37].

#### HIV/AIDS Pathogenesis

Human Immunodeficiency Virus (HIV-1), a retrovirus, attacks human cells bearing the CD4<sup>+</sup> surface marker. A related yet less virulent virus, HIV-2, is prevalent in Western Africa. HIV belongs to a special subclass of retroviruses, lentiviruses, that also cause diseases in cats (FIV), monkeys (SIV), sheep (Visna) and horses (CAEV). All lentiviruses attack the immune system and take months to years to display adverse effects [35-36, 38]. The principal methods of HIV transmission are sexual, parenteral, and mother/fetal exposure. The latter being the largest contributor of new infections [1-2, 11].

HIV virions are 100 – 120 nm in diameter. Virions consist of two copies of singlestranded RNA in a core that is surrounded by a lipid envelope. The envelope is studded with glycoproteins (gp120 and gp41) that play a pivotal role in the invasion of cells, interactions with the immune system and antibody responses. The essential viral enzymes, reverse transcriptase, protease and integrase are contained in the virions [21].

The HIV-1 genome consists of nine genes that encode messages for structural and regulatory proteins. The key regulatory proteins, Rev and Tat, are responsible for replication and

transcription respectively. The key structural genes, Gag, Env and Pol are responsible for the formation of capsid proteins, formation of the viral envelope and production of the enzymes responsible for replication [21].

Like all viruses, HIV can only replicate inside host cells. HIV begins its life cycle when it binds to a CD4 receptor and one of two coreceptors on the surface of a CD4<sup>+</sup> T-lymphocyte. Two additional coreceptors have been identified, c-c chemokine receptor (CCR5) and C-X-C chemokine receptor (CXCR4). The virus then fuses with the host cell. After fusion, the virus releases RNA into the host cell. Once inside the cell, the HIV enzyme reverse transcriptase, converts the single-stranded HIV RNA to double-stranded HIV DNA. The enzyme integrase then splices the HIV DNA into human DNA. This integrated HIV DNA is called a provirus, which may remain inactive for several years, producing few or no new copies of HIV. When proviruses become active they subvert the cellular machinery to reproduce. The provirus utilizes a host RNA polymerase to create copies of the HIV genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of HIV proteins. The long chains of HIV proteins are cut by HIV protease into smaller individual proteins. As the smaller HIV proteins come together with copies of the HIV RNA genetic material, new infectious progeny are formed [11, 21].

HIV primarily infects T-lymphocyte cells (T-cells). In the presence of HIV, the immune system forms antibodies that are pivotal in HIV+ diagnosis. These antibodies attack the virus and HIV-infected cells. Constant destruction and impairment of HIV-infected CD4<sup>+</sup> cells result in the gradual loss of immune system function. T-cell depletion leads to immunodeficiency which predisposes the patient to infection from both "normal" and opportunistic infections. Opportunistic infections include bacterial, viral, protozoal and fungal infections. As well as

malignancies, neurological and other life-complicating conditions. A patient is said to have progressed from HIV+ to having Acquired Immunodeficiency Syndrome (AIDS) when they exhibit at least one opportunistic infection or their T-cell count has dropped below 200 cells/cm<sup>3</sup>

# <u>HIV Treatment</u>

Once someone is diagnosed as HIV+, their physical health determines when they are to begin antiretroviral therapy. The two main determinants of treatment readiness are viral load and T-cell count. The normal T-cell count is 500 – 1500 cells/cm<sup>3</sup>; asymptomatic patients with T-cell counts below 350 often are recommended for treatment. Viral load represents the number of HIV RNA copies per cm<sup>3</sup> of blood. Patients with viral loads above 100,000 copies/ml are recommended to begin treatment. The high viral load will accelerate the depletion of T-cells leading to symptomatic conditions. Therapeutic decision making relies on the aforementioned tests and likelihood of progression to AIDS. Treatment of HIV has advanced from monotherapy to highly active antiretroviral therapy (HAART), which consists of a combination of three or more anti-HIV drugs [11, 20-21, 39-40].

The treatment of HIV/AIDS is one, if not, the most challenging medical enigma of the 20th and 21st centuries. Since the introduction of the first nucleoside reverse transcriptase inhibitor (NRTI), zidovudine, treatment has made countless strides. The development of additional NRTIs, non nucleoside transcriptase inhibitors (NNRTIs), protease inhibitors, fusion inhibitors and integrase inhibitors has resulted in potent antiretroviral therapy. These advances have led to a significant improvement in the mortality and morbidity of HIV infection. The United States and other developed countries have also shown a decrease in death rates [11, 20-21, 40].

Currently, nearly all of the existing antiretroviral agents inhibit the activity of the two central HIV enzymes, reverse transcriptase and protease. The HIV enzyme reverse transcriptase catalyzes the conversion of single-stranded HIV RNA into double-stranded DNA. Inhibition of reverse transcriptase promotes DNA chain termination, thus reduced viral replication. HIV protease is necessary to cleave the viral polyproteins in order to produce new viral copies; protease inhibitors prevent HIV protease from carrying out this process, thus preventing the production of mature, infectious virions. HIV integrase is responsible for incorporating viral DNA into host DNA; integrase inhibitors interrupt this process, thus ending viral replication. Entry inhibitors prevent HIV from entering cells by binding to HIV surface proteins [11, 21].

### Mother to Child Transmission

The demographics of HIV/AIDS have changed drastically in the past 27 years. Now women make up nearly 50% of global HIV/AIDS patients including 61% of HIV positive adults in Sub-Saharan Africa. In America, the rate of infection in African American and Hispanic women has grown to over 60% of all new diagnoses [1-2]. The rise in HIV infection among women has also led to a disparate burden on children. In 2008, children under 15 accounted for 2.5 million people living with HIV, over 400,000 new HIV infections and over 330,000 AIDS-related deaths [1-2].

A vast majority of HIV-positive children were infected by Mother to Child Transmission (MTCT) or Vertical Transmission which can occur *in utero*, at delivery or through breastfeeding. Risk factors include maternal viral load, maternal disease progression, pre-maturity, multiple birth and vaginal delivery, with maternal viral load considered the strongest predictor of mother-to-child transmission [8, 41-44]. The risk of transmission for non-breastfeeding populations is 15-30% without preventative measures, while breastfeeding may increase risk to 45% [2].

Preventative measures include voluntary cesarean delivery, antiretroviral drug treatment and alternatives to breastfeeding. However, in areas where cesarean delivery and alternate feeding are either unsafe or unacceptable, it is necessary to reduce transmission during labor and delivery, which may account for up to two thirds of all vertical transmission. Recent studies in countries with a heavy burden of HIV have shown that prolonged antiviral treatment, beginning in the third trimester, reduces the risk of vertical transmission to less than 5%. Breastfeeding may offset these gains; therefore further studies are currently underway to reduce transmission caused by breastfeeding [2, 45-54]. In the last few years, there has been an increase in political and community involvement in efforts to curb the HIV pandemic. The focus of developed countries is to reduce viral load through treatment, followed by extensive monitoring. However, nations faced with financial constraints must provide short-term and cost-effective methods of intervention [2, 11, 21].

### HIV Treatment in Pregnancy

There are two main goals of treatment of HIV positive women: treatment of maternal HIV infection and prevention of vertical transmission. While it is recommended that pregnant women follow the same guidelines as nonpregnant adults, several considerations must be taken into account [2, 5, 11, 55-58]. These include pharmacokinetic changes due to pregnancy, potential teratogenic effects of drugs, toxicity to mother, infant or both and development of drug resistance. During pregnancy, physiological changes can alter drug absorption, distribution, metabolism and elimination, thus making it difficult to predict pharmacokinetics in pregnant women. Pregnant women have increased body water and fat that is associated with increased cardiac output, ventilation, and liver and renal blood flow. Pregnancy also prolongs gastrointestinal transit time, decreases plasma protein concentration and increases renal sodium

reabsorption. Compartmentalization of drugs in the fetus and placenta, placental transport of drugs, biotransformation of drugs in fetus and placenta and elimination of drugs by the fetus can also alter the pharmacokinetics in pregnant women. Pharmacokinetic studies among pregnant women indicate that no dosing adjustments are required for several antiretroviral agents [2, 44, 58-63].

Risks of the drug on the fetus and newborn are weighed against the benefits of vertical transmission prevention. Several factors influence the potential harm to the fetus from maternal consumption; these include the drug (dose and agent), gestational age of fetus, duration of exposure, and interaction with endogenous and exogenous compounds the fetus is exposed to. Concerns have been raised about several antiretroviral agents. Zalcitabine has been associated with an increased risk of hydrocephalus at very high doses in rodents and delavirdine has been associated with an increased risk of ventricular septal defects in rodents. Significant malformations (anencephaly, anophthalmia, cleft palate) have been observed in infant monkeys with *in utero* exposure to Efavirenz at drug levels similar to therapeutic ranges in humans [2, 11, 21, 59, 64-65]. Efavirenz is classified as FDA Pregnancy Category D; therefore treatment with Efavirenz should be avoided during the first trimester, which is the primary period of fetal organogenesis. The long-term effects of exposure of the infant *in utero* to combination antiretroviral (ARV) regimens require further study [11, 21, 66].

Pregnancy presents unique concerns for toxicity of ARV treatment, including, toxicities that may be magnified by pregnancy and toxicity in the fetus. There is a female preponderance for and pregnancy can mimic some of the early symptoms of the lactic acidosis/hepatic steatosis syndrome or be associated with other disorders of liver metabolism [67]. The dual NRTI combination d4T + ddI should be avoided in pregnancy because the risk of lactic acidosis is

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potentially increased with this combination [2, 11]. In a French study, the combination of AZT and 3TC given to women for preventing mother-to-child transmission (PMTCT) from the early second trimester of pregnancy and to the infant for six weeks was associated with higher rates of infant anemia and neutropenia than were observed in infants exposed to AZT alone [68]. Tenofovir has shown decreased fetal growth, reduced bone porosity and bone demineralization in infant monkeys exposed *in utero*. Several research groups have reported persistent, although clinically asymptomatic, hematological abnormalities in uninfected infants exposed to ART *in utero* [2, 5, 7, 69]. Data suggest that a disorder of mitochondrial fatty acid oxidation in the mother or fetus during late pregnancy may play a role in the development of acute fatty liver of pregnancy and HELLP syndrome (consisting of Hemolytic anemia, Elevated Liver enzymes and Low Platelet count) and possibly contribute to susceptibility to antiretroviral-associated mitochondrial toxicity [70-72].

Antiretroviral drug resistance is one of the primary factors leading to therapy failure in HIV infected persons. Nausea and vomiting during early pregnancy may jeopardize adherence and increase the risk of resistance in pregnant women receiving antiretroviral treatment [2, 64, 73-76]. Treatment of pregnant HIV positive women also makes drug resistance a two-fold problem. Resistance can both limit future maternal treatment and diminish the efficacy of prevention of vertical transmission.

The NRTI drugs with which the most extensive clinical experience has been gained in pregnant women are AZT and 3TC, and these are the preferred NRTIs for pregnant women. The benefits of prophylaxis of MTCT must be weighed against the potential therapeutic risks to the mother and fetus [2, 11, 21, 77-79]. Drug choice should be individualized and is based on available data derived from animal toxicity data, anecdotal experience, registry data, and clinical

trials [2]. Data are limited for antiretroviral drugs, particularly when used in combination therapy. It is necessary for continued evaluation of placental transfer of antiretroviral agents alone and in combination in order to optimize treatment in pregnant women.

### Clinical Trials

Several studies have shown that antiretroviral treatment can greatly reduce mother to child transmission. The Pediatric AIDS Clinical Trials Group (PACTG) protocol 076 in 1994 established that antenatal, intrapartum and postpartum zidovudine (AZT) treatment reduced vertical HIV transmission rates with 68% efficacy [4]. Subsequent to this pivotal study long-term zidovudine guidelines were adapted for MTCT prophylaxis [2, 6, 11, 20-21, 80-82]. However, further trials investigated several shorter and cheaper regimens as well as combination therapy. Shaffer et al. found that an AZT regimen started late in pregnancy (from 36 weeks) and given during labor is 50% effective in prevention of MTCT among non-breastfeeding women [83]. Two West African studies by Dabis et al. and Wiktor et al. showed similar regimens provided 38% and 37% efficacy of the West African studies is largely due to breastfeeding. While these studies provided promising results, they did not achieve the rate of efficacy of a long-term AZT regimen. Subsequent studies examined the efficacy of other short and long-term AZT regimens [5, 9, 41, 56, 83, 85-90].

The next progression of studies focused on whether combination therapy provided improved efficacy. The perinatal transmission (PETRA) study investigated the efficacy of two AZT/3TC regimens Arm 1 (antepartum, intrapartum, and postpartum) and Arm 2 (intrapartum and postpartum). Efficacy of Arm 1 and Arm 2 were 50% and 37%, respectively, thus allowing for future studies of more complex regimens [68]. Studies in resource-rich countries have shown

that triple-drug regimens can reduce MTCT risk to fewer than 2% [2, 11]. Since around 1998, triple-ARV combinations have increasingly been used to prevent MTCT in Europe and North America. Implementation of triple-drug regimens and the elimination of breastfeeding have nearly eliminated MTCT in these settings [2, 11, 79, 82, 91-94].

By far the shortest and least expensive method of MTCT prophylaxis is single dose Nevirapine. In the HIVNET 012 study, single-dose Nevirapine (sd-NVP) was shown have 42% efficacy when administered during labor and postpartum to the infant versus similar treatment with zidovudine. The SAINT trial in South Africa, sd-NVP was shown to have comparable efficacy to AZT/3TC combination therapy. Currently, intrapartum and post partum (infant only) sd-NVP is globally accepted as the minimum treatment for MTCT prophylaxis. Furthermore, sd-NVP has been shown to enhance combination therapy for the reduction of vertical transmission, reducing risk to 2-4% [2-3, 82, 95-97]

#### **Placental Transport**

Historically, the placenta was viewed as a protective barrier and site for nutrient and waste exchange between mother and fetus. Over the past 50 years, further evidence has shown that the placenta provides the link between mother and fetus, and though its main task is to act as a barrier and transport nutrients and oxygen to the fetus. Many foreign compounds are transported across the placenta to some degree and may therefore influence the unborn child. The first evidence of reproductive toxicity caused by a fetal exposure from maternal intake was the "thalidomide disaster" in 1957–1961. Teratogenic effects of thalidomide were evidenced by skeletal malformations of thousands of infants. In the early 1970s, it was scientifically confirmed that prenatal alcohol exposure can cause mental retardation, facial malformations, prenatal and/or postnatal growth retardation. Smoking and recreational drugs have also been

shown to have adverse effects on fetal development. In recent years, there has been a rise in administration of drugs to pregnant woman, creating a special population. Medication during pregnancy is administered for maternal treatment, fetal treatment or both. In all cases the benefit of treatment must outweigh the risk to the fetus. Understanding placental transport is critical to maternal and fetal safety [98-104].

### Placental Anatomy and Transfer

During gestation, the primary role of the placenta is to facilitate the growth and development of the fetus. It serves as an endocrine organ by producing various steroid hormones (e.g., estrogens and progesterone) and polypeptide hormones (e.g., chorionic gonadotropin and placental lactogen) relevant to pregnancy [103]. The human placenta is a complex transport system that acts as a semi-permeable barrier between the maternal and fetal circulation. It functions as an important transport organ that mediates the exchange of nutrients, metabolites and hormones between mother and fetus, as well as export of fetal waste. To facilitate these functions, the placenta expresses numerous transporters; up to now, approximately 20 different drug transport proteins have been determined [100, 102-104]. Transporters are expressed both in the maternal-facing apical (brush border) membrane and fetal-facing basal membrane of the syncytiotrophoblast, the functional unit of the placenta. A variety of endogenous compounds that may be nutrients or metabolic waste products, are recognized as substrates by the placental transporters in a differential manner. Quite understandably exogenous compounds with structural resemblance to endogenous substrates also interact with these transporters. Therefore, if such compounds are present in maternal blood, placental transporters may facilitate the transfer of these compounds from mother to fetus. Similarly, there are transporters in placenta that mediate the efflux of endogenous substrates from fetus into mother and these transporters may prevent the entry of exogenous compounds into the fetus [100, 102-104].

The placental transfer of compounds can occur by passive diffusion, facilitated diffusion, active transport or pinocytosis. The primary mechanism of placental transfer is passive diffusion. Passive diffusion represents penetration of a molecule down the concentration gradient, is not subject to saturation or competitive inhibition, and does not require input of energy [103]. It is dependent only by the factors introduced in Fick's law of passive diffusion:

$$V_{diff} = \frac{D \times S \times (C_M - C_F)}{a}$$

where  $V_{diff}$  = rate of diffusion, D = diffusion coefficient, S = surface area of exchange,  $C_M$  = concentration in the maternal circulation,  $C_F$  = concentration in the fetal circulation, and a = thickness of the placenta. The speed and extent of placental transfer is dependent on the physiochemical and structural characteristics of the compound, concentration of drug and physical characteristics of the maternal-fetal compartment. Molecules weighing under 600 Da, non-ionized and lipid soluble will show unimpeded diffusion. Fetal exposure to a drug depends on various processes, including the rate of drug intake by the mother, the rate of drug elimination by the mother, the drug transfer rate across the placenta in both directions, and the drug distribution rates in mother and fetus. Many pharmacologically active compounds cross the placenta by simple diffusion, although also larger molecules such as antibodies may be transported this way [98-100, 103-106].

Drugs may also be transferred across the placenta by facilitated diffusion, which is carrier mediated transport down a concentration gradient without energy-costs. Unlike passive diffusion, facilitated diffusion can be saturated at high concentrations relative to the Michelis-Menten constant ( $K_m$ ). Only a few drugs have been suggested to be transported this way,

including ganciclovir. Generally, compounds structurally related to endogenous compounds intended for this kind of transport, are assumed to use facilitated diffusion, e.g. hormones and nucleosides. The movement of a substance against a chemical or electrical gradient with energy costs is called active transport. Active transport is carrier mediated with a high degree of competition between structurally related compounds. Active drug transporters are present either in the apical membrane or in the basolateral membrane where they pump drugs into or out of the synctiotrophoblast. An example is the sodium/multivitamin transporter (SMVT) located in the placental brush-border membrane; drugs like carbamazepine may compete with endogenous biotin for the SMVT transporter [98-100, 103-106]. Placental transfer may also involve pinocytosis, in which the compound is invaginated into the cell membrane where it is transferred to the opposite site as a vesicle. However, placental transport studies have shown that this process is too slow to be highly relevant for the transfer of drugs from mother to fetus [98, 103, 105].

#### **Placental Transporter Expression**

Human placenta has been shown to express several active and facilitative transporters; additionally, several transporter families have been shown to exist in both species. Table 2.1 summarizes transporters involved in trans-placental transfer of drugs to the fetus. Transporters in the ATP binding cassette (ABC) superfamily have been found in the placenta, these efflux transporters are responsible for the removal of xenobiotics from the placenta [98, 100, 104-105]. Transporters located on the apical membrane efflux compounds into maternal circulation; while those located on the basolateral membrane efflux compound into fetal circulation. The best characterized and most abundant placental efflux transporter is p-glycoprotein which is encoded by the human multidrug resistance gene *MDR1*. P-gp is considered essential to the mediation of

active efflux of lipophilic drugs from the fetal compartment to maternal circulation, using energy derived by ATP hydrolysis. The presence of p-gp at the apical membrane limits fetal access of xenobiotics including digoxin, anticancer drugs and HIV protease inhibitors [98, 100, 104-105].

In addition to p-glycoprotein, the placenta also expresses another ABC superfamily transporter, multidrug resistance-associated protein (MRP). The preferred substrates of MRPs are organic anions that carry a net negative charge at physiological pH. The placental expression of at least three members of the MRP family, MRP1, MRP2, and MRP3, has been documented. The presence of MRPs in the placenta is somewhat controversial. Recent findings show that the human placenta at term expresses MRP2 on the apical membrane of the syncitiotrophoblast, while MRP1 and MRP3 are expressed on the basolateral membrane and the blood vessel endothelia. MRP1-3 are organic anion transporters which suggest that nucleoside antiviral agents are substrates. MRP4 and 5, although not yet identified in the human placenta, have been shown to transport nucleosides and nucleoside analogs [98, 100, 104-105]. Transcripts of MRP1-6 have been found either in human or rat placenta. The role of MRPs in the placenta has not been established, but it is likely related to efflux of polar conjugates of xenobiotics or metabolites of endogenous compounds [98-100, 104-105, 107]. Another ABC protein highly expressed in the placenta is the breast cancer resistance protein (BCRP). BCRP is localized at the apical sycytiotrophoblast, where it serves as a maternal-fetal barrier to passage of xenobiotics such as anti-cancer topoisomerase I inhibitors. While substrate selectivity of BCRP has not been completely elucidated, there is considerable overlap in substrates between BCRP and P-gp [98-99, 103-104]. The structure, substrate selectivity and localization of BCRP suggest that, like Pgp, it may function in a protective capacity, removing cytotoxic drugs from the fetal tissues. Wang et al. found that the cytotoxicity of AZT and 3TC was reduced in BCRP-overexpressing

cells. These results suggest AZT and 3TC as potential substrates and the protective capacity of BCRP [98].

The Na<sup>+</sup> - independent uptake of numerous organic anionic compounds is mediated by the gene superfamilies, organic anion-transporting polypeptide (OATP) and organic anion transporter (OAT). Two OATPs (B and E) and OAT4 have been identified in the human placenta; however, there is minimal evidence of OAT1-3 and eight OATPs in rat placenta, with the exception of OAT12. The renal excretion of ACV and AZT is reduced by the known inhibitor of organic anion transport, probenecid, thereby suggesting that they are substrates of the OAT system [98-99, 104, 108-109]. Facilitative transporters can function both as influx or efflux transporters depending on the directionality of the concentration gradient. The organic cation transporter OCT3 is a Na<sup>+</sup> and Cl<sup>-</sup> independent monoamine transporter; this transporter is located on the basolateral membrane and responsible for importing dopamine and norepinephrine into the fetal compartment, and is sensitive to inhibition by steroids. The carnitine transporters OCTN2 and OCTN1 have been identified in the placenta. While OCTN2 has been localized at the placental brush-border membrane, the localization of OCTN1 is unknown. OCTN2 transports cations in a Na<sup>+</sup>-independent manner, whereas the transport of zwitterions occurs in a Na<sup>+</sup>-dependent manner. Because OCTN2 transports organic cations, its activity can be inhibited by cationic drug substrates. Ohashi, et al. showed that many zwitterionic and cationic drugs inhibit OCTN2 in human embryonic kidney cells, including tetraethylammonium, pyrilamine, quinidine, verapamil and valproate. The serotonin transporter (SERT) and norepinephrine transporter (NET) mediate the placental clearance of serotonin and catechoalamines at the apical and basal syncytiotrophoblast, respectively. OCT3, SERT and NET are the transport systems most implicated in the effects of abusable drugs [98-99, 104, 108-109].

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Transporter	Placental Localization	Direction of Transport	Physiological Function	References				
ATP-Dependent Efflux Pumps								
P-glycoprotein	Apical	Efflux	Removal of xenobiotics from fetal compartment	[98-100, 104, 108]				
MRPs (ABCC1- ABCC6)	Apical or Basolateral	Efflux	Removal of xenobiotics from fetal compartment	(10, 102, 107, 114, 126)				
BCRP	Apical	Efflux	Removal of xenobiotics and from fetal compartment	(10, 102, 107, 114, 126)				
Organic Anion Solute Carriers								
OATP-B,E	Basolateral	Influx/Efflux	Mediate Na <sup>+</sup> -independent uptake of organic anions	(102, 107, 114)				
OAT-4	Unknown	Influx/Efflux	Mediate Na <sup>+</sup> -independent uptake of organic anions	(102, 107, 114)				
	Or	ganic Cation Solut	te Carriers					
OCTN2	Apical	Influx/Efflux	Transport of Carnitine	(102, 107, 114)				
OCTN1	Unknown	Influx/Efflux	Transport of organic cations	(102, 107, 114)				
OCT3	Basolateral	Influx/Efflux	Transport of organic cations	(102, 107, 114)				
NET	Apical	Influx	Transport of dopamine and norepinephrine	(102, 107, 114)				
SERT	Apical	Influx	Transport of serotonin	(102, 107, 114)				
	Equila	brative Nucleoside	e Transporters					
ENT1 (SLC29A1)	Apical	Influx/Efflux	Na <sup>+</sup> -independent transport of purine and pyrimidine nucleosides	(102, 107, 114)				
ENT2 (SLC29A2)	Unknown	Influx/Efflux	Na <sup>+</sup> -independent transport of purine and pyrimidine nucleosides	(102, 107, 114)				
Concentrative Nucleoside Transporters								
CNT1 (SLC28A1)	Apical	Influx/Efflux	Na <sup>+</sup> -dependent transport of pyrimidine nucleosides	(102, 107, 114)				
CNT2 (SLC28A2)	Unknown	Influx/Efflux	Na <sup>+</sup> -dependent transport of purine nucleosides	(102, 107, 114)				
CNT3 (SLC28A3)	Unknown	Influx	Na <sup>+</sup> -dependent transport of purine and pyrimidine nucleosides	(102, 107, 114)				

Table 2.1. Transporters Involved in Maternal-Fetal Transfer of Drugs

The Na<sup>+</sup>-independent equilibrative nucleoside transporters (ENT1 and ENT2) have been cloned from the human and rat placenta. ENT1 is thought to be situated on the brushborder membrane of the placental syncytiotrophoblasts, the placental localization of ENT2 is currently unknown. Both ENT1 and ENT2 have a broad specificity for nucleosides and nucleoside drugs and both transport purine and pyrimidine nucleosides but with different affinities; ENT1 transports cytidine, guanosine, thymidine and adenosine with a higher affinity than ENT2, while ENT2 transports inosine with a higher affinity than ENT1. These transporters likely move nucleosides and nucleoside analogs across the placenta from the mother to the fetus or vice versa, depending on the direction of the concentration gradient. Nucleoside drugs such as gemcitabine and ribavirin are efficiently transported by ENT1 and ENT2 (102, 107, 114). Three different Na<sup>+</sup>-dependent concentrative nucleoside transporters (CNT1, CNT2 and CNT3) have been cloned from human and murine organs, including the placenta. Actual placental location of CNTs is much debated, with CNT1 possibly located on the apical side, where it works in concert with ENTs (102, 107, 114). CNT1 is highly selective to pyrimidine nucleosides, yet it also transports the purine nucleoside adenoside in a highly selective and low capacity manner (126). CNT 2 is selective to purine nucleosides, while CNT3 is broadly selective and transports both purine and pyrimidine nucleosides (102, 107, 114). Several nucleoside antiretroviral drugs such as zidovudine, lamivudine, didanoside and zalcitabine have been shown to be substrates for CNT1-3 (126).

### Models to Study Drug Transfer

It is of utmost importance to study the transfer of chemicals and drugs across the placenta. Most commonly risk assessment is based on results from *in vitro* and *in vivo* studies. *In vitro* methods include: monolayer cell cultures, human placenta cell lines, placental explants,

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placental microsomes, membrane vesicles, and perfused placental cotyledon. In vitro models have the potential of replacing or reducing the number of animals used for toxicological testing. Though in vitro models cannot fully account for all the physiological and biochemical variables in the mother, placenta and fetus and how these variables change throughout gestation, they should be the first in line when a new substance is to be investigated (71). The placenta perfusion model is often praised as a non-invasive and ethical method. The perfusion model has been used to validate HIV protease inhibitors. Human placental perfusion test system offers information about transplacental transfer, placental metabolism, storage, acute toxicity and potential role of transporters, vascularization, and fetal exposure (54, 70, 71, 101, 102, 117). Placental microsomes are used as an enzyme activity model thereby providing means for studying chemical metabolism in the placenta thus this system can be used to address both early and late placental enzyme activity. Human placental choriocarcinoma cells, BeWo, JAr and JEG cells share many of the same properties but noteworthy differences in characteristics have been identified. Consequently, they are commonly used in toxicology to study transplacental transport and metabolism in vitro. They have a wide variety of use, such as placental transport, metabolism, endocrine function, enzyme function, cellular proliferation and differentiation (71).

*In vivo* models of placental transport are used for determination of drug clearance. Sampling of human maternal and cord blood at the same time and further analysis for presence of compounds is a fairly simple and ethically agreeable method. This technique is used to illustrate the drug concentration between the two circulations. The study of human coelocentesis is another method by which it is possible to obtain information of the transfer across the early placenta. The coelocentesis method, however, does not allow use of kinetic models based on blood and plasma samples. Kinetic models are necessary to allow for extrapolating quantitative drug transfer to the fetus and subsequent risk assessment (71, 92, 112, 113).

Due to the aforementioned ethical reasons, in vivo placental transfer studies are investigated using animal models as opposed to pregnant women. Animal models for placental transfer of drugs include rodents, sheep and non-human primates (5, 6, 12, 19, 44, 53, 83, 85, 86, 105, 112, 125). Primates such as rhesus, macaques and baboons are used since they have hemochorial placentation similar to the human placenta. It was shown by Patterson, et al. that the anti-HIV compound, d4T, and its active and inactive metabolites were transferred across the rhesus macaques late term placenta (83). However, there are limitations to such studies including cost, small litter size, long gestation period and requirement of specialized experimental conditions. Animal studies are invaluable in determination of potential risks of a drug or chemical, however difficulties in extrapolation of results to humans still exist (48, 71).

### Comparative Placental Structure

Placental transfer of numerous compounds, including nucleoside analogs have been successfully studied using the pregnant rat model (4-6, 12, 19, 20, 31, 44, 53, 108, 125). The efficiency and/or the rate of transfer of materials between the mother and embryo are affected by placental characteristics of the rat and human. Both the human and rodent placentas are hemochorial, in which trophoblastic cells are in direct contact with maternal blood without an intervening endothelium (4, 12, 19, 31, 53, 125). Although, there are structural similarities between rat and human placenta, human placenta contains one syncytiotrophoblast while rat placenta contains syncytiotrophoblast layers I and II). While the rat placenta is structurally different than that of humans, it possesses comparable basic hemochorial organization and types of cells. Additionally, results reported from *in vitro* human placenta studies and

pharmacokinetics studies in monkeys are similar to those found using the pregnant rat model (44, 48, 112).

The aforementioned anatomical similarities between rat and human placenta make the pregnant rat model ideal for the investigation of human placental transfer. The pregnant rat model also presents several advantages including short gestation period (22 days), easy handling, quick turnaround and minimal housing requirements. Furthermore, the large litter size and the containment of each fetus, placenta, and amniotic fluid in individual fetal sacs allows serial sampling which can be used to create a viable concentration time profile for pharmacokinetic studies. Additionally, rodent pups allow for determination of both amount and concentration of the drug in the fetus. This is due to their small size and inability to eliminate or metabolize drug, thus allowing calculation of maternal/fetal disposition parameters without the need for fetal dosing (4-6, 12, 19, 20, 31, 44, 53, 125).

#### In Vivo Placental Transport of Nucleoside Analogs

The nucleoside reverse transcriptase inhibitor, zidovudine is the most widely utilized and studied drug in pregnancy. The placental transfer of AZT monotherapy has been studied by several groups, thus proving that AZT crosses the placenta by passive diffusion and is transported significantly to fetal tissues (44, 81, 82, 84, 112). Accumulation in amniotic fluid has been recorded in placental studies of antiretrovirals, however, high concentrations in amniotic fluid has been recorded, suggesting its role as a slowly equilibrating reservoir (44, 82, 84, 112). Several groups have studied potential interactions in combination therapy, with conflicting results. Odenics et al. investigated the interaction between various antiviral drugs, confirming that passive diffusion is the primary mechanism of transfer (82). When studying the effects of combination therapy in pregnant pigtailed macque, the group reported that AZT had no effect on

the placental transfer of d4T. Likewise, the group did not find interaction between AZT and ddI when simultaneously administered to pregnant pigtailed macque. Yet, there was a significant increase in the ratio of AZT concentration in amniotic fluid to that in the fetus, when compared to that of AZT monotherapy (84, 85, 112). The group hypothesized that the presence of ddI may hinder fetal reabsorption of AZT from the amniotic fluid; leading to accumulation of AZT and higher concentration ratio.

While several studies showed no interaction between antivirals when coadministered during pregnancy, other studies have identified noticeable interaction between various antiviral agents. Brown et al. reported considerable alteration in the disposition of ACV and AZT when the two drugs were coadministered in pregnant rats. Concentration of AZT in all tissues decreases in the presence of ACV; while, combination therapy showed a three-fold increase of ACV exposure in amniotic fluid and fetal tissue with the combination therapy (12). Lewis et al. reported that pharmacokinetics of 3TC is altered when coadministered with AZT in pregnant rats, decreasing the half-life by 20%. Additionally, relative exposure of AZT decreased in the presence of 3TC, while the relative exposure of 3TC increased in combination therapy, suggesting that transporters play a complex role in the disposition of these compounds (52). A study in healthy monkeys showed that the pharmacokinetics of AZT is altered in the presence of ddI, lowering the steady-state volume of distribution and clearance of AZT (34).

#### Pharmacokinetics Modeling During Pregnancy

Pharmacokinetic modeling via computer simulation is imperative for better comprehension of the pharmacokinetic mechanisms present in the feto-maternal unit. This process is enhanced by analysis with compartmental models, where mother and fetus are considered separate units, with localized pharmacokinetic properties. Several studies of the placental transfer of nucleoside analogs using pharmacokinetic models have been published (4-6, 12, 19, 20, 31, 44, 52, 53, 125). All compartmental models of the feto-maternal unit are configured with a maternal plasma and fetus compartment. More advanced models may include placenta, amniotic fluid, maternal peripheral compartment and/or fetal peripheral compartment.

Pereira *et al.* developed a three compartment model consisting of maternal plasma, fetal, and amniotic fluid compartments to describe the effects of AZT on ddI in pigtailed macques (84). Huang *et al.* developed a five-compartment pharmacokinetic model that described AZT distribution in maternal plasma (central and peripheral), amniotic fluid, placenta, and fetal tissue of the pregnant rat (44). A recent study by Lewis *et al.* used a five compartment model consisting of maternal plasma, maternal tissue, placental, fetal and amniotic fluid compartments to characterize the distribution of AZT and 3TC in the pregnant rat (52).

#### Preclinical Pharmacokinetics in Drug Discovery

A pivotal step in drug discovery is the performance of preclinical animal studies. Animal studies have provided invaluable pharmacological and toxicological information for drug development. Only one in 10 Investigational New Drugs (IND) will be approved for clinical use (96, 99). Reasons for this low rate of success are due to low efficacy or evidence of toxicity in animal studies. The development of animal studies must attempt to provide adequate correlation to humans in efficacy studies and toxicity. While animal studies cannot guarantee the safety and efficacy of the drug in humans, they can enhance the dependability and extrapolative value. Rodents are the preferred models (mice and rats), while other species are used including hamsters, guinea pigs, rabbits, dogs and non-human primates. All animals are bred and housed in a controlled environment, under pathogen-free conditions. Experimental use of animals is controlled under Good Laboratory Practice (GLP) and protocols are submitted to the Animal

Research Ethics Committee for approval. Experiments may only proceed with Ethics Committee approval (32, 79, 96, 99).

Various areas of drug development are dependent on pharmacokinetic and metabolic studies. These include drug discovery and lead optimization, pharmacology and safety evaluation, clinical development and marketing. *In vitro* and preclinical animal studies are necessary to establish the biological activity, toxicology and safety, pharmacokinetics and pharmacodynamics data necessary to develop clinical trials of an investigational drug. Pharmacokinetics can also help support the choice of animal species and dosing regimen and can facilitate interpretation of findings in toxicology studies to allow rapid entry into clinical trials (79, 96, 99).

Animal models and *in vitro* are commonly utilized during discovery and preclinical development to characterize pharmacokinetics, metabolism and pharmacodynamics. Preclinical studies are also employed to help identify agents with desirable pharmacological and biological properties. In order to perform robust pharmacokinetic studies, analytical methods to quantitate the parent and metabolite(s) in relevant biological fluids must be developed. For novel compounds analytical methods may not always be available and it may not be prudent to invest a lot of resources to develop a method. After a bioanalytical method is developed and validated, serial blood samples are collected from dosed animals, processed and analyzed by the method. The plasma concentration/time profile after intravenous dosing provides preliminary information on the distribution and disposition kinetics of the drug (79, 96, 99). These results confirm the utility of the assay method for determination of the drug and assist in the development of more definitive animal pharmacokinetic experiments. Plasma concentration/time profiles after oral dosing provide information on absorption kinetics and absolute bioavailability of the drug.

Allometry is used to predict pharmacokinetic behavior in humans based on animal data. The power relationship equation is used for allometric pharmacokinetic predictions:

$$Y = aX^b$$

Where Y is the dependent variable (e.g. half-life, clearance, etc.), X is the independent variable (usually species body weight), and a and b are allometric parameters. Robust allometric relationships are used to determine the initial dose in clinical trials and dose escalation schemes. Suitable animal studies allow a better understanding of the relationship between drug concentration and drug effects. Pharmacokinetic/pharmacodynamic animal studies are utilized in dosing regimen comparisons, investigation of drug interactions and understanding the relationship between drug concentration and drug effect (96, 99).

# Integrase Inhibition

There have been many advances in the treatment of HIV. Currently, there are over 20 FDA-approved drugs available for HIV treatment. With the exception of two fusion inhibitors (Fuzeon and Selzentry) and the integrase inhibitor, raltegravir, all approved drugs inhibit reverse transcriptase or protease (8, 25, 30, 32, 111). Highly Active Antiviral Treatment (HAART) has altered the course and prognosis of Acquired Immunodeficiency Syndrome (AIDS). Robust treatment can lead to near normal T-cell counts and undetectable viral counts. However, resistant viruses and toxicity are limiting factors in therapy consisting of reverse transcriptase and protease inhibitors, which may lead to suboptimal clinical outcomes. In addition, while inhibition of reverse transcriptase and protease can suppress viral replication, it is not eradicated. Therefore, it is logical to study other antiviral targets. HIV-Integrase has been identified as a potential target, due to its vital role in viral replication (8, 17, 32, 38, 72, 74).

Along with protease and reverse transcriptase, the retroviral enzyme integrase is responsible for replication of human immunodeficiency virus (HIV-1). Subsequent to reverse transcription viral DNA is incorporated into the host DNA, thus serving as a fundamental step in the viral lifecycle. HIV integrase catalyses the insertion of the reverse-transcribed viral genome into the host DNA in a multistep process. After assembling onto viral DNA, HIV integrase cleaves the two terminal nucleotides from each 3'-end. Following nuclear entry, the viral 3'-ends are ligated into the host genome (strand transfer). In the cytosol, intergrase trims the 3'-ends of both strands of the viral-DNA (vDNA) immediately downstream of a conserved CA dinucleotide, leaving a 2-base overhang at each 5'-end. HIV genome integration yields a provirus that can go on to produce new virus or lie dormant in a latent state. The integration process is necessary for the production of progeny viruses, and mutations that inactivate the IN have deleterious effect on the capability of HIV to replicate. Inhibition of HIV integrase results in arrest of the HIV life cycle, and represents therefore an attractive target for the treatment of HIV infection (8, 17, 32, 38, 72, 74).

The discovery of clinically relevant inhibitors of HIV integrase for antiviral therapy has proven to be a challenging task. Several groups have discovered compounds that exhibit integrase inhibition capabilities, however early assay conditions and minimal availability of the integrase-inhibitor pharmacophore made it difficult. While various structurally diverse compounds have exhibited inhibition of integrase, a small group of  $\beta$ -diketo acids represent the most convincing, biologically validated inhibitors of integrase. Many of these compounds selectively inhibit the strand transfer reaction of the enzyme. Nair et al. discovered new  $\beta$ -diketo acids with purine nucleobase scaffolds that are potent inhibitors of both the strand transfer and 3<sup>'</sup>processing steps of HIV-1 integrase (8, 15-17, 32, 38, 72-77).

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## **OBJECTIVES**

There have been numerous breakthroughs in the treatment of HIV over the past three Morbidity and mortality have decreased exponentially since the introduction of decades. Zidovudine However, thousands of children are infected every year due to vertical transmission. One of The World Health Organization's (WHO) main goals is to eradicate vertical transmission. Several groups have investigated the efficacy of various monotherapy and combination therapy regimens in the prevention of vertical transmission. While vertical transmission has nearly been eliminated in resource rich countries, further evaluation of treatment methods must be made. It is pertinent to understand the pharmacokinetics, placental transport and interactions at the placental level in order to develop optimum treatment of mother and child. Highly active antiviral therapy (HAART) consisting of reverse transcriptase inhibitors and protease inhibitors has provided beneficial treatment to many HIV-positive patients. However, due to drug resistance and toxicity, patients can be faced with limited therapeutic outcomes. Hence, it is logical to investigate new targets, such as HIV-Integrase, the enzyme necessary for viral replication. The objectives of the current research are as follows:

Aim 1: To develop an assay for the determination of stavudine in maternal plasma, amniotic fluid, fetal and placental tissues using high-performance liquid chromatography with ultraviolet detection.

Aim 2: To determine the pharmacokinetics and fetal disposition of stavudine in the pregnant rat and comparison of pharmacokinetics to another antiviral agent.

Aim 3: To develop an assay for the determination of a novel integrase inhibitor in rat plasma and determine its pharmacokinetic profile.

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Aim 4: To develop an assay for the determination of a novel intergrase inhibitor in mouse tissue and determine tissue disposition.

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# CHAPTER 3

# DETERMINATION OF STAVUDINE IN PLASMA, AMNIOTIC FLUID AND

RAT TISSUES BY HPLC WITH ULTRAVIOLET DETECTION  $^{\rm 1}$ 

<sup>&</sup>lt;sup>1</sup>Shawn K. Blue, Michael G. Bartlett, and Catherine A. White. To be submitted to *Journal of* 

## ABSTRACT

A simple high-performance liquid chromatography (HPLC) method has been developed and validated for determination of Stavudine (D4T) in maternal plasma, amniotic fluid, placental and fetal tissues. Liquid-liquid extraction enhanced by salting out with saturated ammonium sulfate was used for extraction of D4T and the internal standard, lamivudine (3TC), from placental and fetal tissue samples. Protein precipitation with 2M perchloric was used for extraction from maternal plasma and amniotic fluid samples. Reverse phase chromatography was performed with a Phenyl column (5 µm, 150 mm x 2.0 mm i.d.) equipped with a Phenomenex guard column. A flow rate of 0.2 ml/min and detection wavelength of 265 nm was used. A 20 mM dibasic phosphate buffer consisting of 5% methanol was used for the mobile phase. The method was validated over the range of 0.1-50 µm/ml for D4T for all four biological matrices. The absolute recovery of stavudine ranged from 74.9 to 86.3 %, in the different biological matrices. The internal standard recovery ranged from 80.7 to 83.4 %. Acceptable intra- and inter-day assay precision (< 15% R.S.D.) and accuracy (< 15% error) were observed over 0.1-50 µg/ml for all four matrices. The method was utilized in the analysis of the placental transport of D4T in pregnant rats.

## **INTRODUCTION**

Globally there are over 33 million people living with HIV/AIDS, including nearly 2.5 million children under 15. Women account for nearly half of all HIV positive adults with a large majority in their reproductive years. In 2008 approximately 420,000 children were newly infected with HIV worldwide [1]. The primary mode of infection for children is vertical transmission which can occur in utero, at delivery or through breastfeeding. The risk of transmission for non-breastfeeding populations is 15-30% without preventative measures while breastfeeding may increase risk to 45%. Preventative measures include voluntary cesarean delivery, antiretroviral drug treatment and alternatives to breastfeeding. However, in areas where cesarean delivery and alternate feeding are not options it is necessary to reduce transmission during labor by antiviral treatment [2].

Several studies have shown that antenatal, intrapartum and postpartum antiretroviral treatment can greatly reduce mother to child transmission (MTCT). In 1994, the Pediatric AIDS Clinical Trials Group (PACTG) protocol 076 established, the nucleoside reverse transcriptase inhibitor, zidovudine (AZT) as an effective method of reducing perinatal HIV transmission by 68% [3]. Since then, AZT has become the standard prophylactic method to reduce vertical transmission. However, due to resistance, toxicity, preference or lack of resources, alternatives to zidovudine are necessary [4-6]. While the pharmacokinetics and pharmacodynamics of antiretroviral drugs is well documented, there is limited knowledge of the efficacy of these drugs in the reduction of vertical transmission [7-15]. Therefore, it is necessary to understand placental transport of other NRTIs such as stavudine.

The nucleoside reverse transcriptase inhibitor, stavudine (D4T) has been shown to have high activity against human immunodeficiency virus [16] types 1 and 2. It is a thymidine nucleoside analogue which must undergo phosphorylation to its triphosphate form for activation. Stavudine has a simple regimen and is well tolerated; therefore treatment with D4T is desirable. D4T is currently recommended for both postexposure prophylaxis and treatment of HIV in combination therapy. According to the FDA, stavudine is classified as a pregnancy category C drug, thus animal studies have shown fetal risk and human studies are inconclusive. However, it may be administered if benefits outweigh the risks [12-15].

There are limited studies of the pharmacokinetics of D4T in pregnant women. Placental and fetal drug distribution is difficult to study [17]. This is due to the ethical exclusion of pregnant women in clinical trials coupled with difficulty in obtaining fetal concentration data. Ergo, it is necessary to find a viable animal model. Anatomical similarities between rat and human placenta make the pregnant rat model ideal for the investigation of human placental transfer [17-22]. The large litter size allows serial sampling, which can be used to create a viable concentration time profile for pharmacokinetic studies. The pregnant rat model has been utilized in maternal-fetal drug transfer studies of various compounds, including nucleoside analogs [4, 17-25].

Several HPLC methods have been developed for determination of stavudine in various biological matrices [4, 17-25]. However, there are no methods developed for the quantification of D4T in complex matrices such as amniotic fluid, placental and fetal homogenates. In order to study the placental transfer of D4T, our group has developed and validated an HPLC method to determine D4T in pregnant rat plasma, amniotic fluid, placental and fetal tissues. This method will be used to build a pharmacokinetic profile to illustrate the transport of D4T across the placenta.

#### **EXPERIMENTAL**

## **Chemicals and Reagents**

Stavudine (D4T) was purchased from Sigma (St. Louis, MO, USA). Lamivudine (3TC) was obtained from Glaxo Smith Kline (RTP, NC, USA). HPLC-grade acetonitrile, methanol and sodium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium sulfate was obtained from J.T Baker Inc. (Philipsburg, NJ, USA). The deionized water used was produced using a Continental Deionized Water System (Natick, MA, USA).

#### **Chromatographic System**

Chromatographic studies were performed by an Agilent 1100 HPLC (Santa Clara, CA) equipped with a variable wavelength UV detector, quaternary pump, degasser and autosampler. An YMC phenyl column (5 μm, 150mm×2mm i.d., Waters, Milford, MA, USA) equipped with a Phenomenex C18 guard column (Torrance, CA, USA) was used to achieve all the chromatographic separations.

# **Chromatographic Conditions**

The mobile phase consisted of 5% methanol in 20mM dibasic sodium phosphate (pH 6), pH was adjusted using phosphoric acid and NaOH concentrated solutions. The flow rate was 0.2 ml/min and the detection wavelength was 256 nm. The injection volume of plasma and amniotic fluid samples was 20  $\mu$ l, while the injection volume of placental and fetal samples was 15  $\mu$ l. HPLC run time for each sample was 20 min.

# **Preparation of Stock and Standard Solutions**

Stock solutions of 3TC and D4T (1 mg/ml) were individually prepared in deionized water. Stavudine standard solutions with concentrations of 500, 250, 100, 10, 7.5, 2 and 1  $\mu$ g/ml were prepared from the 1 mg/ml stock solution by serial dilution with deionized water. 3TC

standard solution at the concentration 50  $\mu$ g/ml was prepared by dilution from the 1 mg/ml stock solution with deionized water. Precision and accuracy standards of 400, 50, 5 and 1  $\Box$ g/ml were prepared in the same manner. Stock solutions (1 mg/ml) were kept refrigerated when not in use. Fresh standard solutions were prepared each day of analysis and validation.

# **Calibration Curves**

Blank plasma, amniotic fluid, placenta and fetal tissues were collected from untreated animals. Placental and fetal homogenates were produced homogenizing tissues with 2 volumes of distilled water (v/w). Calibration points for plasma, placental and fetal samples were prepared by spiking 100  $\mu$ l of the biological matrices with 10  $\mu$ l of each D4T standard solution and 10  $\mu$ l of the 50  $\mu$ g/ml 3TC solution. However, amniotic fluid calibration points were prepared using 50  $\mu$ l of the biological matrix by spiked with 5 $\mu$ l of each D4T standard solution and 5  $\mu$ l of the 50 $\mu$ g/ml 3TC standard solution. The calibration curves of all matrices were in the range of 0.1–50  $\mu$ g/ml with individual calibration points of 50, 25, 10, 1, 0.75, 0.2 and 0.1  $\mu$ g/ml, while the internal standard concentration was 5  $\mu$ g/ml for all samples. After each matrix was spiked, it was subject to further sample preparation before analysis.

## **Precision and Accuracy**

Validation for this method was demonstrated by using four QC points for each calibration curve. Intra-day accuracy and precision was determined by analysis of five replicates of each QC point each day. In order to determine the inter-day accuracy and precision this process was repeated 3 times over 3 days. The QC points for all matrices were 0.1, 0.5, 5 and 40 µg/ml.

# **Sample Preparation**

For plasma and amniotic fluid, extraction of the analytes was performed by protein precipitation, via acid precipitation with 2M perchloric acid. This was achieved by adding 15  $\mu$ l of acid to 100  $\mu$ l of the sample. Samples were then vortexed and centrifuged at 10,000 rpm for 10 min. The supernatant was aspirated and the pellet was discarded.

For placental and fetal homogenates, the analyte was extracted by a salting out technique. One hundred and eighty microliters of saturated ammonium sulfate solution and 360  $\mu$ l of cold acetonitrile were added to 100  $\mu$ l samples, vortexed and centrifuged at 10,000 rpm for 10 min. The upper organic layer was aspirated and dried under vacuum. Prior to analysis, samples were then reconstituted in 100  $\mu$ l of deionized water. Injection volumes of 20  $\mu$ l for plasma and amniotic fluid samples and 15  $\mu$ l placental and fetal samples were used.

## **Sample Collection**

The UGA Animal Use and Care Committee approved the use of animals. The rats were housed one animal per cage in the College of Pharmacy animal facility (AAALAC accredited). The environment was controlled (20-22° C, 14 h of light/day) with daily feedings of standard chow pellets and water ad libitum.

Timed pregnant female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing an average of 340 g were anesthetized intramuscularly with ketamine:acepromazine:xylazine (50:3.3:3.4 mg/kg) and dosed intravenously via jugular canula with 25 mg/kg of D4T dissolved in 0.1 N NaOH in physiological saline (pH 7.4) on the 19th day of gestation. Venous cannulation was performed for dosing and blood sampling. A laparotomy is done for access to the amniotic fluid, placenta and fetus. Blood samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min into heparinized tubes. Amniotic fluid, fetuses and placentas were collected concurrent with maternal blood sampling. All samples were stored on ice until processed. Plasma was collected after centrifuging blood samples at 10,000 rpm for 10 min. Fetal and placental tissues were homogenized in two volumes of distilled water. All samples were stored at  $-20^{\circ}$  C until analysis. Prior to analysis, samples from all were spiked with the internal standard (3TC) solution to yield a concentration 5 µg/ml.

#### RESULTS

The chemical structures of stavudine and lamivudine are represented in Figure 3.1. In order to achieve the best separation from endogenous matrix peaks, the organic content and pH of the mobile phase were examined. Satisfactory resolution of D4T and 3TC from endogenous peaks in all biological matrices was achieved with 5% methanol in buffer. At pH 6, D4T and 3TC eluted at 11 and 14 min, respectively, which provided ideal separation from endogenous peaks. Different UV wavelengths were also investigated; the wavelength which gave the best response for D4T was used. Chromatograms of each blank matrix and each matrix spiked with D4T (0.1  $\mu$ g/ml) and 3TC (5  $\mu$ g/ml) are shown in Figure 3.2.

Several liquid-liquid extraction and protein precipitation methods were investigated for the extraction of stavudine and the internal standard, lamivudine, from the different biological matrices. Although the literature provides extraction methods used for D4T, none were suitable for the complexity of the fetal and placental matrices in our application. Therefore, it is imperative to utilize a clean up method which eliminates the overlapping of endogenous peaks with the analyte. A salting out technique using saturated ammonium sulfate solution and acetonitrile provided the best extraction technique for fetal and placental homogenates. Acid precipitation using perchloric acid achieved satisfactory separation of the analytes from the biological content for plasma and amniotic fluid. These techniques have been successfully used for extraction of other antivirals in the pregnant rat [4, 18, 21, 23].

The calibration curves showed acceptable linearity ( $R^2 > 0.99$ ) over the range 0.1–50 µg/ml for all matrices. Linear regression equations were generated with Microsoft Excel software using a 1/x-weighting scheme for each day of validation and analysis.

The extraction efficiencies for D4T and 3TC from various matrices are expressed in terms of absolute recovery. Plasma, amniotic fluid, fetal and placental homogenate samples were spiked with 40, 5, 0.5 and 0.1  $\mu$ g/ml solutions of D4T and 5  $\mu$ g/ml solutions for 3TC samples. The absolute recoveries were calculated by comparing the peak areas of spiked samples to the corresponding peak areas of the untreated standard solutions (n=15). Stavudine recoveries ranged from 74.9 to 86.3 % in the different biological matrices. Recovery of the internal standard, lamivudine, ranged from 80.7 to 83.4 %. The recoveries are shown in Table 3.1.

Calculation of accuracy and precision was performed over 3 days. Precision, as expressed by % R.S.D., and accuracy as expressed by % error for D4T in the four biological matrices are shown in Table 3.2. Over three separate days, measurement of five samples at each QC point was used to determine intra-day (n = 5) precision and accuracy. The pooled data over three days was used to determine inter-day (n = 15) precision and accuracy. The calibration curve and each of the four QC points 40, 5, 0.5 and 0.1 µg/ml were used for these calculations. Intra-day precision (% R.S.D.) and accuracy (% error) of D4T ranged from 0.02 to 13.8 and 1.42 to 13.9%, respectively. Inter-day precision and accuracy of D4T ranged from 0.38 to 8.3 and 1.3 to 12%,

Stability testing was performed for D4T and 3TC at the concentration level of 0.5  $\mu$ g/ml. Spiked matrix samples (20 samples) went through three consecutive freeze/thaw cycles over the

period of four days. Five samples were extracted and analyzed as described before. The remaining spiked matrix samples were stored at -20°C. Each of the following three consecutive days, spiked matrix samples were thawed, and five more were extracted and analyzed. The day-to-day measured peak areas of D4T and 3TC were compared and the results listed in Table 3.4. The % R.S.D. between the average peak areas of D4T and 3TC each day was less than 10 %. The stability of extracted matrix samples in the autosampler was also evaluated. At time 0, three samples of each matrix were injected onto the HPLC column and analyzed. In another 24 hr, the same samples from each matrix were injected again. The peak areas for D4T and 3TC in each injection were compared. The % R.S.D. between samples was less than 10 % for all three compounds and no obvious decline in peak areas between each injection was observed.

## **ANIMAL STUDIES**

To demonstrate the utility of this assay, a pregnant rat was given an i.v. bolus dose of D4T (25 mg/kg each). Plasma, amniotic fluid, placental and fetal tissues were processed and analyzed as mentioned. A calibration curve from each matrix was prepared on the day of analysis to calculate the concentrations of D4T present in the real samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 5  $\mu$ g/ml of the internal standard. In addition, real samples were spiked with the appropriate amounts of deionized water (10  $\mu$ l for plasma, fetal, and placental homogenates, 5  $\mu$ l for amniotic fluid) to account for the addition of D4T and 3TC in the calibration points. The sample peak area ratios of D4T and the internal standard, 3TC, were used to calculate the concentrations of D4T in each sample. The concentration-time profile of D4T in all matrices is shown in Figure 3.3. Plasma data was analyzed using a two-compartment model using WinNonlin (Pharsight, Mountain View, CA, USA). For D4T, estimates for half-life (t<sub>1/2</sub>), volume of distribution at steady state

 $(V_{ss})$ , and clearance (CL) in maternal plasma were 35.3 min, 621 mL/kg, and 12.2 mL/min-kg, respectively. These values were in close agreement with earlier reported data for D4T (8).

#### CONCLUSION

A sensitive and accurate method was developed and validated for the quantification of D4T in rat maternal plasma, amniotic fluid, placental and fetal tissues. The use of acid precipitation and salting out techniques provided an inexpensive and convenient method of sample preparation. This method yielded high recoveries, good linearity, and precision and accuracy in the range of  $0.1 - 50 \mu g/ml$ . This method will be utilized for pharmacokinetic studies to investigate the fetal and maternal disposition of D4T in the pregnant rat.

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Table 3.1. Absolute recoveries of D4T and 3TC from plasma, amniotic fluid, placenta, and fetus (n=15)

Analyte	Concentration	Plasma	<b>Amniotic Fluid</b>	Placenta	Fetus
	40	$86.3\pm0.03$	$86.8\pm0.07$	$78.3\pm0.05$	$74.9\pm0.02$
DAT	5	$80.4\pm0.07$	$80.3\pm0.06$	$82.9 \pm 0.1$	$83.4\pm0.02$
D4 I	0.5	$81.3 \pm 0.04$	$84.5 \pm 0.06$	$79.9\pm0.06$	$85.8\pm0.09$
	0.1	$80.2\pm0.02$	$88.5 \pm 0.06$	$89.1 \pm 0.07$	$86.6 \pm 0.04$
3TC	5	$83.4\pm0.1$	$82.2 \pm 0.7$	$80.7\pm0.7$	$82.8\pm0.07$

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40	$37.92 \pm 3.13$	5.20	8.27	$43.65 \pm 3.13$	9.120	7.161	$40.01 \pm 2.01$	0.03	5.02	$40.53 \pm 3.57$	1.32	8.81
S	$4.78 \pm 0.40$	4.37	8.37	$4.46 \pm 0.28$	10.79	6.189	$4.64\pm0.24$	7.13	5.26	$4.63\pm0.32$	7.43	6.94
0.5	$0.50\pm0.05$	0.67	10.79	$0.57\pm0.04$	13.30	7.753	$0.46\pm0.04$	7.39	7.99	$0.51\pm0.06$	2.19	11.95
0.1	$0.09\pm0.01$	13.82	10.62	$0.11 \pm 0.01$	9.25	6.190	$0.10\pm0.004$	1.97	3.98	$0.10\pm0.01$	0.87	11.98
					AI	nniotic F	luid					
40	$39.62 \pm 4.1$	0.96	10.36	$39.99 \pm 0.57$	0.02	1.42	$39.40 \pm 1.10$	1.49	2.80	$39.67 \pm 0.53$	0.82	5.81
S	$4.63\pm0.47$	7.48	10.08	$4.92\pm0.10$	1.60	1.97	$4.90 \pm 0.16$	2.08	3.18	$4.81\pm0.37$	3.72	6.26
0.5	$0.54\pm0.05$	8.22	8.84	$0.51 \pm 0.01$	1.01	2.15	$0.49\pm0.02$	1.66	3.79	$0.51\pm0.03$	2.53	6.90
0.1	$0.11 \pm 0.01$	12.52	10.34	$0.11 \pm 0.01$	5.79	8.09	$0.10\pm0.01$	1.46	5.78	$0.11 \pm 0.01$	6.59	8.98
						Placent	я					
40	$42.05 \pm 0.17$	5.12	0.41	$40.95 \pm 0.33$	2.36	0.79	$41.94 \pm 0.11$	4.85	0.26	$41.64 \pm 2.31$	4.11	1.33
S	$4.36\pm0.08$	12.74	1.75	$5.12 \pm 0.05$	2.44	0.91	$5.11 \pm 0.06$	2.12	1.12	$4.86\pm0.30$	2.73	7.63
0.5	$0.49\pm0.05$	2.28	9.89	$0.51 \pm 0.01$	2.31	1.76	$0.49\pm0.01$	1.17	1.55	$0.50\pm0.03$	0.38	5.71
0.1	$0.11 \pm 0.01$	7.73	9.37	$0.11 \pm 0.01$	6.97	12.73	$0.11 \pm 0.004$	8.23	3.43	$0.11 \pm 0.01$	7.64	8.63
						Fetus						
40	$42.39 \pm 0.06$	5.98	0.14	$41.15 \pm 0.06$	2.88	0.15	$40.68\pm0.09$	1.70	0.21	$41.41 \pm 0.75$	3.52	1.81
5	$4.88\pm0.02$	2.37	0.50	$5.47 \pm 0.51$	9.44	9.28	$5.23 \pm 0.49$	4.55	9.41	$5.42 \pm 0.51$	8.33	9.40
0.5	$0.52\pm0.02$	3.54	3.63	$0.53\pm0.01$	6.08	1.54	$0.50\pm0.02$	0.60	4.16	$0.52\pm0.02$	3.41	3.77
0.1	$0.10 \pm 0.01$	1.18	13.37	$0.11 \pm 0.02$	11.49	13.86	$0.10 \pm 0.01$	3.63	6.01	$0.10\pm0.01$	4.65	12.03

Table 3.3. Results of freeze/thaw stability of D4T, and internal standard, 3TC, in maternal plasma, amniotic fluid, placenta, and fetus, represented by area  $\pm$  S.D. (n=5) of each day and % R.S.D. of the area between days

	Maternal Plasma	Amniotic fluid	Placenta	Fetus
D4T				
Day 1	$76.0 \pm 5.21$	$114.1 \pm 3.36$	$96.9 \pm 3.81$	$117.2 \pm 13.5$
Day 2	$75.5 \pm 3.62$	$114.8 \pm 6.43$	$98.3 \pm 6.48$	$118.0 \pm 11.6$
Day 3	$76.0 \pm 3.90$	$113.2 \pm 13.7$	$96.7 \pm 11.4$	$110.9 \pm 4.78$
Day 4	$73.4 \pm 0.56$	$110.2 \pm 1.16$	$97.3\pm0.09$	$94.83 \pm 3.71$
% R.S.D.	8.76	7.60	2.05	8.58
3TC				
Dav 1	$93.9 \pm 3.52$	$84.9 \pm 4.51$	$73.0 \pm 2.84$	$93.8 \pm 9.43$
Day 2	$93.2 \pm 4.27$	$85.3 \pm 9.27$	$74.1 \pm 3.84$	$92.8 \pm 8.78$
Day 3	$93.9 \pm 6.25$	$84.9 \pm 13.8$	$72.7 \pm 8.36$	$88.5 \pm 3.72$
Day 4	$94.5 \pm 0.14$	$80.7 \pm 1.30$	$72.5\pm0.28$	$76.2\pm2.95$
% R.S.D.	8.68	5.05	6.31	4.67



Figure 3.1. Chemical structures of stavudine (d4T) and lamivudine (3TC).



Figure 3.2. (a) Chomatographs of blank maternal plasma (top) and maternal plasma spiked with 0.1  $\mu$ g/ml D4T (I) and 5  $\mu$ g/ml 3TC (II) (bottom). (b) Chromatographs of blank amniotic fluid (top) and amniotic fluid spiked with 0.1  $\mu$ g/ml D4T (I) and 5  $\mu$ g/ml 3TC (II) (bottom). (c) Chromatographs of blank placental homogenate (top) and placental homogenate spiked with 0.1  $\mu$ g/ml D4T (I) and 5  $\mu$ g/ml 3TC (II) (bottom). (c) Chromatographs of blank placental homogenate (top) and placental homogenate spiked with 0.1  $\mu$ g/ml D4T (I) and 5  $\mu$ g/ml 3TC (II) (bottom). (d) Chromatographs of blank fetal homogenate (I) (top) and fetal homogenate spiked with 0.1  $\mu$ g/ml D4T (I) and 5  $\mu$ g/ml 3TC (II) (bottom).





Figure 3.3. Concentration-time profile

# CHAPTER 4

# EFFECTS OF ZALCITABINE ON THE PLACENTAL DISPOSITION OF STAVUDINE IN PREGNANT RATS<sup>1</sup>

<sup>&</sup>lt;sup>1</sup>Blue, Shawn K., Xu, M, Bartlett, M. G., and White, C. A. To be submitted to *Antimicrobial Agents and Chemotherapy*.

#### ABSTRACT

The purpose of this study was to compare the pharmacokinetics and placental transfer of stavudine (D4T) alone and in combination with zalcitabine (DDC) following IV administration. Thus, the pharmacokinetics and placental transfer of D4T was compared with the pharmacokinetics and placental transfer of D4T/DDC. D4T and D4T/DDC were administered IV bolus at a dose of 25 mg/kg to timed-pregnant Sprague-Dawley rats on day 19 of gestation via a jugular cannula. Maternal plasma, placenta, fetus and amniotic fluid samples were collected over a period of six hours post-dose. Concentrations in each matrix were determined by All pharmacokinetic parameters were determined using WinNonlin. HPLC-UV. A two compartment model with first order elimination was used to fit all maternal plasma data. All tissue data was analyzed by noncompartmental analysis. In addition, a five-compartment model and pharmacokinetic parameters and relative was fitted to the data exposures (AUC<sub>tissue</sub>/AUC<sub>maternal plasma</sub>) were determined.

Maternal plasma pharmacokinetics of D4T alone and in combination with DDC exhibited significant differences in Cmax (49.05  $\pm$  10.6 vs. 70.9  $\pm$  4.3 mg/L) and CL<sub>T</sub> (0.43  $\pm$  0.06 vs 0.30  $\pm$  0.07 L/hr-kg). While changes in AUC (59.1  $\pm$  8.5 vs. 86.1  $\pm$  22.3) and V<sub>ss</sub> (1.32  $\pm$  0.3 vs. 0.82  $\pm$  0.3) were not significant. Relative exposure for D4T alone in the amniotic fluid (0.25  $\pm$  0.04 vs. 0.15  $\pm$  0.04) and fetus (0.24  $\pm$  0.04 vs. 0.10  $\pm$  0.02) were significantly higher than D4T given in combination with DDC. The fetal and amniotic fluid uptake of D4T was significantly lowered in the presence of DDC. This suggests upregulation of efflux transporters which help pump D4T back to placenta from fetus and inhibition of transporters between placenta and amniotic fluid when dosed in combination with DDC.

Keywords: Pharmacokinetics, Stavudine, Zalcitabine, Placental Transfer

#### **INTRODUCTION**

Currently there are over 30 million people infected with HIV, throughout the world, over 2 million of whom are children under 15 (1). This included over 400,000 newly infected children (1). Vertical transmission accounts for nearly 90% of new HIV cases globally in children (2-6). Mother to Child Transmission (MTCT) can occur either intrauterine, during delivery or through breastfeeding (7-9). The rate of HIV infection in women has risen dramatically over the past twenty five years (1). Adult women have gone from under 10% to nearly half of all HIV positive adults (1). In order to curb these trends, it is necessary to understand the kinetics of HIV medication administered during pregnancy as well as the placental distribution of the drug (5, 10-13).

The Pediatric AIDS Clinical Trials Group (PACTG) protocol 076 established in 1994, the nucleoside reverse transcriptase inhibitor, zidovudine (AZT) is an effective method of reducing perinatal HIV transmission rates from over 25% to less than 10% (14). The standard treatment of HIV consists of highly active antiretroviral therapy (HAART); consisting of at least 2 nucleoside reverse transcriptase inhibitor (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (PI) (15). HAART has led to a significant improvement in the mortality and morbidity of HIV infection. Other studies have shown that prenatal, intrapartum and postpartum combination therapy may provide an even greater reduction in vertical transmission risk (15-22). Previous studies have shown that anti-HIV drugs cross the placenta via passive diffusion, reducing the chance of placental drug-drug interactions (15-22). However, additional studies have shown interactions between co-administered nucleosides (23). Xu *et al.* have shown that D4T significantly alters the placental disposition of DDC in pregnant

mice. Their study suggests that D4T either blocks the influx of DDC to the placenta or upregulates efflux transporters which transfers DDC from placenta to maternal plasma (24).

Human and rat placenta have been proven to express several bidirectional transporters. The Na<sup>+</sup>-independent equilibrative nucleoside transporters (ENT1 and ENT2) and Na<sup>+</sup>- dependent concentrative nucleoside transporters (CNT1, CNT2 and CNT3) have been cloned from the human and rat placenta. Nucleoside drugs such as gemcitabine and ribavirin are efficiently transported by ENT1 and ENT2. Several nucleoside antiretroviral drugs such as zidovudine, lamivudine, didanoside and zalcitabine have been shown to be substrates for CNT1-3 (25-27). It is hypothesized that transporters, likely ENTs or CNTs, may have a role in the placental disposition of D4T and DDC when coadministered. While combination therapy has been proven to reduce vertical transmission of HIV, drug-drug interactions may not allow maximum efficacy. Therefore it is necessary to understand the pharmacokinetics and placental disposition of HIV drugs alone and in combination (5, 10-13, 28).

Nucleoside reverse transcriptase inhibitors, stavudine (D4T) and zalcitabine have been shown to have high activity against HIV types 1 and 2 (29). Like all other nucleoside analogues D4T and DDC must undergo phosphorylation to their triphosphate forms for activation (29). Both D4T and DDC are well tolerated and have a simple regimen; therefore treatment with D4T and DDC is desirable. D4T and DDC have been shown to be synergistic or additive in combination (29). According to the FDA, stavudine and zalcitabine are classified as pregnancy category C drugs, thus animal studies have shown fetal risk and human studies are inconclusive (16, 30-32). However, they may be administered if benefits outweigh the risks.

There is limited understanding of the pharmacokinetics of D4T and DDC in combination, in pregnant women. It is difficult to study placental and fetal drug distribution in humans(12). This is due to the ethical exclusion of pregnant women in clinical trials coupled with difficulty in obtaining fetal concentration data (33). Therefore, it is necessary to find a viable animal model. Previous studies have shown that the pregnant rat model is ideal for estimating human placental transport of several nucleosides alone and in combination (12, 34-42). The anatomical similarities between rat and human placenta, both belonging to the same hemochorial type, make the pregnant rat model ideal for the investigation of human placental transfer (12, 35, 37, 39-42). The large litter size allows for serial sampling which enables the formation of a complete pharmacokinetic profile. This study examines the effects of DDC on placental transfer of D4T.

## MATERIALS AND METHODS

#### **Chemicals and Reagents**

Stavudine (D4T) and Zalcitabine were purchased from Sigma (St. Louis, MO, USA). Lamivudine (3TC) was obtained from Glaxo Smith Kline (RTP, NC, USA). HPLC-grade acetonitrile, methanol and sodium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium sulfate was obtained from J.T Baker Inc. (Philipsburg, NJ, USA). The deionized water used was produced using a Continental Deionized Water System (Natick, MA, USA).

#### Animal Study

The UGA Animal Use and Care Committee approved the use of animals. The rats were housed one animal per cage in the College of Pharmacy animal facility (AAALAC accredited). The environment was controlled (20-22° C, 14 h of light/day) with daily feedings of standard chow pellets and water ad libitum.

Timed pregnant female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing an average 340 g were anesthetized intramuscularly with ketamine:acepromazine:xylazine

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(50:3.3:3.4 mg/kg) and dosed intravenously via jugular canula with 25 mg/kg of D4T (n=4) and D4T/DDC (n=5) dissolved in 0.1 N NaOH in physiological saline (pH 7.4) on the 19th day of gestation. Venous cannulation was performed for dosing and blood sampling. A laparotomy is done for access to the amniotic fluid, placenta and fetus. Blood samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min into heparinized tubes. Amniotic fluid, fetuses and placentas were collected concurrently with maternal blood sampling. All samples were stored on ice until processed. Plasma was collected after centrifuging blood samples at 10,000 rpm for 10 min using a Micro-centrifuge Model 235V (Fisher Scientific, USA). Fetal and placental tissues were homogenized in two volumes of deionized water (wt/vol) using a Ultra-Turbax T8 (IKA Labortechnik, Germany). All samples were stored at -20° C until analysis. Prior to analysis, samples were spiked with the internal standard (3TC) solution to yield a concentration of 5  $\mu$ g/ml.

### HPLC Analysis

D4T concentrations were determined by an HPLC-UV method developed previously. Plasma and amniotic fluid samples were prepared using 15  $\mu$ l of 2 M perchloric acid. Fetal and placental samples were processed using a salting out technique using 180  $\mu$ l saturated ammonium sulfate solution and 360  $\mu$ l of cold acetonitrile. The calibration curves of plasma and amniotic fluid were in the range of 0.1 – 50  $\mu$ g/ml and 0.2 – 50  $\mu$ g/ml for placental and fetal homogenates. The internal standard concentration was 5  $\mu$ g/ml (3TC) for all samples. An Agilent 1100 HPLC (Santa Clara, CA) equipped with a variable wavelength UV detector, quaternary pump, degasser and autosampler performed chromatographic studies. An YMC phenyl column (5  $\mu$ m, 150mm×2mm i.d., Waters, Milford, MA, USA) equipped with a Phenomenex C18 guard column (Torrance, CA, USA) was used to achieve all the

chromatographic separations. The mobile phase consisted of 5% methanol in 20mM dibasic sodium phosphate (pH 6), pH was adjusted using phosphoric acid and NaOH concentrated solutions. The flow rate was 0.2 ml/min and the detection wavelength was 256 nm. The injection volume of plasma and amniotic fluid samples was 20 µl, while the injection volume of placental and fetal samples was 15 µl. HPLC run time for each sample was 20 min.

D4T and DDC concentrations were determined by a previously HPLC-UV developed method (42). All samples were processed using a salting out technique using 200  $\mu$ l saturated ammonium sulfate solution and 1  $\mu$ l of cold acetonitrile. The calibration curves of plasma, amniotic fluid, placental and fetal homogenates were in the range of 0.1 – 50  $\mu$ g/ml. The internal standard concentration was 1  $\mu$ g/ml (3TC) for all samples. An Agilent 1100 HPLC (Santa Clara, CA) equipped with a variable wavelength UV detector, quaternary pump, degasser and autosampler performed chromatographic analyses. A Spherisorb S3W silica column (4.6×3.0mm i.d., Waters, Milford, MA, USA) equipped with a Phenomenex C18 guard column (Torrance, CA, USA) was used to achieve chromatographic separation. The mobile phase consisted of 3% acetonitrile in 22mM formic acid. The flow rate was 0.5 ml/min and the detection wavelength was 265 nm. The injection volume of samples was 40  $\mu$ l and the HPLC run time for each sample was 22 min.

#### **Data Analysis**

WinNonlin (Pharsight, Mountain View, CA, USA) was used for compartmental analysis of plasma data. Plasma data from D4T monotherapy and D4T-DDC combination therapy rats, was fitted using a two-compartment model with first-order elimination. A 1/ŷ-weighting scheme was used throughout the analysis. Noncompartmental analysis was used for amniotic fluid,

placenta, and fetus data. In order to determine the relative exposure of each matrix to D4T and DDC, the  $AUC_{(0-t)}$  values for each tissue were compared to the  $AUC_{(0-t)}$  value for the maternal plasma data. Truncated area under concentration-time profile is used due to the inability to calculate accurate tissue half-lives of D4T.

To determine the distribution of D4T alone and in combination with DDC in maternal plasma ( $C_c$ ), maternal tissue ( $C_t$ ), placental ( $C_p$ ), fetal ( $C_f$ ) and amniotic fluid ( $C_a$ ) compartments three five-compartmental models were evaluated. Based on goodness of fit criteria, the five-compartment model that best fit D4T data is shown in Figure 4.2. The five-compartment model estimated bidirectional transfer between maternal plasma and placenta, placenta and fetus, placenta and amniotic fluid, and maternal plasma and tissue compartment. Differential equations were simultaneously fitted to the data. The equations for the model are presented here (Equations 1-5).

$$\frac{dC_c}{dt} = \left(\frac{k_{pc}V_p}{V_b}\right)C_p + \left(\frac{k_{tc}V_t}{V_b}\right)C_t - (k_{cp} + k_{ct} + k_{c0})C_c$$
(1)

$$\frac{dC_p}{dt} = \left(\frac{k_{cp}V_b}{V_p}\right)C_c + \left(\frac{k_{fp}V_f}{V_p}\right)C_f + \left(\frac{k_{ap}V_a}{V_p}\right) - (k_{pc} + k_{pf} + k_{pa})C_p$$
(2)

$$\frac{dC_f}{dt} = \left(\frac{k_{pf}V_p}{V_f}\right)C_p - k_{fp}C_f$$
(3)

$$\frac{dC_a}{dt} = \left(\frac{k_{pa}V_p}{V_{ba}}\right)C_p - k_{ap}C_a \tag{4}$$

$$\frac{dC_t}{dt} = \left(\frac{k_{ct}V_b}{V_t}\right)C_c - k_{tc}C_t$$
(5)

A fixed four-compartment model (Figure 4.2) was used to estimate bidirectional transfer between maternal plasma and placenta, placenta and fetus, and placenta and amniotic fluid. The equations for the model are presented here (Equations 6-8).

$$\frac{dC_p}{dt} = \left(\frac{k_{cp}V_b}{V_p}\right)C_c + \left(\frac{k_{fp}V_f}{V_p}\right)C_f + \left(\frac{k_{ap}V_a}{V_p}\right) - (k_{pc} + k_{pf} + k_{pa})C_p$$
(6)

$$\frac{dC_f}{dt} = \left(\frac{k_{pf}V_p}{V_f}\right)C_p - k_{fp}C_f \tag{7}$$

$$\frac{dC_a}{dt} = \left(\frac{k_{pa}V_p}{V_{ba}}\right)C_p - k_{ap}C_a \tag{8}$$

Where, plasma concentration ( $C_c$ ) is fixed to the placenta. After 2-compartment analysis of the maternal plasma, the parameters A, B,  $\alpha$  and  $\beta$  are used in the biexponential equation for plasma concentration (Equation 9).

$$Cc = A * e^{-\alpha_{*} * t} + B * e^{-\beta^{*} t}$$
(9)

WinNonlin generated parameter estimates and intercompartmental clearance values (CL) were calculated from the product of the intercompartmental rate constants and tissue volumes. The pharmacokinetic parameters generated for each dosing group and the relative exposure numbers were compared by using an unpaired *t* test (P < 0.05) to detect statistically significant differences. To determine which model best fit the D4T data the goodness of fit tests for model selection Akaike Information and Schwarz Bayesian Criterion (AIC and SBC), Condition Number and residual sum of squares were used.

#### **RESULTS**

Figure 4.3 shows the mean plasma concentration vs. time profile and fitted curves for D4T dosed alone (n=4) and in combination with DDC (n=4). D4T maternal plasma

concentration declines in a biexponential fashion after IV dosing alone and in combination with DDC, with an average terminal half-life of  $2.5 \pm 0.9$  and  $2.0 \pm 0.8$  hr, respectively. Maternal plasma was analyzed via a two-compartmental model. Table 4.1 presents the plasma pharmacokinetic parameters for D4T. While not significant, there was a decrease in half-life and steady state volume of distribution (Vss) of D4T in combination with DDC as compared to D4T alone. However, there is a significant decrease in total clearance and of D4T when given in combination with DDC, suggesting competition in renal elimination. D4T exhibits extensive distribution due to relatively high Vss values. Maternal peak plasma concentrations were significantly higher for D4T in combination with DDC. While not statistically significant, there was also an increase in the AUC for D4T when given with DDC.

The mean concentration versus time profiles in placenta, amniotic fluid and fetus are shown in Figure 4.4. Pharmacokinetic parameters determined by noncompartmental analysis of the placenta, fetus and amniotic fluid data are shown in Table 4.2. The concentrations of D4T in maternal plasma and placenta decline in parallel. D4T exhibits rapid placental uptake in both situations; with the peak concentration occurring at 5 minutes. Uptake of D4T in the fetus and amniotic fluid was less rapid in relation to the placenta, which may suggest saturation of active/facilitative transport. The  $T_{max}$ , for amniotic fluid of D4T in combination with DDC is significantly slower than for D4T alone (3.4 vs. 1.0 hr). Under all dosing conditions, the amniotic fluid could serve as a depot for the drug. Recirculation due to fetal swallowing and micturition could explain the high concentration of D4T in the amniotic fluid (5, 14). Peak concentrations in the fetus and amniotic fluid were lower in rats dosed D4T and DDC in combination, with the difference only being significant in the fetus.

There is not a significant difference in the RE or AUC in the placenta (0.32 vs. 0.26) and (19.3 vs. 20.8 hr-mg/L), respectively. The AUC of D4T in amniotic fluid and fetus were lower in the presence of DDC. Therefore, relative exposure (RE) for D4T in combination with DDC in amniotic fluid (0.25 vs. 0.15) and fetus (0.24 vs. 0.1) were significantly lower than RE for D4T alone.

The maternal plasma, placenta, fetus, and amniotic fluid were also simultaneously fitted to a five-compartment model (Figure 4.2). As indicated earlier, three 5-compartmental models were tested for goodness of fit. One failed model incorporated bidirectional transfers between placenta and fetus, placenta and amniotic fluid and fetus and amniotic fluid. Another model did not include placental and amniotic fluid transfer. The model developed for D4T maternal-fetal transport incorporates bidirectional transfer between fetus and placenta as well as between amniotic fluid and placenta. The model-predicted D4T concentration versus time profiles in maternal plasma, placenta, fetus, and amniotic fluid closely to the observed data (Figure 4.5).

Intercompartmental clearances are presented in Table 4.3. D4T exhibits rapid transfer from maternal plasma to the placenta; consistent with the short  $T_{max}$ . The significant difference between  $CL_{cp}$  and  $CL_{pc}$  for D4T alone and in combination suggests that active transporters played a role in transplacental transfer of D4T by pumping D4T from placenta back into maternal plasma. In addition,  $CL_{cp}$  is significantly lowered in the presence of DDC indicating inhibition of transporters between maternal plasma and placenta. Clearance of D4T between maternal tissues and maternal central were significantly lowered in the presence of DDC.

Clearance between the tissue compartment and maternal plasma ( $CL_{tc}$ ) is significantly decreased in combination with DDC again suggesting transporter inhibition.  $CL_{fp}$  was higher than  $CL_{pf}$  for D4T in combination with DDC. While not significant these findings suggest

upregulation of transporters which help pump D4T back to the placenta from the fetus when dosed in combination with DDC. Intercompartmental clearances,  $CL_{ap}$  and  $CL_{pa}$ , suggests facilitative diffusion between the placenta and amniotic fluid, for D4T alone and with DDC.  $CL_{pa}$  and  $Cl_{ap}$  for D4T were significantly lower in the presence of DDC, suggesting inhibition of transport proteins between the placenta and amniotic fluid.

The four-compartment model-predicted D4T concentration versus time profiles in maternal plasma, placenta, fetus, and amniotic fluid closely to the observed data (Figure 4.6). Intercompartmental clearances are presented in Table 4.4. D4T exhibits rapid transfer from maternal plasma to the placenta; consistent with the short  $T_{max}$ . The difference between  $CL_{cp}$  and  $CL_{pc}$  for D4T alone and in combination suggests that active transporters played a role in transplacental transfer of D4T by pumping D4T from placenta back into the maternal plasma.

For the fixed model, significant differences existed between  $CL_{pf}$  and  $CL_{fp}$  for D4T in combination with DDC. However, there was no significant difference between  $CL_{pf}$  and  $CL_{fp}$  for D4T monotherapy. These findings suggest upregulation of transporters which help pump D4T back to the placenta from the fetus when dosed in combination with DDC.  $CL_{pa}$  and  $Cl_{ap}$  for D4T were significantly lower in the presence of DDC, suggesting inhibition of transporters between the placenta and the amniotic fluid.

When compared to the five-compartmental model, the intercompartmental clearances produced by the fixed model were not significantly different. The bidirectional clearance between the placenta and fetus in rats dosed with D4T alone was the only significantly different parameter, with  $CL_{pf}$  and  $Cl_{fp}$  significantly higher in the fixed model. The goodness of fit criterion AIC (59.0 ± 13.5 vs. 122 ± 20.4) and SBC (67.3 ± 14.1 vs. 137.6 ± 20.6) were significantly lower for D4T only rats. AIC (74.05 ± 21.6 vs. 182 ± 49.6) and SBC (82.3 ± 22.3)

vs.  $182 \pm 49.6$ ) were also significantly lower in D4T/DDC combination rats. Based on the superior goodness of fit criterion, the fixed model was better for explaining the D4T data as shown on Table 4.4.

#### DISCUSSION

In this study, the effects of combination therapy (D4T-DDC) on the placental disposition of D4T were examined. Several groups have studied potential interactions in combination therapy, with conflicting results. Odenics et al. investigated the interaction between various antiviral drugs, confirming that passive diffusion is the primary mechanism of transfer(43). When studying the effects of combination therapy in pregnant pigtailed macque, the group reported that AZT had no effect on the placental transfer of d4T. Likewise, the group did not find interaction between AZT and ddI when simultaneously administered to pregnant pigtailed macque. Yet, there was a significant increase in the ratio of AZT concentration in amniotic fluid to that in the fetus, when compared to that of AZT monotherapy (44-46). The group hypothesized that the presence of ddI may hinder fetal reabsorption of AZT from the amniotic fluid; leading to accumulation of AZT and higher concentration ratio.

Brown *et al.* reported considerable alteration in the disposition of ACV and AZT when the two drugs were coadministered in pregnant rats. Concentration of AZT in all tissues decreases in the presence of ACV; while, combination therapy showed a three-fold increase of ACV exposure in amniotic fluid and fetal tissue with the combination therapy (12). Lewis et al. reported that 3TC altered pharmacokinetics of AZT in pregnant rats, decreasing the half-life by 20%. Additionally, relative exposure of AZT in fetus and amniotic fluid decreased in the presence of 3TC, while the relative exposure of 3TC increased in the placenta and fetus in combination therapy, suggesting that transporters play a complex role in the disposition of these compounds (23). Xu *et al.* identified that D4T alters the pharmacokinetics of DDC when given in combination (24).

Maternal plasma pharmacokinetics of D4T is altered in the presence of DDC. There was a decrease in half-life, steady state volume of distribution (Vss) and total clearance of D4T when given in combination with DDC. The decrease in clearance suggests that elimination is saturated in the presence of DDC. Maternal peak plasma concentrations were significantly higher for D4T in combination with DDC. Peak concentration in the placenta occurs at 5 minutes. Peak concentrations in the fetus and amniotic fluid were lower in rats dosed with D4T and DDC in combination, with the difference only being significant in the fetus. Relative exposure (RE) for D4T in combination with DDC in amniotic fluid and fetus were significantly lower than RE for D4T alone. There is a 3-fold increase in the  $T_{max}$  in the amniotic fluid for D4T in combination with DDC when compared to D4T alone.

The presence of active transporters can be assumed when there is a significant difference between two intercompartmental clearances. Differences are noted between  $CL_{cp}$  and  $CL_{pc}$  for D4T alone and in combination with DDC. These differences were present for both the 5compartment model and the fixed 4-compartment model, suggesting that active transport plays a role in the transplacental transfer of D4T. For the 5-compartment model, the difference between  $CL_{pf}$  and  $CL_{pa}$  and  $CL_{ap}$  were not significant for D4T dosed alone or in combination, suggesting passive diffusion. However, for the fixed model,  $CL_{pf}$  and  $CL_{fp}$  are significantly different in combination with DDC, suggesting upregulation of transporters.

Many classes of transporters have been proven to be expressed in the placenta. Nucleoside antiviral agents have been shown to be substrates of several placental transporters(25-27). Efflux transporters such as the multidrug resistance proteins (MRPs) and

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the breast cancer resistance protein (BCRP) function to move xenobiotics from the placenta to maternal circulation. The bidirectional transporters, Na<sup>+</sup>-independent equilibrative nucleoside transporters (ENT1 and ENT2) and Na<sup>+</sup>-dependent concentrative nucleoside transporters (CNT1, CNT2 and CNT3), organic anion transporter (OAT4), organic anion transporting polypeptides (OATPs) and organic cation transporters (OCT2, OCT3 and OCTN2) have been identified in human and rat placenta. Nucleoside drugs such as gemcitabine and ribavirin are efficiently transported by ENT1 and ENT2. Several nucleoside antiretroviral drugs such as zidovudine, lamivudine, didanoside and zalcitabine have been shown to be substrates for CNT1-3 (25-27). It is hypothesized that transporters, likely ENTs or CNTs, may have a role in the placental disposition of D4T and DDC when coadministered.

The disposition of D4T is significantly altered in the presence of DDC. The fetal uptake of D4T was significantly lowered in the presence of DDC. Intercompartmental clearances between the placenta and amniotic fluid were significantly decreased in the presence of DDC. These results were consistent with our findings with the two-compartment and noncompartmental modeling, which showed a significant difference in the relative exposure in amniotic fluid and fetus for D4T/DDC combination dosing. This suggests that transporters and passive diffusion play a role in the tissue disposition of these drugs. As aforementioned, human and rat placentas have several bidirectional transporters; including ENTs and CNTs. Zalcitabine, among other NRTIs, has been shown to be substrates for CNT1-3 (25-27). However, the identity, function and location of these transporters are unknown. Therefore, cell culture studies or other *in vitro* methods are necessary to identify which transporters, their location and their function in the placental uptake of these nucleoside analogs. After comparing a five-compartment model and a four-compartment model fixed to plasma concentrations, the overall difference in

intercompartmental clearance was not significant. Yet, the fixed model provides a better fit based on the goodness of fit criteria (AIC, SBC, WRSS and Condition #).

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46. Pereira CM, Nosbisch C, Baughman WL, Unadkat JD. 1995. Antimicrob Agents Chemother 39: 343-5 Table 4.1. Maternal Plasma Pharmacokinetic parameters generated from compartmental analysis of plasma data collected from D4T and D4T - DDC in pregnant rats (25 mg/kg).

Parameter	D4T	D4T - DDC
Half-life (hr)	$2.5\pm0.9$	$2.0\pm0.8$
AUC (hr-mg/L)	59.1 ± 8.6	86.1 ± 22.3
CL <sub>T</sub> (L/hr-kg)	$0.43\pm0.06$	$0.30 \pm 0.07^{*}$
V <sub>ss</sub> (L/kg)	$1.32\pm0.3$	$0.82\pm0.3$
C <sub>max</sub> (mg/L)	$49.05\pm10.6$	$70.9\pm4.3^*$

\* Indicates significant difference between D4T dosed alone and in combination with DDC (P<0.05)

	Place	nta	F	etus	Amnio	tic Fluid
rarameter	D4T	D4T - DDC	$\mathbf{D4T}$	D4T - DDC	D4T	D4T - DDC
Half-life (hr)	$4.31 \pm 0.8$	$4.61 \pm 2.3$	$6.95\pm0.9$	$3.66\pm0.9^*$	$8.35\pm1.0$	$5.53\pm1.6^*$
$AUC^{a}$	$19.3 \pm 2.8$	$20.8 \pm 4.7$	$14.1 \pm 2.9$	$8.11\pm1.0^*$	$14.5 \pm 1.1$	$12.6 \pm 2.0$
Cmax <sup>b</sup>	$7.73 \pm 1.4$	$12.1 \pm 3.4$	$4.21\pm0.9$	$2.94\pm0.3^*$	$4.04 \pm 1.0$	$3.72 \pm 0.8$
Tmax (hr)	$0.08\pm0.0$	$0.08 \pm 0.0$	$0.94\pm0.4$	$0.53 \pm 0.2$	$1.00 \pm 1.2$	$3.37 \pm 1.5^{*}$
Relative Exposure <sup>c</sup>	$0.33\pm0.05$	$0.26\pm0.09$	$0.24\pm0.04$	$0.10\pm0.02^*$	$0.25 \pm .04$	$0.15\pm0.05^*$

Exposures (mean  $\pm$  standard deviation) for D4T and D4T - DDC in pregnant rats (25 mg/kg). Table 4.2. Placental, Fetal and Amniotic Fluid Pharmacokinetic Parameters and Relative

<sup>a</sup> Expressed as hr·mg/kg for placenta and fetus and as hr·µg/mL for amniotic fluid

<sup>b</sup> Expressed as  $\mu g/g$  for placenta and fetus and as  $\mu g/mL$  for amniotic fluid <sup>c</sup> Relative Exposure = AUCTissue/AUCMaternal Plasma \* Indicates significant difference between D4T dosed alone and in combination with DDC (P<0.05)

Table 4.3. Intercompartmental Clearance estimates (mean  $\pm$  standard deviation) for D4T alone and in combination with DDC in pregnant rats (25 mg/kg).

Parameter	D4T	D4T-DDC
$CL_{cp}$	$1.37\pm0.38$	$0.76\pm0.15$
$CL_{pc}$	$2.84 \pm 1.06$	$2.51\pm0.77$
$CL_{pf}$	$2.68\pm0.85$	$2.01 \pm 1.79$
$CL_{\mathrm{fp}}$	$3.13 \pm 0.85$	$3.74 \pm 2.9$
$CL_{pa}$	$0.15 \pm 0.03$	$0.05 \pm 0.02*$
$CL_{ap}$	$0.16 \pm 0.03$	$0.03 \pm 0.01*$
CL <sub>ct</sub>	$1.05 \pm 1.14$	$0.25\pm0.20$
CL <sub>tc</sub>	$3.92 \pm 1.66$	$0.50 \pm 0.85^{*}$
$CL_T$	$0.27 \pm 0.10$	$0.12 \pm 0.15$

\* Indicates significant difference between D4T dosed alone and in combination with DDC (P<0.05)

Table 4.4. Intercompartmental Clearance estimates (mean  $\pm$  standard deviation) for D4T alone and in combination with DDC in pregnant rats (25 mg/kg) Fixed Model.

Parameter	D4T	D4T-DDC
$CL_{cp}$	$2.04\pm0.59$	$1.38 \pm 0.75$
$CL_{pc}$	$4.61 \pm 1.75$	$4.19 \pm 2.49$
$\mathrm{CL}_{\mathrm{pf}}$	$5.11 \pm 1.70^{\#}$	$2.97\pm0.97$
$\mathrm{CL}_{\mathrm{fp}}$	$6.23 \pm 1.46^{\#}$	$6.39 \pm 1.39$
CL <sub>pa</sub>	$0.15\pm0.03$	$0.04 \pm 0.01^{*}$
CL <sub>ap</sub>	$0.17\pm0.04$	$0.05 \pm 0.02^{*}$
AIC	$59.0 \pm 13.5^{a}$	$74.0 \pm 21.6^{a}$
SBC	$67.3 \pm 14.0^{a}$	$82.3 \pm 22.3^{a}$
WRSS	$5.1 \pm 1.6^{a}$	$8.9\pm4.4^{a}$
Condition #	$112.5 \pm 43.4$	431.6 ± 345.6

\* Indicates significant difference between D4T dosed alone and in combination with DDC (P < 0.05)

<sup>#</sup> Indicates significant difference between 5-Compartment and 4-Compartment model (P<0.05)

<sup>a</sup>Indicates significant improvement from 5-Compartment model (P<0.05)



Figure 4.1: Chemical Structure of Stavudine and Zalcitabine



Figure 4.2: Schematic representation of the (a) five-compartment and (b) fixed pharmacokinetic model used to describe the disposition of D4T alone and in combination with DDC in placental, fetal, and amniotic fluid compartments after IV injection to pregnant rats.



Figure 4.3: Plasma Concentration versus time profile (a) for D4T alone and in combination with DDC; Fitted concentration versus time profiles of (b) D4T alone and (c) in combination with DDC



Figure 4.4: Concentration (mean plus standard deviation) versus time profiles of D4T alone and in combination with DDC from (a) amniotic fluid, (b) fetus and (c) placenta



Figure 4.5: Measured (a) D4T and (b) D4T - DDC concentration versus time data for maternal plasma, placenta, fetal tissue, and amniotic fluid fitted simultaneously to the five compartment model.




a)



Figure 4.6: Measured (a) D4T and (b) D4T - DDC concentration versus time data for maternal plasma, placenta, fetal tissue, and amniotic fluid fitted simultaneously to a fixed four compartment model.

# CHAPTER 5

# BIOAVAILABILITY OF A NOVEL INTEGRASE INHIBITOR IN $\operatorname{RATS}^1$

<sup>&</sup>lt;sup>1</sup> Blue, Shawn K., Nair V., and White, C. A. To be submitted to *Antimicrobial Agents and Chemotherapy* 

# ABSTRACT

The β-diketo acid integrase inhibitor 4-(1,3-dibenzyl-1,2,3,4-tetrahydro-2,4novel dioxopyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid (DA inhibitor) has been shown to have potent activity against human immunodeficiency virus (HIV-1). An HPLC method has been developed and validated for determination of DA inhibitor in male Sprague-Dawley rat plasma. Liquid-liquid extraction enhanced by salting out with saturated ammonium sulfate was used for extraction of DA inhibitor and the internal standard 4-(1-dibenzyl-1,2,3,4-tetrahydro-2,4dioxopyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid from plasma samples. Reverse phase chromatography was performed with a Nova-Pak C18 analytical column (3.9 x 300 mm, 5 µ particle size) equipped with a Phenomenex C18 guard column. A flow rate of 1 ml/min and detection wavelength of 341 nm was used. The mobile phase consisted of acetonitrile and water under a gradient (10-90% ACN). The method was validated over the range of 0.1-50  $\mu$ m/ml for plasma. The absolute recovery of DA inhibitor ranged from 64 to 79%. The internal standard recovery was above 80%. Acceptable intra- and inter-day assay precision (< 15% R.S.D.) and accuracy (< 15% error) were observed over 0.1-50 µg/ml in rat plasma. The pharmacokinetics and bioavailability of DA inhibitor was studied in male Sprague-Dawley rats following IV and oral administration. Rats were used in an intravenous/oral crossover study to determine oral bioavailability. DA inhibitor was administered IV bolus at doses of 5, 10 and 12.5 mg/kg to male Sprague-Dawley rats via a jugular cannula. DA inhibitor was administered orally at doses of 5, 10 and 12.5 mg/kg to male Sprague-Dawley rats via oral gavage. After a 36-hour washout period, rats received the same dose via the opposite administration (i.e. Day 1 oral/Day 2 iv). Plasma samples were collected over a period of eight hours post-dose. Concentrations in each matrix were determined by the developed HPLC method. All pharmacokinetic parameters were determined using WINNONLIN. A one compartment model with first order elimination was used to fit plasma data. Oral bioavailability for DA inhibitor for 5, 10 and 12.5 mg/kg dosing were 55.3%, 51.1% and 41.3%, respectively. AUC and  $C_{max}$  were significantly altered by dose escalation and oral administration.

Keywords: Integrase Inhibitor, Bioavailability, Pharmacokinetics

# INTRODUCTION

There have been many advances in the treatment of HIV. Currently, there are over 20 FDA-approved drugs available for HIV treatment. With the exception of two fusion inhibitors (Fuzeon and Selzentry) and the integrase inhibitor, raltegravir, all approved drugs inhibit reverse transcriptase or protease [1-5]. Highly Active Antiviral Treatment (HAART) has altered the course and prognosis of Acquired Immunodeficiency Syndrome [6-7]. Robust treatment can lead to near normal T-cell counts and undetectable viral counts [3-4, 7-9]. However, resistant viruses and toxicity are limiting factors in therapy consisting of reverse transcriptase and protease inhibitors, which may lead to suboptimal clinical outcomes. In addition, while inhibition of reverse transcriptase and protease can suppress viral replication, it is not eradicated. Therefore, it is logical to study other antiviral targets. HIV-Integrase has been identified as a potential target, due to its vital role in viral replication [3-4, 7-15].

Along with protease and reverse transcriptase, the retroviral enzyme integrase is responsible for replication of human immunodeficiency virus (HIV-1). Subsequent to reverse transcription viral DNA is incorporated into the host DNA, thus serving as a fundamental step in the viral lifecycle. HIV integrase catalyses the insertion of the reverse-transcribed viral genome into the host DNA in a multistep process [4, 7-8, 11-13, 16-17]. After assembling onto viral DNA, HIV integrase cleaves the two terminal nucleotides from each 30-end. Following nuclear entry, the viral 30-ends are ligated into the host genome (strand transfer). In the cytosol, intergrase trims the 3' ends of both strands of the viral-DNA (vDNA) immediately downstream of a conserved CA dinucleotide, leaving a 2-base overhang at each 5'- end. HIV genome integration yields a provirus that can go on to produce new virus or lie dormant in a latent state. The integration process is necessary for the production of progeny viruses, and mutations that

inactivate the integrase enzyme have deleterious effect on the capability of HIV to replicate. Inhibition of HIV integrase results in arrest of HIV life cycle, and represents therefore an attractive target for the treatment of HIV infection [4, 8, 12, 18-19].

The discovery of clinically relevant inhibitors of HIV integrase for antiviral therapy has proven to be a challenging task. Several groups have discovered compounds that exhibit integrase inhibition capabilities, however early assay conditions and minimal availability of the integrase-inhibitor pharmacophore made it difficult [1-2, 4-5, 7-9, 17, 19-30]. While various structurally diverse compounds have exhibited inhibition of integrase, a small group of  $\beta$ -diketo acids represent the most convincing, biologically validated inhibitors of integrase. Many of these compounds selectively inhibit the strand transfer reaction of the enzyme. Nair *et al.* discovered new  $\beta$ -diketo acids with purine nucleobase scaffolds that are potent inhibitors of both the strand transfer and 3'-processing steps of HIV-1 integrase [12, 17-18]. Subsequent to activity studies, our group embarked on preclinical pharmacokinetic animal studies of the novel integrase inhibitor developed by Nair *et al.* 

Animal studies are integral to the development of investigational drugs, pharmacokinetics being central [31-33]. However, in order to study the pharmacokinetics of any drug, an analytical method must first be developed. This paper will discuss the development and validation of a precise and accurate method to quantify 4-(1,3-dibenzyl-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid (DA inhibitor) in rat plasma. The purpose of the current study is to examine the pharmacokinetics of the novel integrase inhibitor, referred to as DA inhibitor, comparing oral and intravenous administration.

# **EXPERIMENTAL**

# **Chemicals and Reagents**

DA inhibitor and 4-(1-dibenzyl-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid were synthesized and provided by the Center for Drug Discovery, The University of Georgia (Athens, GA, USA). Acetonitrile, acetic acid and dimethyl sulfoxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals and solvents were ACS analytical or HPLC grade.

# **Chromatographic System**

Chromatographic studies were performed on an Agilent 1100 HPLC (Palo Alto, CA) equipped with a variable wavelength UV detector, quaternary pump, degasser and autosampler. A Waters Nova-Pak C18 analytical column ( $3.9 \times 300 \text{ mm}$ ,  $5 \mu$  particle size) (Milford, MA, USA) equipped with a Phenomenex C18 guard column (Torrance, CA, USA) was used to achieve chromatographic separation.

# **Chromatographic Conditions**

The mobile phase consisted of acetonitrile and water using a linear gradient (10-90% ACN) over 20 min. The flow rate was 1 ml/min and the detection wavelength was 341 nm. The injection volume was 20 µl. HPLC run time for each sample was 20 min.

# **Preparation of Stock and Standard Solutions**

Stock solutions (1 mg/ml) of DA inhibitor and the internal standard were individually prepared in dimethyl sulfoxide. DA inhibitor standard solutions with concentrations of 500, 400, 250, 100, 10, 7.5, 5 and 1  $\mu$ g/ml were prepared from the 1 mg/ml stock solution by serial dilution with dimethlyl sulfoxide. Internal standard solution at the concentration 50  $\mu$ g/ml was prepared by dilution from the 1 mg/ml stock solution with dimethyl sulfoxide. Precision and accuracy

standards of 400, 50, 5 and 1  $\mu$ g/ml were prepared in the same manner. Stock solutions (1 mg/ml) were kept refrigerated when not in use. Fresh standard solutions were prepared each day of analysis and validation.

# **Calibration Curves**

Blank plasma was obtained from Harlan (Indianapolis, IN, USA). Plasma calibration points were prepared by spiking 100  $\mu$ l of the plasma with 10  $\mu$ l of each DA inhibitor standard solution and 10  $\mu$ l of the 50  $\mu$ g/ml internal standard solution. The calibration curve of plasma was in the range of 0.1–50  $\mu$ g/ml with individual calibration points of 50, 25, 10, 1, 0.75, 0.2 and 0.1  $\mu$ g/ml while the internal standard concentration was 5  $\mu$ g/ml for all samples. Subsequent to being spiked each sample was subject to further sample preparation before analysis.

# **Precision and Accuracy**

This method was validated using four QC points for the calibration curve. To determine the intra-day accuracy and precision five replicates of each QC point were analyzed each day. Inter-day accuracy and precision were determined by repeating this process 3 times over 3 days. The QC points for plasma were 0.1, 0.5, 5 and 40  $\mu$ g/ml.

# **Sample Preparation**

DA inhibitor extraction from plasma was performed via salting out. A saturated ammonium sulfate solution (180 $\mu$ l) and 180 $\mu$ l of cold acetonitrile were added to 100  $\mu$ l samples, vortexed for 20 sec. and centrifuged at 10,000 rpm for 10 min. The upper organic layer was aspirated and dried under vacuum. Samples were then reconstituted in 100  $\mu$ l of dimethyl sulfoxide, prior to analysis.

# **Animal Study**

The UGA Animal Use and Care Committee approved the use of animals for this study. Groups of male Sprague-Dawley rats (~280mg) were dually cannulated (venous and arterial) prior to dosing. Rats were randomized into a crossover study. There was a 48-hour washout period between the two doses. The venous cannula was used for bolus injection and the arterial cannula was used for withdrawal of blood samples. Following bolus injection, the cannula was washed with a heparinized saline solution to avoid the possibility of clotting. Orally dosed rats received an equivalent dose at the same time via oral gavage. After the washout period, rats received the same dose via the opposite administration. The rats were housed one animal per cage in the College of Pharmacy animal facility (AAALAC accredited). The environment was controlled (20-22° C, 14 h of light/day) with daily feedings of standard chow pellets and water ad libitum.

Rats were dosed with exact volumes of DA inhibitor /DMSO solution doses of 5, 7.5 and 10 mg/kg as intravenous injections or as oral gavages. Blood samples (0.3 mL) were drawn at 0, 5, 15, 30, 45, 60, 90, 120, 240, 300, 360, and 480 min post- dose and collected in heparinized tubes. They were centrifuged at 10000 g for 10 min and plasma was harvested and kept at -20° until analyzed.

# **Data Analysis**

Compartmental analysis using WinNonlin (Pharsight, Mountain View, CA, USA) was used to determine pharmacokinetic parameters for both administration routes. DA inhibitor exhibits 2-compartmental model kinetics after intravenous dosing. However, a one compartmental model was adequate for analysis of concentration-time data after oral dosing. A  $1/\hat{y}^2$ -weighting scheme was used throughout the analysis. Oral bioavailability was determined by comparing the AUC<sub>po</sub> and AUC<sub>iv</sub>:

$$F = \frac{AUC_{po}}{AUC_{iv}}$$

where, F = bioavailability,  $AUC_{po} = area$  under the concentration-time curve after oral administration and  $AUC_{iv} = area$  under the concentration-time curve after intravenous administration. Clearance and Volume of distribution for oral dosing were adjusted to account for bioavailability. The pharmacokinetic parameters generated for each dosing group were compared using one-way ANOVA with Tukey's multiple comparison tests to identify statistically significant differences (P<0.05).

#### RESULTS

# **Analytical Method**

The chemical structures of DA inhibitor and internal standard are represented in Figure 5.1. In order to achieve the best separation from endogenous peaks, several mobile phases were investigated. Buffers at varying organic content and pH were tested; however these did not provide adequate separation. Successive exploration revealed satisfactory resolution of DA inhibitor and compound B from endogenous peaks was obtained with ACN/H<sub>2</sub>O under a gradient (10-90% ACN). Under this gradient DA inhibitor and internal standard eluted at 8 and 7.5 min, respectively, which provided ideal separation from endogenous peaks. The wavelength which gave the best response for DA inhibitor was 341 nm. Chromatograms of blank plasma and spiked plasma with DA inhibitor (0.1  $\mu$ g/ml) and internal standard (5  $\mu$ g/ml) are shown in Figure 5.2.

Linear regression equations were generated with Microsoft Excel software using a 1/xweighting scheme for each day of validation and analysis. The calibration curves showed acceptable linearity ( $R^2 > 0.99$ ) over the range 0.1-50 mg/ml for plasma.

The extraction efficiencies for DA Inhibitor and internal standard from plasma are expressed in terms of absolute recovery. Samples were spiked with 40, 5, 0.5 and 0.1 µg/ml solutions of DA inhibitor and 5 µg/ml solutions for internal standard. The absolute recoveries were calculated by comparing the peak areas of spiked plasma samples to the corresponding peak areas of the untreated stock solutions. Absolute recoveries of DA inhibitor (n = 15) ranged from 64 to 79%. Recovery of the internal standard, internal standard, was above 80%. The recoveries are shown in Table 5.1

Calculation of accuracy and precision was performed over 3 days. Precision, as expressed by % R.S.D., and accuracy as expressed by % error for DA Inhibitor in plasma is illustrated in Table 5.2. Over three separate days, measurement of five samples at each QC point was used to determine intra-day (n = 5) precision and accuracy. The pooled data over three days was used to determine inter-day (n = 15) precision and accuracy. The calibration curve and each of the four QC points 40, 5, 0.5 and 0.1 µg/ml were used for these calculations. Intra-day precision (% R.S.D.) and accuracy (% error) of DA inhibitor ranged from 0.6 to 7.6 % and 2.3 to 11.3%, respectively. Inter-day precision and accuracy of DA inhibitor ranged from 0.85 to 3.4 % and 4.7 to 11.00%, respectively.

Stability testing was performed for DA Inhibitor and the internal standard at the concentration level of 0.5 and 5  $\mu$ g/ml, respectively. Spiked matrix samples (20 samples) went through three consecutive freeze/thaw cycles over the period of four days. Five samples were extracted and analyzed as described before. The remaining spiked matrix samples were stored at

-20°C. Each of the following three consecutive days, spiked matrix samples were thawed, and five more were extracted and analyzed. The day-to-day measured peak areas of DA Inhibitor and internal standard were compared and the results listed in Table 5.3. The % R.S.D. between the average peak areas of DA Inhibitor and internal standard each day was less than 15%. The stability of extracted matrix samples in the autosampler was also evaluated. At time 0, three samples of plasma were injected onto the HPLC column and analyzed. In another 24 hr, the same samples from plasma were injected again. The peak areas for DA Inhibitor and internal standard in each injection were compared. The % R.S.D. between samples was less than 15% for both compounds and no obvious decline in peak areas between each injection was observed.

# **Pharmacokinetics and Bioavailability**

Mean concentration-time profiles of DA inhibitor in rat plasma following intravenous and oral administration of 5, 10 and 12.5 mg/kg doses are shown in Figure 5.3. The plasma DA inhibitor concentrations declined in a biexponential fashion after IV administration, indicating a 2-compartmental model. The pharmacokinetic parameters generated via WINONLIN 2-compartmental analysis of plasma are shown in Table 5.4 after intravenous dosing. The plasma DA inhibitor concentrations declined in a monoexponential fashion after oral administration and exhibited shallow absorption, indicating a 1-compartmental model. DA Inhibitor is slowly absorbed from the gastrointestinal tract, with peak plasma concentrations being reached after 90 min. for all doses. Subsequent to reaching peak concentration, elimination of DA inhibitor acts similarly to IV dosing. The pharmacokinetic parameters generated via WINNONLIN 1-compartmental analysis of plasma is shown in Table 5.5 for oral dosing. Graphical comparisons of IV and oral dosing for half-life, clearance and AUC are shown in Figure 5.4. Dose proportionality was not shown in AUC or Cmax for DA Inhibitor for IV and oral dosing.

The volume of distribution decreased as the dose increased for IV dosing. The volume of distribution for all 3 doses indicates extensive distribution and tissue binding, which is consistent with the lipophilic nature of this compound. DA Inhibitor is characterized by low clearance which is consistent with prior preclinical studies of Diketo-Acid integrase inhibitors (26, 42). Clearance decreased as dose increased (164.8 to 97.4 ml/hr-kg), indicating saturation of drug elimination. Since this compound is extensively metabolized, saturation of hepatic enzymes is the most likely explanation for the observed decrease in clearance. There was no significant change in elimination half-life of DA Inhibitor with dose escalation after intravenous dosing, as expected with the coupled decreases in clearance and volume of distribution. For 5 mg/kg dosing,  $K_{12}$  is significantly higher than  $K_{21}$ , suggesting active transport from the central compartment into the peripheral compartment. However, for higher doses, compartmental transfer approaches equilibrium. This change may be due to saturation of cell uptake as dose increases and/or sequestering in tissues.

The oral pharmacokinetic parameters of DA Inhibitor are consistent with published preclinical pharmacokinetic studies of diketo integrase inhibitors [34-36]. After oral dosing, the elimination half-life increased with escalated dosing. Half-life was significantly longer in 10 and 12.5 mg/kg doses when compared to initial dose (5 mg/kg). Also, elimination half-life after oral dosing is longer than IV dosing. A trend for oral bioavailability and absorption rate constant to decrease as dose increased was observed. DA inhibitor exhibits moderate bioavailability (41-55%), which is consistent with previous preclinical pharmacokinetic studies of integrase inhibitors [34-36].

The decrease in bioavailability with increasing dose may result, in part, from saturation of influx transporters and/or solubility issues in the GI tract due to the hydrophobicity of the compound.

There was also an insignificant decrease in absorption rate constant with increased dosing.

# DISCUSSION

While various structurally diverse compounds have exhibited inhibition of integrase, a small group of  $\beta$ -diketo acids represent the most convincing, biologically validated inhibitors of integrase [4, 7, 23-25, 37-41]. Many of these compounds selectively inhibit the strand transfer reaction of the enzyme. Nair *et al.* discovered new  $\beta$ -diketo acids with purine nucleobase scaffolds that are potent inhibitors of both the strand transfer and 3'-processing steps of HIV-1 integrase [8, 12, 17-18, 21, 42-43]. In this study, the effects of dose escalation and oral dosing of DA Inhibitor are investigated. The pharmacokinetic parameters (slow clearance and moderate bioavailability) of DA Inhibitor in rodents were comparable to previous preclinical studies of integrase inhibitors [34-36]. AUC and C<sub>max</sub> increased linearly with dose escalation for IV and oral dosing. Clearance decreased with increased dosing, likely due to saturation of drug elimination. An increase in half-life with oral dosing was noticed in this study is; suggesting that absorption may still occur after T<sub>max</sub> is reached. Additionally, a trend for oral bioavailability and absorption rate constant to decrease as dose increased was observed.

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Table 5.1: Absolute recov	veries of DA inhibit	or and internal sta	undard from plasma	(n=15)
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Analyte	Concentration (µg/ml)	Recovery
	40	$75.5 \pm 9.7$
DA inhihitan	5	$79.4\pm7.8$
DA Innibitor	0.5	$78.9 \pm 12$
	0.1	$64.1 \pm 5.5$
Internal Standard	5	$80.1\pm9.9$

Table 5.2: Intra-day (n=5) and inter-day (n=15) precision (% R.S.D.) and accuracy (% error) measured for QC points for DA inhibitor from plasma. T.C. = Theoretical Concentration and E.C. = Experimental Concentration

	D	ay 1		Õ	ay 2		Ő	ay 3		Int	erday	
T.C.	E.C.	R.S.D	Error	E.C.	R.S.D	Error	E.C.	R.S.D	Error	E.C.	R.S.D	Error
40	$40.25 \pm 2.64$	0.625	6.56	$40.78 \pm 1.18$	1.94	2.89	$40.71 \pm 2.00$	1.79	4.91	$40.58\pm1.89$	1.45	4.68
5	$5.23\pm0.39$	4.645	7.39	$5.05\pm0.13$	1.02	2.53	$5.22\pm0.17$	4.47	3.28	$5.1 \pm 0.25$	3.38	4.87
0.5	$0.48\pm0.03$	4.18	5.77	$0.51\pm0.01$	1.376	2.34	$0.526\pm0.03$	5.35	5.65	$0.50\pm0.03$	0.85	6.02
0.1	$0.11 \pm 0.01$	9.02	8.5	$0.11 \pm 0.01$	6.59	6.77	$0.092 \pm 0.01$	7.55	11.27	$0.10 \pm 0.01$	2.69	11.00

Table 5.3: Results of freeze/thaw stability of DA inhibitor (0.5  $\mu$ g/ml) and internal standard (5  $\mu$ g/ml) plasma represented by area  $\pm$  S.D. (n=5) of each day and % R.S.D. of the area between days

DA inhibitor	Area
Day 1	$24.66 \pm 2.27$
Day 2	$27.01\pm3.29$
Day 3	$28.86 \pm 1.33$
Day 4	$25.78\pm2.16$
% R.S.D.	12.9
Internal Standard	Area
Internal Standard Day 1	<b>Area</b> 19.86 ± 0.47
Internal Standard Day 1 Day 2	<b>Area</b> 19.86 ± 0.47 19.67 ± 0.87
Internal Standard Day 1 Day 2 Day 3	Area 19.86 ± 0.47 19.67 ± 0.87 19.37 ± 1.13
Internal Standard Day 1 Day 2 Day 3 Day 4	Area $19.86 \pm 0.47$ $19.67 \pm 0.87$ $19.37 \pm 1.13$ $18.51 \pm 0.21$

Table 5.4: Pharmacokinetic parameter estimates for DA inhibitor at 5, 10 and 12.5 mg/kg IV bolus dosing (2-compartment model)

Parameter	5 mg/kg	10 mg/kg	12.5 mg/kg
Half-life (hr)	$14.5 \pm 5.1$	$5.9 \pm 0.1^{*}$	$9.1\pm0.7$
AUC (hr*ug/mL)	$31.4\pm6.0$	$79.2 \pm 6.1$	$128.3\pm3.0$
CL (mL/hr/kg)	$164.8 \pm 38.9$	$126.8\pm10.05$	$97.4 \pm 2.3^{*}$
C <sub>max</sub> (ug/mL)	$7.3 \pm 1.5$	$19.6 \pm 2.3$	$24.0 \pm 1.3$
V <sub>d</sub> (mL/kg)	$708.9 \pm 144.5$	$515.6\pm 64.3$	$521.2 \pm 63.1$
$K_{12}(hr^{-1})$	$0.53\pm0.06$	$1.06\pm0.4^*$	$1.02\pm0.1$
$K_{21}(hr^{-1})$	$0.22 \pm 0.1$	$1.05 \pm 0.03^{*}$	$0.79 \pm 0.07^{*\#}$
Dist Half-life (hr)	$0.76 \pm 0.1$	$0.32 \pm 0.07^{*}$	$0.36 \pm 0.03^{*}$

<sup>\*</sup> Indicates significant difference from 5 mg dosing (P < 0.05) <sup>#</sup> Indicates significant difference from 10 mg dosing (P < 0.05)

Table 5.5: Pharmacokinetic parameter estimates for DA inhibitor at 5, 10 and 12.5 mg/kg oral dosing (1-compartment model)

Parameter	5 mg/kg	10 mg/kg	12.5 mg/kg
Half-Life (hr)	$3.3 \pm 0.1$	$4.33\pm0.2^{\ast a}$	$4.51 \pm 0.6^{*}$
AUC (hr*ug/mL)	$16.6 \pm 1.93^{a}$	$40.3 \pm 3.4$	$53.11 \pm 4.3$
CL (mL/hr/kg)	$165.0\pm38.8$	$126.9\pm7.04$	$97.4 \pm 1.2^{*}$
C <sub>max</sub> (ug/mL)	$3.0\pm0.29^{a}$	$5.6 \pm 0.21$	$7.2 \pm 1.1$
V <sub>d</sub> (mL/kg)	$788.45\pm220.9$	$791.24 \pm 42.1$	$635.1\pm 64.9$
F	$0.55 \pm 0.2$	$0.51\pm0.04$	$0.41\pm0.01$
$K_a (hr^{-1})$	$4.5\pm0.74$	$3.86\pm0.53$	$3.65\pm0.12$
Absorption Half-Life (hr)	$0.16\pm0.03$	$0.18\pm0.02$	$0.19\pm0.01$

<sup>\*</sup> Indicates significant difference from 5 mg dosing (P < 0.05) <sup>#</sup> Indicates significant difference from 10 mg dosing (P < 0.05) <sup>a</sup> Indicates significant difference between i.v. and oral dosing at same dose (P < 0.05)



Figure 5.1: Structures of DA inhibitor and internal standard



Figure 5.2: Chomatographs of blank plasma (top) and plasma spiked with 1  $\mu$ g/ml internal standard (I) and 0.5  $\mu$ g/ml DA inhibitor (II) (bottom).



Figure 5.3: Concentration (mean) vs. time profiles of DA inhibitor for plasma following IV bolus and oral administration at (a) 5 mg/kg (b) 10 mg/kg and (c) 12.5 mg/kg



Figure 5.4: Comparison of (a) Half-life (b) Clearance and (c) AUC means in IV and Oral Administration

# CHAPTER 6

# PHARMOCOKINETICS AND TISSUE DISPOSITION OF A NOVEL INTEGRASE INHIBITOR IN MOUSE TISSUES<sup>1</sup>

<sup>&</sup>lt;sup>1</sup> Shawn K. Blue, Vasu Nair, Jason Zastre and Catherine A. White. To be submitted to *Antimicrobial Agents and Chemotherapy* 

# ABSTRACT

A simple high performance liquid chromatography (HPLC) method has been developed and validated for determination of the novel integrase inhibitor 4-(1,3-dibenzyl-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid (DA inhibitor) in mouse tissues (liver, lung, kidney, brain and heart). Liquid-liquid extraction with acetonitrile was used for extraction of DA inhibitor from tissue samples. Reverse phase liquid chromatography was performed with a Nova-Pak C18 analytical column (3.9 x 300 mm, 4 µ particle size) equipped with a Phenomenex C18 guard column. A flow rate of 1 ml/min and detection wavelength of 341 nm was used. The mobile phase consisted of acetonitrile and water under a gradient (10-90% ACN). The method was validated over the range of 0.05-20 µg/ml for plasma. The absolute recovery of DA inhibitor ranged from 59.4 to 89.1%. Acceptable intra- and inter-day assay precision (< 20% R.S.D.) and accuracy (< 15% error) were observed over 0.05-20  $\mu$ g/g in mouse tissues. The method was applied to determine the tissue distribution of DA inhibitor. Half-life, volume of distribution and oral clearance were not significantly different for Pregnane X Receptor knockout (PXR-KO) and human Pregnane X Receptor transgenic (hPXR) mice with a yielding half-lives (6.0 vs. 5.5 hr), Vd (29.5 vs. 25.2 L/kg) and CL/F (56.7 vs. 52.6 ml/min/kg). The high volume of distribution indicates extensive tissue distribution.

Keywords: Integrase Inhibitor, Pregnane X Receptor, Tissue Disposition

# **INTRODUCTION**

The HIV/AIDS pandemic is one of the greatest ordeals in the world. Nearly 30 million have died from AIDS since 1981 and globally over 33 million people are currently living with HIV [1-4]. There have been great advances in the treatment of HIV/AIDS since the introduction of the first nucleoside reverse transcriptase inhibitor (NRTI), zidovudine in 1987 [4-6]. The introduction of combination therapies has led to a substantial improvement in the quality of life for HIV positive individuals [4-6]. The development of additional NRTIs, non nucleoside transcriptase inhibitors, protease inhibitors, fusion inhibitors and recently integrase inhibitors has resulted in potent antiretroviral therapy [2-4, 7-9]. The United States and other developed countries have also shown a significant decrease in death rates [1].

Robust treatment can lead to near normal T-cell counts and undetectable viral counts. However, resistant viruses and toxicity are limiting factors in therapy consisting of reverse transcriptase and protease inhibitors, which may lead to suboptimal clinical outcomes [2-3, 7, 9-16]. Nearly all of the existing antiretroviral agents inhibit the activity of the two central HIV enzymes, reverse transcriptase and protease [2-3, 13, 15, 17-18]. To advance the treatment of HIV additional targets must be identified. HIV-Integrase has been identified as a potential target, due to its vital role in viral replication. Integrase inhibitors block the action of integrase, a viral enzyme, which integrates HIV DNA into the target cells [6, 12-17, 19-23]. Currently there is only one integrase inhibitor commercially available for treatment [16, 24-25].

While various structurally diverse compounds have exhibited inhibition of integrase, a small group of  $\beta$ -diketo acids represent the most convincing, biologically validated inhibitors of integrase [3, 6-7, 20-21, 24, 26-29]. Many of these compounds selectively inhibit the strand transfer reaction of the enzyme. Nair *et al.* discovered new  $\beta$ -diketo acids with purine

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nucleobase scaffolds that are potent inhibitors of both the strand transfer and 3'-processing steps of HIV-1 integrase [11, 14-15, 30-33].

Animal studies are integral to the development of investigational drugs, pharmacokinetics being central [8-9, 34-36]. Pharmacokinetic studies include determination of pharmacokinetic parameters, as well as understanding metabolic pathways. Cytochrome P450 enzymes (CYPs), a large group of hemoproteins, are key in the body's metabolism of endogenous and exogenous compounds[34]. The nuclear pregnane X receptor (PXR) has been proven to be key in the activation of CYP3A4, CYP2C8, CYP2C9, CTP2C19 and CYP2B6 [37-38]. PXR also regulates of the Phase II conjugating enzymes glutathione S-transferase, aldehyde dehydrogenases, alcohol sulfotransferase 2A1 and UDP-glucuronosyltransferase A1 [37, 39-40]. The transport uptake and efflux proteins OATP1, OATP2, MDR1 and MRP1-3 are also targets of PXR [37].

Therefore, to better understand the pharmacokinetics and tissue disposition of the novel integrase inhibitor, DA inhibitor, we have investigated the tissue distribution of DA inhibitor in mouse tissues (brain, heart, lung, kidney and liver) post oral dosing. The purpose of this study is to develop a HPLC method to quantify DA inhibitor in mouse tissues (liver, kidney, lung, heart and brain) for the purpose of determining pharmacokinetic parameters and tissue distribution of DA inhibitor in mice. As previously stated PXR is integral in the activation and regulation of several metabolizing enzymes and uptake proteins. By investigating the tissue distribution of DA inhibitor in PXR-KO and hPXR transgenic mice we are able to estimate its metabolic pathway.

# EXPERIMENTAL

# **Chemicals and Reagents**

DA Inhibitor and 4-(1-dibenzyl-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid were synthesized and provided by the Center for Drug Discovery, The University of Georgia (Athens, GA, USA). Acetonitrile, acetic acid and dimethyl sulfoxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals and solvents were ACS analytical or HPLC grade.

# Chromatographic System

Chromatographic studies were performed on an Agilent 1100 HPLC (Palo Alto, CA) equipped with a variable wavelength UV detector, quaternary pump, degasser and autosampler. A Waters Nova-Pak C18 analytical column (3.9x300mm; 4 µ particle size) (Milford, MA, USA) equipped with a Phenomenex C18 guard column (Torrance, CA, USA) was used to achieve chromatographic separation.

# **Chromatographic Conditions**

The mobile phase consisted of acetonitrile and water using a linear gradient (10-90% ACN) over 20 min. The flow rate was 1 ml/min and the detection wavelength was 341 nm. The injection volume was 20  $\Box$ 1. HPLC run time for each sample was 20 min.

# **Preparation of Stock and Standard Solutions**

Stock solutions (1 mg/ml) of DA inhibitor and internal standard were individually prepared in dimethyl sulfoxide. DA inhibitor standard solutions with concentrations of 200, 100, 25, 10, 5, 2.5, 1 and 0.5  $\mu$ g/ml were prepared from the 1 mg/ml stock solution by serial dilution with dimethlyl sulfoxide. Internal standard solution at the concentration 10  $\mu$ g/ml was prepared by dilution from the 1 mg/ml stock solution with dimethyl sulfoxide. Precision and accuracy

standards of 10, 5, 1 and 0.5  $\mu$ g/ml were prepared in the same manner. Stock solutions (1 mg/ml) were kept refrigerated when not in use. Fresh standard solutions were prepared each day of analysis and validation.

# **Calibration Curves**

Blank liver, lung, kidney, brain and heart tissues were obtained from untreated animals. Tissue calibration points were prepared by spiking 50  $\mu$ l of the tissue homogenate with 5  $\mu$ l of DA inhibitor standard solution and 5  $\mu$ l of the 10  $\mu$ g/ml internal standard solution. The calibration curve of tissues were in the range of 0.05–20  $\mu$ g/ml with individual calibration points of 20, 10, 2.5, 1, 0.5, 0.1 and 0.05  $\mu$ g/ml while the internal standard concentration was 1  $\mu$ g/ml for all samples.

# **Precision and Accuracy**

This method was validated using four QC points for the calibration curve. To determine the intra-day accuracy and precision five replicates of each QC point were analyzed each day. Inter-day accuracy and precision were determined by repeating this process 3 times over 3 days. The QC points for tissues were 0.05, 0.1, 1 and 5  $\mu$ g/ml.

# **Sample Preparation**

Liver, lung, kidney, brain and heart were isolated from the rats and homogenized in two volumes of distilled water with a Tissumizer (Tekmar, Cincinnati, OH, USA). Liquid- liquid extraction with acetonitrile was used for extraction of DA inhibitor from tissues. Cold acetonitrile ( $100\Box 1$ ) was added to 50 µl samples, vortexed for 20 sec. and centrifuged at 10,000 rpm for 10 min. The upper organic layer was aspirated and dried under vacuum. Samples were then reconstituted in 100 µl of water, prior to analysis.

# **Pharmacokinetic Study**

The UGA Animal Use and Care Committee approved the use of animals for this study. The mice were housed one animal per cage in the College of Pharmacy animal facility (AAALAC accredited). The environment was controlled (20-22° C, 14 h of light/day) with daily feedings of standard chow pellets and water *ad libitum*.

In order to determine plasma pharmacokinetics and tissue disposition, groups of female PXR-KO and hPXR mice (~28 g) were dosed via oral gavage (10 mg/kg) with exact volumes of DA inhibitor/DMSO solution. Mice (n=3 per time point) were euthanized via CO<sub>2</sub> asphyxiation at 0.25, 0.5, 1, 2, 6, 9, and 12 hr post-dose. Plasma and tissues (liver, lung, kidney, brain and heart) were prepared using methods previously described.

Compartmental and noncompartmental analysis using WinNonlin (Pharsight, Mountain View, CA, USA) was used to determine pharmacokinetic parameters. DA inhibitor exhibits one-compartmental model plasma kinetics. Non-compartmental analysis was used for tissue kinetics, allowing the use of truncated AUC in further analysis. A  $1/\hat{y}^2$ -weighting scheme was used throughout the analysis. The pharmacokinetic parameters generated for each dosing group were compared using an unpaired *t*-test (P < 0.05) to identify statistically significant differences.

#### RESULTS

# **Analytical Method**

The chemical structures of DA inhibitor and internal standard are represented in Figure 6.1. In order to achieve the best separation from endogenous peaks, several mobile phases were investigated. Buffers at varying organic content and pH were tested; however these did not provide adequate separation. Successive exploration revealed satisfactory resolution of DA

inhibitor and internal standard from endogenous peaks was obtained with ACN/H<sub>2</sub>O under a gradient (10-90% ACN). Under this gradient DA inhibitor and internal standard eluted at 9.7 and 7.6 min, respectively, which provided ideal separation from endogenous peaks. Chromatograms of blank plasma and spiked plasma with DA inhibitor (0.5  $\mu$ g/ml) and internal standard (1  $\mu$ g/ml) are shown in Figure 6.2.

Linear regression equations were generated with Microsoft Excel or JMP statistical software using a 1/x-weighting scheme for each day of validation and analysis. The calibration curves showed acceptable linearity ( $R^2 > 0.99$ ) over the range 0.05-20 mg/ml for plasma.

The extraction efficiencies for DA inhibitor and internal standard from each matrix (liver, kidney, lung, heart and brain) are expressed in terms of absolute recovery. Samples were spiked with 20, 10, 2.5, 1, 0.5, 0.1 and 0.05 solutions of DA inhibitor and 1  $\mu$ g/ml solutions for internal standard samples. The absolute recoveries were calculated by comparing the peak areas of spiked tissue samples to the corresponding peak areas of the untreated stock solutions. Absolute recoveries of DA inhibitor (n = 15) ranged from 59.4 to 89.1%. Recovery of the internal standard, ranged from 67.5 to 85.0%. The recoveries are shown in Table 6.1

Calculation of accuracy and precision was performed over 3 days. Precision, as expressed by % R.S.D., and accuracy as expressed by % error for DA inhibitor in each tissue is illustrated in Table 6.2. Over three separate days, measurement of five samples at each QC point was used to determine intra-day (n = 5) precision and accuracy. The pooled data over three days was used to determine inter-day (n = 15) precision and accuracy. The calibration curve and each of the four QC points 5, 1, 0.5 and 0.05 µg/ml were used for these calculations. Intra-day precision (% R.S.D.) and accuracy (% error) of DA inhibitor ranged from 0.22 to 16.3 and 0.76 to 16.2%,
respectively. Inter-day precision and accuracy of DA inhibitor ranged from 0.66 to 10.4 and 1.4 to 10.1%, respectively.

Stability testing was performed for DA inhibitor and internal standard in mouse tissue at the concentration level of 0.1 and 1 µg/ml, respectively. Spiked matrix samples (25 samples) went through three consecutive freeze/thaw cycles over the period of four days. Five samples were extracted and analyzed as described before. The remaining spiked matrix samples were stored at -20°C. Each of the following three consecutive days, spiked matrix samples were thawed, and five more were extracted and analyzed. The day-to-day measured peak areas of DA inhibitor and internal standard were compared and the results listed in Table 6.3. The % R.S.D. between the average peak areas of DA inhibitor and internal standard each day was less than 15%. The stability of extracted matrix samples in the autosampler was also evaluated. At time 0, three samples of each matrix were injected onto the HPLC column and analyzed. In another 24 hr, the same samples from each matrix were injected again. The peak areas for DA inhibitor and internal standard in each injection were compared. The % R.S.D. between samples was less than 15% for both compounds in each matrix and no obvious decline in peak areas between each injection was observed.

#### **Pharmacokinetics and Tissue Disposition**

The concentration-time profiles of DA inhibitor (10 mg/kg) in all matrices are shown in Figure 6.3. The plasma DA inhibitor concentrations declined in a monoexponential fashion after oral administration, indicating a 1-compartment model. The pharmacokinetic parameters generated from one-compartmental analysis are presented in Table 6.4. The  $k_a$  values of DA Inhibitor indicated fast absorption from the gastrointestinal tract. No significant changes are noted in half-life, Vd/F and CL/F. Extensive distribution into total body water (0.43 L/kg) is

indicated by the very large Vd/F (26.5 L/kg) of DA inhibitor [41]. This is due to, in part, to the lipophilic nature of this compound. Preceding studies performed by our group examined plasma pharmacokinetics of orally administered DA Inhibitor in rats. Comparing the results of this study, the major pharmacokinetic parameters, AUC and  $C_{max}$  are significantly lower in both hPXR and PXR-KO mice when compared to DA inhibitor pharmacokinetics in rats. Additionally, half-life is significantly longer in hPXR and PXR-KO mice when compared to our previous rat study. According to unpublished research by Nair *et al.*, pharmacokinetic differences between rats and mice are consistent with  $\beta$ -diketo acid integrase inhibitors

The tissue distribution of DA inhibitor determined by noncompartmental analysis of liver, kidney, brain heart and lungs for PXR-KO and hPXR mice is summarized in Table 6.5. DA inhibitor concentrations declined in parallel to the plasma, which suggests hepatic metabolism. DA inhibitor levels in the kidney, lung and brain decrease very slowly, suggesting that the drug may be sequestering in these tissues. The slow elimination from tissues may provide a longer duration of antiviral activity, which is favorable for anti-HIV drugs.

In PXR-KO mice, DA inhibitor in the liver, kidney and lung reached peak concentrations of 0.17, 0.19 and 0.17  $\mu$ g/g, respectively, at 0.75 hr. While, peak concentrations of 0.21 and 0.15  $\mu$ g/g, respectively, were reached in 1 hr for heart and brain. Peak concentrations of DA Inhibitor were reached at 1 hr for liver, kidney, lung and heart for hPXR mice. Peak concentration in the brain was reached in 0.75 hr. for hPXR mice. The C<sub>max</sub> of DA inhibitor in the lung is higher in PXR-KO mice when compared to hPXR mice, while no difference was shown in the kidney and brain. Conversely, the C<sub>max</sub> in the heart and liver are lower in PXR-KO mice in comparison to hPXR mice. Yet, peak concentration was similar for other tissues. Kidney and brain concentrations exceed the concentration exhibited in the plasma after the 9 hr time point for PXR-KO mice. While in hPXR mice, concentrations in the heart exceeded plasma levels after the 9 hr time point.

The AUC in the liver and heart was lower in PXR-KO mice as compared to hPXR mice. However, the change in AUC was minimal in the kidney; another PXR expression site (26). Relative exposure (RE) is determined by ratio of the truncated (0-time) area under concentrationtime curves (AUC<sub>tissue</sub>/AUC<sub>plasma</sub>). Relative exposure in the liver and heart is lower in PXR-KO mice in comparison to hPXR mice. This suggests that PXR is involved in the upregulation of transporters responsible for uptake of DA Inhibitor in the liver and heart. Conversely, higher RE is found for the brain, kidney and lung in PXR-KO mice when compared to hPXR mice, which indicates competitive uptake into these tissues in the presence of PXR.

## CONCLUSION

A sensitive and accurate method was developed and validated for the quantification of DA inhibitor in mouse tissues. Liquid-liquid extraction with acetonitrile provided rapid and inexpensive sample preparation. This method yielded high recoveries, good linearity, and precision and accuracy in the range of  $0.05 - 20 \mu g/g$ .

Various structurally diverse compounds have exhibited inhibition of integrase; the most convincing, biologically validated inhibitors of integrase are represented by a small group of  $\beta$ -diketo acids [3, 6-7, 20-21, 24, 26-29]. Many of these compounds selectively inhibit the strand transfer reaction of the enzyme. Nair *et al.* discovered new  $\beta$ -diketo acids with purine nucleobase scaffolds that are potent inhibitors of both the strand transfer and 3'-processing steps of HIV-1 integrase [11, 14-15, 30-33]. The pharmacokinetics and tissue disposition of DA inhibitor were investigated.

Pregnane X Receptor is integral in the activation and regulation of several metabolizing enzymes and uptake proteins [38-40]. By investigating the tissue distribution of DA inhibitor in PXR-KO and hPXR transgenic mice we are able to estimate its metabolic pathway. In this study, we investigate the tissue disposition for PXR-KO mice and hPXR mice. Plasma pharmacokinetics proves that DA inhibitor has extensive tissue distribution. Our study shows that PXR has no significant bearing on the plasma pharmacokinetics. However, PXR is shown to play a role in the tissue disposition of DA inhibitor. This is exhibited in the higher AUC and relative exposure in hPXR mouse heart and liver, suggesting upregulation of transporters targeted by hPXR. While higher RE is found for the brain, kidney and lung in PXR-KO mice, indicating competitive uptake in the presence of PXR.

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Analyte	Concentration (□g/ml)	Liver	Kidney	Lung	Heart	Brain
	1	$89.09 \pm 2.6$	$88.64 \pm 1.1$	$85.02 \pm 2.4$	$84.91 \pm 3.7$	$71.11 \pm 1.3$
	0.5	$84.59 \pm 3.1$	$74.03 \pm 2.9$	$79.30 \pm 4.8$	$72.08 \pm 3.1$	$65.89 \pm 1.3$
DA inhibitor	0.1	$66.94 \pm 2.9$	$78.98 \pm 1.8$	$61.85 \pm 2.5$	$73.33 \pm 4.4$	$81.73 \pm 2.4$
	0.05	$59.36 \pm 1.4$	$79.07\pm4.3$	$66.34\pm2.0$	$67.05\pm3.2$	$70.23\pm3.2$
Internal						
Standard	1	$70.68\pm4.5$	$67.47 \pm 2.9$	$85.01\pm2.4$	$84.91 \pm 3.7$	$71.11 \pm 1.3$

Table 6.1: Absolute recoveries of DA inhibitor from tissues (n=15)
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Table 6.2: Intra-day (n=5) and inter-day (n=15) precision (% R.S.D.) and accuracy (% error) measured for QC points for DA inhibitor from tissues. T.C. = Theoretical Concentration and E.C. = Experimental Concentration

		Day 1			Day 2			Day 3		, ,	Interday	
T.C.	E.C.	R.S.D	Error	E.C.	R.S.D	Error Liver	E.C.	R.S.D	Error	E.C.	R.S.D	Error
1	0.98	1.97	0.76	1.04	3.68	2.425	1.01	1.12	3.14	1.01	0.95	3.22
0.5	0.49	2.63	10.92	0.47	5.25	1.131	0.48	3.24	7.95	0.48	3.71	7.40
г.	0.10	3.49	10.72	0.10	3.16	8.280	0.10	3.41	9.51	0.10	1.02	9.39
.05	0.05	1.61	7.55	0.06	16.28	6.559	0.06	14.42	7.46	0.05	9.70	10.01
						Kidney						
1	1.00	0.23	1.13	0.98	1.54	1.521	0.99	0.66	0.98	0.99	0.66	1.36
0.5	0.49	1.10	6.25	0.47	5.45	2.102	0.48	4.38	3.49	0.48	3.64	4.51
г.	0.11	5.83	4.93	0.11	5.62	3.860	0.10	3.95	3.19	0.10	5.13	3.85
.05	0.05	5.26	16.20	0.05	8.45	14.894	0.045	9.23	12.92	0.04	7.65	13.82
						Lung						
1	1.01	1.44	2.53	1.07	7.36	1.64	1.04	4.39	1.30	1.04	4.34	2.96
0.5	0.47	6.31	11.67	0.47	6.76	2.36	0.47	6.53	4.91	0.46	6.53	6.89
г.	0.11	10.46	7.39	0.11	14.43	10.29	0.11	10.14	6.63	0.11	11.68	7.90
.05	0.06	13.37	5.05	0.05	1.23	6.10	0.05	7.30	5.13	0.05	7.30	6.92
						Heart						
1	0.99	0.59	7.02	0.99	1.00	3.95	0.9921	0.79	2.45	0.99	0.79	4.50
0.5	0.51	2.33	6.47	0.48	2.97	0.81	0.4984	0.32	3.15	0.49	0.32	4.54
.1	0.10	2.65	11.54	0.10	8.18	5.76	0.1054	5.41	7.21	0.10	5.42	8.11
.05	0.04	7.33	8.42	0.05	5.00	11.83	0.0469	6.16	5.37	0.05	6.17	8.37
						Brain						
1	1.01	0.91	1.79	1.00	0.47	1.75	1.02	1.64	1.44	1.01	1.01	1.62
0.5	0.48	4.25	3.25	0.50	0.68	1.11	0.49	1.36	1.55	0.49	2.09	2.57
г.	0.11	8.42	2.24	0.11	10.54	4.76	0.11	12.19	2.63	0.11	10.38	3.47
.05	0.04	12.85	4.84	0.05	6.83	9.00	0.05	5.39	4.05	0.04	8.36	6.95

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Table 6.3: Results of freeze/thaw stability of DA inhibitor (0.1  $\Box$ g/ml) in tissues represented by area ± S.D. (n=5) of each day and % R.S.D. of the area between days

		Ar	ea		
DA inhibitor	Liver	Kidney	Lung	Heart	Brain
Day 1	$19.0 \pm 0.9$	$17.8 \pm 0.5$	$17.0 \pm 0.8$	$14.0 \pm 1.2$	$12.4 \pm 0.2$
Day 2	$18.5 \pm 1.1$	$17.4 \pm 0.6$	$17.0 \pm 1.8$	$15.4 \pm 1.3$	$14.1 \pm 0.2$
Day 3	$18.5 \pm 1.1$	$17.4 \pm 1.0$	$16.6 \pm 1.0$	$15.1 \pm 0.9$	$13.9 \pm 0.7$
Day 4	$18.7\pm1.0$	$17.5 \pm 0.6$	$16.9 \pm 1.2$	$14.8 \pm 1.1$	$13.5 \pm 0.3$
% R.S.D.	11.3	10.6	6.1	7.8	11.5

Table 6.4: Plasma pharmacokinetic parameter estimates for DA inhibitor at 10 mg/kg dosing (mean  $\pm$  SEM)

Parameter	PXR-KO	hPXR
T 1/2 (hr)	$5.9\pm0.30$	$5.7\pm0.35$
AUC (hr*ug/ml)	$2.9\pm0.09$	$3.2 \pm 0.11$
C <sub>max</sub> (ug/ml)	$0.31\pm0.01$	$0.34\pm0.01$
Tmax (hr)	$0.81\pm0.08$	$0.96\pm0.07$
V/F (L/kg)	$29.1 \pm 1.12$	$25.7 \pm 1.25$
CL/F (mL/hr)	$3.44 \pm 0.11$	$3.12 \pm 0.11$
$K_a(hr^{-1})$	$4.68\pm0.65$	$3.64\pm0.51$

Table 6.5: Tissue Distribution after oral administration of DA Inhibitor (10 mg/kg) in PXR-KO and hPXR mice

		PXR-KC	Mice		
Parameter	Liver	Kidney	Lung	Heart	Brain
$C_{max}$ (ug/g)	0.17	0.19	0.17	0.21	0.15
Tmax (hr)	0.75	0.75	0.75	1.00	1.00
AUC (hr*ug/ml)	0.83	1.85	1.58	1.52	1.50
RE	0.38	0.85	0.72	0.69	0.69
		hPXR I	Mice		
Parameter	Liver	Kidney	Lung	Heart	Brain
$C_{max}$ (ug/g)	0.22	0.19	0.15	0.26	0.14
Tmax (hr)	1.00	1.00	1.00	1.00	0.75
AUC (hr*ug/ml)	1.39	1.74	1.42	2.51	1.49
RE	0.56	0.70	0.57	1.00	0.60



Figure 6.1: Structures of DA inhibitor and internal standard











Figure 6.2: Chomatographs of blank tissue and tissue spiked with 1 mg/ml of internal standard (I) and 0.1  $\mu$ g/ml of DA inhibitor (II) spiked tissues (a) brain, (b) heart, (c) lung, (d) kidney and (e) liver









# CHAPTER 7

# CONCLUSIONS

### **Placental Transport Study**

The treatment of HIV/AIDS has plagued the therapeutic community for nearly 3 decades. Today, women account for over 50% of HIV-positive patients. The reduction of vertical transmission is one of The World Health Organization's (WHO) key objectives. Medication during pregnancy is administered for maternal treatment, fetal treatment or both. In all cases, the benefit of treatment must outweigh the risk to the fetus. Resource rich countries have nearly eradicated vertical transmission; however, further evaluation of treatment methods must be made. Studies of the efficacy of various monotherapy and combination therapy regimens in the prevention of vertical transmission are well documented. It is pertinent to understand the pharmacokinetics, placental transport and interactions at the placental level in order to develop optimum treatment of mother and child. For obvious ethical reasons, *in vivo* placental transfer studies are investigated using animal models as opposed to pregnant women. The purpose of these experiments was to investigate the mechanisms of placental transport of Stavudine and compare it to that of another NRTI (Zalcitabine) using a pregnant rat model.

The premier step in performing animal studies is the development and validation of a bioanalytical method. A sensitive and accurate method was developed and validated for the quantification of D4T in rat maternal plasma, amniotic fluid, placental and fetal tissues. This method was utilized for pharmacokinetic studies to investigate the fetal and maternal disposition of D4T (25 mg/kg) in the pregnant rat. A five-compartmental model was used to determine the

transport mechanisms of stavudine which were then compared to stavudine in combination with zalcitabine. The disposition of D4T is significantly altered in the presence of DDC. The fetal uptake of D4T was significantly lowered in the presence of DDC. Intercompartmental clearances between the placenta and amniotic fluid were significantly decreased in the presence of DDC. These results were consistent with our findings with the two-compartment and noncompartmental modeling, which showed a significant difference in the relative exposure in amniotic fluid and fetus for D4T/DDC combination dosing. This suggests that transporters and passive diffusion play a role in the tissue disposition of these drugs. After comparing a five-compartment model and a four-compartment model fixed to plasma concentrations, the overall difference in intercompartmental clearance was not significant. Yet, the fixed model provides a better fit based on the goodness of fit criteria (AIC, SBC, WRSS and Condition #).

### **Integrase Inhibitor Study**

Preclinical animal studies are essential in drug discovery. Animal studies have provided invaluable pharmacological and toxicological information for drug development. After a bioanalytical method is developed and validated, serial blood samples are collected from dosed animals, processed and analyzed by the method. Animal models are commonly utilized during discovery and preclinical development to characterize pharmacokinetics, metabolism and pharmacodynamics.

Antiviral therapy consisting of reverse transcriptase inhibitors and protease inhibitors has provided beneficial treatment to many HIV-positive patients. However, due to drug resistance and toxicity, patients can be faced with limited therapeutic outcomes. Hence, it is logical to investigate new targets, such as HIV-Integrase, the enzyme necessary for viral replication. In conjunction with Nair *et al.*, who have developed the investigational integrase inhibitor, DA

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inhibitor, the purpose of these experiments was to determine the pharmacokinetic profile of DA inhibitor.

A bioanalytical method was developed and validated for the quantification of DA inhibitor in rat plasma. This method was used to determine pharmacokinetic profile of DA inhibitor after intravenous and oral dosing. 2-compartmental (IV) and 1-compartmental (oral) analysis via WinNonlin were used to determine pharmacokinetic parameters and bioavailability was also determined. DA inhibitor is extensively distributed which is consistent with the lipophilic nature of this compound. The clearance decreased as the dose increased indicating saturation of drug elimination. Since this compound is extensively metabolized saturation of liver clearance is the most likely explanation. The decrease in bioavailability with increasing dose may result, in part, from saturation of influx transporters, saturation of first pass metabolism and/or solubility issues in the GI tract due to the hydrophobicity of the compound. A decrease in half-life with oral dosing was noticed in this study is. Additionally, a trend for oral bioavailability and absorption rate constant to decrease as dose increased was observed.

In order to determine the tissue disposition of DA inhibitor, an additional method was developed and validated for determination in mouse tissues (liver, kidney, lung, heart and brain). Plasma pharmacokinetics was obtained through 1-compartmental analysis, thus proving that DA inhibitor has extensive tissue distribution. Our study showed that PXR has no significant bearing on the plasma pharmacokinetics. After noncompartmental analysis of tissues via WinNonlin it was shown that DA inhibitor has extensive tissue distribution. Higher AUC and relative exposure in hPXR mouse heart and liver, suggesting upregulation of transporters targeted by hPXR. While higher RE is found for the brain, kidney and lung in PXR-KO mice, indicating competitive uptake in the presence of PXR