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Pharmacokinetics of Antiviral Agents in Veterinary Medicine
(Under the Direction of Dr. F. Douglas Boudinot)

The pharmacokinetics and brain distribution of zidovudine (3'-azido-3'-deoxythymidine, AZT) and lamivudine ((-)- 2', 3'-dideoxy-3'-thiacytidine, 3TC) were investigated in healthy cats.

Plasma concentrations of AZT declined rapidly with a terminal half-life of 1.4 ± 0.19, 1.4 ± 0.16 and 1.6 ± 0.26 hr following IV, IG and PO administration of 25 mg/kg AZT to six cats in a three way cross-over study. The area under the curve (AUC) following IV, IG, and PO administration was 64.7 ± 16.6, 42.5 ± 9.41 and 60.5 ± 17.0 mg×h/L, respectively

In a three way cross-over study, the half-lives of 3TC were 1.9 ± 0.21, 2.6 ± 0.66 and 2.7 ± 1.5 hr following IV, IG and PO administration of 25 mg/kg 3TC to six cats. The corresponding AUC was 130 ± 55.2, 115 ± 97.5 and 106 ± 94.9 mg×hr/L.

Both AZT and 3TC were well-absorbed following IG and PO administration.

To determine the distribution of AZT to cerebrospinal fluid (CSF) and brain, plasma, CSF, and brain samples were obtained from five cats that received a 7.0 mg/kg AZT loading dose, followed by three-hour infusion at 14.5 mg/kg/h. Mean (± STD) plasma, CSF, and brain concentrations of AZT at steady state were 26.3 ± 4.21, 8.27 ± 1.21, 6.37 ± 0.61 mg/L, respectively. In six cats, after 7.0 mg/kg loading dose of 3TC, followed by three-hour infusion at 5.5 mg/kg/hr, 3TC concentrations in plasma and CSF at steady states were 17.09 ± 5.26 and 1.73 ± 0.56 mg/L. No 3TC was detected in cat brain.

Pharmacokinetics of (-)-β-D-2,6-diaminopurine dioxolane (DAPD) and its metabolite, (-)-β-D-dioxolane guanosine (DXG) were estimated following administration of 50 mg/kg DAPD intravenously or orally to four adolescent ducks. Most DAPD concentrations were below the limit of quantitation. The AUC of DXG was 17.08 and 13.86 mg·hr/L following intravenous administration of DAPD and 18.26 and 23.11 mg·hr/L following oral administration. The half-life of DXG was 0.33 and 0.56 hr after intravenous DAPD and 0.85 and 0.86 hr following oral dosing of DAPD.

INDEX WORDS: Pharmacokinetics, Feline immunodeficiency virus (FIV), Cats, Lamivudine (L-2',3'-Dideoxy-3'-thiacytidine, 3TC), Zidovudine (3'-azido-3'-deoxythymidine, AZT), Ducks, DAPD ((-)-β-D-2,6-diaminopurine dioxolane), DXG ((-)-β-D-dioxolane guanosine)

PHARMACOKINETICS OF ANTIVIRAL AGENTS IN VETERINARY MEDICINE

by

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To my parents, and sisters

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Lentivirus

The identification and characterization of human immunodeficiency virus (HIV) as the cause of acquired immunodeficiency syndromes (AIDS) in 1983/1984 (Barre-Sinoussi *et al* 1983, Gallo *et al* 1984, Popovic *et al* 1984) soon led to the isolation of similar viruses from a variety of different species. Among these viruses, simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) are closely related to HIV and are used as animal models for HIV (Schellekens and Horzinek 1989, Hamburg *et al* 1993). HIV, SIV, and FIV belong to the same lentivirus subfamily of *Retroviridae*. The *Retroviridae* family is so called due to the presence of a unique enzyme, reverse transcriptase (RT), the key enzyme that facilitates the production of a deoxyribose nucleic acid (DNA) copy from a ribonucleic acid (RNA) genome. HIV, SIV and FIV share the characteristics as members of *Retroviridae*, including RNA carried genetic information, three polyprotein genes called group specific antigen (*gag*), polymerase (*pol*) and envelope (*env*) which control the viral structure, and a life cycle involving insertion of the viral genome into the genetic material of the host cell. As *'Lenti'* means slow in Greek, the lentivirus typically produce infections with long periods of latency, with many of them affecting cells of the immune system and the brain, causing neurological and immunological diseases. Another characteristic of lentivirus is its ability to rapidly change itself (Schoub 1994).

Viral life cycle

Though the details of how lentivirus replicates remains unclear, the following steps are believed to be essential for HIV, SIV, and FIV replication (Figure 1.1.). First

the virus is attached to the host cell, the core of the virus enters the cell and genetic material, RNA, is released into cell. Next a DNA copy is made under the function of reverse transcriptase (RT) and integrated into host cell DNA. Then, RNA and protein are synthesized according to the integrated viral gene. Finally, the protein and genetic material assemble together and released from the host cell as new virus that is able to infect new cells and replicate. Protease plays pivotal role in the assembly of protein in this final step. Interruption of any steps in the replication cycle will terminate the virus replication.

Anti-HIV compounds

Currently available antiretroviral compounds target two crucial enzymes in the replication cycle: reverse transcriptase (RT) and protease. There are three classes of anti-HIV compounds commercially available: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Since the first antiretroviral drug, 3'-azido-3'-deoxythymidine (zidovudine, AZT or ZDV) was introduced in 1987, fourteen agents have been approved by the Food and Drug Administration (FDA) for the treatment of HIV infection. These drugs include six NRTIs (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, and abacavir), three NNRTIs (nevirapine, delavirdine, and efavirenz), and five PIs (saquinavir, ritonavir, indinavir, nelfinavir, and amprenavir). Several promising agents are under development as well.

FIV and its similarity with HIV

FIV was first isolated in northern California from cats showing similar symptoms as AIDS in human (Pedersen *et al* 1987). Cats with FIV-infection show a similar pattern of early cellular and humoral responses, period of latency, and subsequent development of clinical disease as humans infected with HIV. FIV resembles HIV in terms of morphologic, physical and biochemical characteristics (Pedersen *et al* 1987, North *et al* 1989, Dua *et al* 1994). Similar to HIV, FIV (structure shown in Figure 1.2.) is 105 to 125 nm in diameter, roughly spherical or oval, and composed of a lipid envelope and the RNA containing core. One of the important features of FIV is that FIV reverse transcriptase (RT) is similar to RT from HIV-1 in physical properties, catalytic activities, template specificity, requirement of Mg^{2+}, and sensitivities to active forms of several RT-targeted antiretroviral compounds (North *et al* 1989, North *et al* 1990a, North *et al* 1990b).

Epidemiology and transmission of FIV

Soon after the discovery, FIV was identified in various domestic and wild feline species worldwide. The prevalence of FIV infection in the cat population depends on the geography location. Though as high as a 26% and 28.9% FIV-positive rate has been reported in the healthy cat population in Australia and Japan (Friend *et al* 1990), the general rate is around 1-12% throughout the world (Grindem *et al* 1989, Hopper *et al* 1991, Hosie *et al* 1989, Ishida *et a*l 1988, O'Connor Jr *et al* 1991, Shelton *et al* 1989, Yamamoto *et al* 1989). In sick and wild cat populations, the prevalence is dramatically higher, reaching 48% in the sick cat population, and even 83% among wild cat species in

South Africa, East Africa, and North America (Olmsted *et al* 1992). In the United States, FIV affects 1-4% of the "healthy" cat, and 13-15% of the sick cat populations (Yamamoto *et al* 1989). FIV-infection is more often observed in free roaming cats, multi-cat households, and male cats (Pedersen *et al* 1989). FIV is present in the blood, serum, plasma, cerebrospinal fluid, brain tissue and saliva of FIV-infected cats (Yamamoto *et al* 1988, Dow *et al* 1990). How FIV is transmitted remains to be elucidated. Though one case of mother to kitten infection in post-partum period was reported (Callanan *et al* 1991), biting is thought to be the main transmission route (Pedersen *et al* 1989). Studies showed that the saliva may be involved in the transmission of FIV (Yamamoto *et al* 1989).

Clinical staging

Experimental inoculation studies demonstrated that FIV related disease is composed of three stages (Bennett and Smyth 1992, Ishida *et al* 1989, Pedersen *et al* 1991). In the initial phase of FIV infection, lymphadenophy occurs in two to six weeks and persists six to nine month, sometimes accompanied by fever, malaise, mild pyrexia, depression, and leukopenia (Pedersen *et al* 1987, Pedersen *et al* 1989, Bennett and Smyth 1992). The followed stage is a latent period, in which the FIV-infected cats appear relative normal without clinical evidence of infection. This period can last 1-5 years before the cats become terminally ill. In the terminal stage, FIV infected cats demonstrate an AIDS-like illness characterized by a number of chronic infections, such as stomatitis, upper respiratory infections, enteritis, dermatomycosis, and neurologic disease (Pedersen *et al* 1987, Pedersen *et al* 1989, Bennett and Smyth 1992. Cats with

AIDS-like syndrome usually survive only one to six months, though supportive treatments are given (Ishida *et al* 1990, Pedersen *et al* 1991).

Current status of anti-FIV therapy

Since its discovery, FIV has been considered as a useful model for HIV (North *et al* 1989, Dua *et al* 1994), however, cats have seen little benefit from these investigations (Pedersen *et al* 1991, Egberink *et al* 1991). As fourteen compounds are available for HIV-infection that have significantly improved and prolonged living for humans with HIV, most clinical ill FIV-infected cats are only treated with empiric and supportive care and the effectiveness of the treatment decreases as FIV progresses. Seeking safe and effective anti-FIV compounds is critical to combat this significant health threat to the cat population. Though there are limited systemically scientific studies to support applications of anti-HIV compounds for the treatment of FIV infection, NRTIs and NNRTIs have been tried by individuals on their sick cats. Almost all available NRTIs, including AZT, (-)-2'-deoxy-3'-thiacytidine (lamivudine, 3TC), 2',3'-dideoxyinosine (didanosine, ddI), 2',3'-dideoxycytidine (zalcitabine, ddC), 2',3'-didehydro-2',3'-didexoythymidine (stavudine, D4T), (-)-6-cyclopropylamino-2',3'-dihydro-2',3'-dideoxyguanosine (abacavir, ABC) and 9-(2-phosphonylmethoxyethyl) adenine (PMEA), and one NNRTIs (nevirapine) have been applied to treat FIV-infection by individual veterinary medicine practice, either alone or in combination (animalhealthchannel.com, www.specent.com/artg).

Previous studies

The similarity between FIV and HIV not only makes FIV an attractive *in vitro* and *in vivo* animal model for investigation of HIV, but also provides the rationale for considering currently available anti-HIV chemotherapy agents in veterinary practice for the treatment of FIV infection. Among the three types of antiretroviral compounds, most HIV protease inhibitors (PIs) can not be applied toward FIV infection. FIV and HIV protease inhibitors have the identical catalytic mechanism and superimposable active site structure (Wlodawer *et al* 1995), however; FIV protease has a more restricted binding subsite that resembles many drug-resistant HIV proteases (Lee *et al* 1998). This property makes FIV protease a perfect model to study drug-resistant HIV protease and to develop new protease inhibitors. But, it also means that current PIs for HIV are not good candidates for the control of FIV infection. Soon after the discovery of FIV, a number of NRTIs were tested against FIV *in vitro* and *in vivo.* As early as in 1989, North *et al* demonstrated that the susceptibility of FIV in FIV-infected Crandell feline kidney cells (CrFK) to AZT is similar to that of HIV-1, which is completely inhibited by 1 to 3 μM AZT (North *et al* 1989). Tanabe-Tochikura *et al* reported similar results for AZT activity towards FIV later while studying the effect of AZT and ddI on FIV replication in 3201/FIV cells (Tanabe-Tochikura *et al* 1992). In 1991, sensitivities of FIV to AZT, 3'-azido-2',3'-dideoxyuridine (AZDU), ddI and D4T were tested in CrFK cells (Remington *et al* 1991), the EC_{50} (50% effective concentration) values for AZT, AZDU, ddI and D4T were 1.4, 71.6, 2.1, and 12.3 μM respectively. Cronn *et al* investigated the inhibition of FIV replication by PMEA in CrFK cells and reported the EC_{50} value to be 1.0 μM (Cronn *et al* 1992). In 1994, Smyth *et al* studied eighteen antiviral agents for anti-FIV activity in CrFK cells (Smyth *et al* 1994). Among the antiviral agents tested, NRTIs were among

the most effective agents against FIV and the least toxic, with EC_{50}s for AZT, ddC, ddI, d4T, 2',3'-dideoxyadenosine (ddA), and 3TC of 0.2, 0.15, 0.87, 2.6, 1.8, 0.04 mg/L, respectively. In a series of studies reported by Hartmann and Egberink, AZT and PMEA were shown to be potent and selective inhibitors of FIV in feline thymocytes with EC_{50} values of 0.05 and 0.60 μM, and CD_{50} (50% cytotoxic concentration, the concentration of compound reducing the number of viable cells by 50%) values of 120 and 80 μM (Egberink *et al* 1991, Hartmann *et al* 1992, Hartmann *et al* 1995). Both compounds (2.5 and 5 mg/kg/12h of PMEA, 5 and 10 mg/kg/12h of AZT) were found to have significant therapeutic effect as indicated by improving clinical signs and raising the CD4/CD8 ratio in naturally infected cats (Hartmann *et al* 1992, Hartmann *et al*1995). The toxicity of AZT in cats includes anemia, neutropenia (Hartmann *et al* 1992, Gregory *et al* 1997). This toxicity was found to be dose-dependent and reversible. The hematological side effects caused by PMEA were more severe than those caused by AZT (Hartmann *et al* 1992). Besides the toxicity, there are still controversies related to the effectiveness of AZT and PMEA in cats (Vahlenkamp *et al* 1995, Gregory *et al* 1997, Hayes *et al* 1993, Smyth *et al* 1994, Hayes 2000, Meers 1993). Smyth *et al*. reported that giving AZT 0.2, 1.0, 5, 25, or 50 mg/kg/day for 29 days to experimentally infected cats was much less effective than expected (Smyth *et al* 1994a). There are several other groups that showed similar results (Hayes *et al* 1993, Hayes *et al* 1995, Meers *et al* 1993) . In a series of efforts, Hayes *et al.* reported that AZT monotherapy reduced the FIV viral burden and the acute CD4 lymphocyte loss in young cats inoculated with FIV during the drug treatment, but did not prevent infection and later CD4 lymphocyte decline. Further thymic histopathology studies implied that it did not reduce thymic inflammation (Hayes

et al 1993, Hayes *et al* 1995, Hayes *et al* 2000). Similar to humans, the thymus of FIV-infected cats carries a heavy viral burden and thymic function is compromised as extensive pathological change was observed in the tissue. Meanwhile, the development of AZT-resistant virus has also been a concern during the application of this drug (Remington *et al* 1991, Remington *et al* 1994). Later the effectiveness of AZT, PMEA, and (R)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine ((R)-PMPDAP, a derivative of PMEA) against FIV was studied in primary feline thymocytes isolated from specific-pathogen-free cats and in CrFK cells (Vahlenkamp *et al* 1995). It was found the EC_{50} and CD_{50} were dramatically different in these two types of cells. The EC_{50}s were 0.05, 0.5, 0.07 μM in thymocytes, and were 4.0, 1.5, 0.1 μM in CrFK cells. The CD_{50}s were 110, 80, 190 in thymocytes, and >200, 90, >200 in CrFK cells. The results also suggested (R)-PMPDAP was a more potent and less toxic inhibitor of FIV *in vitro* and *in vivo.* The cell dependent antiviral activity by 2',3'-dideoxycytidine on feline leukemia virus (FeLV) had been reported previously (Polas *et al* 1990).

Recently, activities of AZT, 3TC, (-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC), and DDC were assessed in feline peripheral blood mononuclear cells for a clinical isolate of FIV (FIV-Maxam), a pathogenic molecular clone, FIV-pPPR, and site-directed mutatant of FIV-pPPR, M183V (McCrackin Stevenson *et al* 2001). The EC_{50}s of AZT, 3TC, ddC were 0.07, 0.14, 0.86 μM for the clinical isolate FIV-Maxam respectively. The EC_{50}s of AZT, 3TC, (-)-FTC were 0.03, 0.46, 0.18 and 1.21 μM for FIV-pPPR respectively. The EC_{50}s towards M183V, which resembles the M184V mutant in HIV, were 0.04, 38.5, 49.8, 1.04 μM for AZT, 3TC, (-)-FTC, and ddC. Besides the individual compound activity to different strains of FIV, these results also indicate that M183V

decreases the sensitivity of FIV to 3TC and (-)-FTC without changing the activity to AZT and ddC.

Since combination chemotherapy significantly improved the therapeutics of HIV infection, it has also been considered in treatment of FIV infection and showed promising results. Preliminary *in vitro* studies showed that combination chemotherapy using AZT, 3TC, and an FIV-specific protease inhibitor was more effective in inhibiting FIV infection than AZT monotherapy in peripheral blood mononuclear cells (PBMC) (Arai *et al* 1998). In a very recent *in vitro* study, the combination of AZT, 3TC, and abacavir, (-)-6-cyclopropylamino-2',3'-dihydro-2',3'-dideoxyguanosine (abacavir, ABC or 1592U89) effectively inhibited FIV replication (Bisset *et al* 2002). The effectiveness of using AZT and 3TC in combination in FIV-infected cats also been indicated in a limited *in vivo* study when applied together with bone marrow transplantations (BMT) (Yamamoto *et al* 1998).

These studies indicate that FIV-infection is a complicated condition and that more research needs to be done to find an effective therapeutic strategy. Meanwhile, different types of cells respond differently to the same antiviral agents. Further, the NRTIs represent an abundant resource to consider for treatment of FIV infection as some members of NRTIs have shown promising *in vitro* and *in vivo* activity toward FIV with relative low toxicity. In addition, combination chemotherapy may be more effective than mono- chemotherapy.

Purpose of current study

In this dissertation research, as part of the efforts to seek effective treatment for FIV infection, two NRTIs (AZT and 3TC) were investigated for their pharmacokinetic characteristics in cats after intravenous (IV), oral (PO) and intragastric (IG) administration. IG administration was investigated as an alternative route for long term administration of these antiviral agents in cats. Further, the cerebrospinal fluid (CSF) and brain tissue distribution after intravenous infusion was studied at steady state. Distribution to CSF and brain tissue of antiviral compounds is of interest since FIV, like HIV, is a neurotropic lentivirus. There is evidence showing penetration of FIV into the central nervous system (CNS) of infected cats (Pedersen *et al* 1989, Yamamoto *et al* 1998, Dow *et al* 1990, Dow *et al* 1992). The presence of FIV in CNS accounts for the observed brain lesions and diffuse gliosis in FIV-infected cats. This physiological damage may explain the abnormal behaviors observed in a subset of FIV-infected cats. One third of FIV-infected cats showed neurologic dysfunction in a three-year study with the most common neurologic abnormality being altered behavior (Dow *et al* 1992). Symptoms may be slight or severe as obvious central and peripheral neurologic disease such as seizures, striking behavior changes, anisocoria, or paresis. Therefore, whether the antiviral compound can reach CNS is a critical point to evaluate its *in vivo* effectiveness.

Mechanism of antiviral activity of NRTIs

The antiviral activity of NRTIs, including AZT, ddI, ddC, D4T, 3TC, and ABC, is accomplished by the corresponding 5'-triphophate metabolites of NRTIs. Intracellular

enzymes are critical for the metabolic activation of NRTIS to the active 5'-triphosphate forms. The 5'-triphosphates are competitive with RT, and incorporated into and terminate the viral DNA strand since they don't have a free 3'-hydroxyl end, which results in the termination of viral replication.

AZT

3'-Azido-3'-deoxythymidine (zidovudine or AZT) (Figure 1.3.) was the first drug approved for the treatment of HIV infection in humans and is the most well studied compound to date. As mentioned above, AZT has shown activity against FIV in vitro and *in vivo.* In feline thymocytes, the EC_{50} was reported to be 0.05-0.07 μM, and the CD_{50} was 100-120 μM (Egberink *et al* 1991, Vahlenkamp *et al* 1995, McCrackin Stevenson *et al* 2001) . These results indicated that AZT is a potent and selective inhibitor of FIV *in vitro*. In FIV-infected cats AZT improved the immune status by raising the CD4/CD8 ratio which subsequently led to an improvement in FIV-related stomatitis, conjunctivitis, and alopecia (Hartmann *et al* 1992, Hartmann *et al* 1995). The enhancement in the cats' overall clinical condition increased the quality of life, and prolonged life expectancy. Though the possible toxicity and emergence of drug resistance has limited the application of AZT, this compound remains one of the most important components in combination chemotherapy for HIV. The pharmacokinetics of AZT have been well documented in mice (Doshi *et al* 1989), rats (Mays *et al* 1991), and monkeys (Boudinot *et al* 1990); however, few studies have attempted to characterize the pharmacokinetics of AZT in cats. In humans, AZT is almost completely absorbed in GI tract with the achievement of peak plasma concentration in 0.5 to 1.5 h after dosing

(Dollery 1999). AZT is rapidly distributed throughout the body with a volume of distribution of 1.6 L/kg. AZT is also able to penetrate the blood brain barrier with a CSF/plasma ratio at 0.53 (Klecker *et al* 1987). The clearance is of AZT is very high at 180 L/h (Dollery 1999). The mean plasma half-life of AZT is approximately 1 h.

3TC

(-)- 2',3'-Dideoxy-3'-thiacytidine (lamivudine, 3TC) (Figure 1.3.) is a dideoxynucloside analogue with potent antiviral activities against HIV-1, HIV-2, hepatitis B virus (HBV) and FIV (Soudeyns *et al* 1991, Coates *et al* 1992a, Coates *et al* 1992b, Smyth *et al* 1994b). 3TC is approved to be administered in combination with other antiviral agents for the treatment of HIV infection and as monotherapy for the treatment of HBV infection. Compared to other available nucleoside reverse transcriptase inhibitors, 3TC has less toxicity and synergic activity with other anti-HIV compounds (Soudeyns *et al* 1991, Coates *et al* 1992a, Coates *et al* 1992b, Chang *et al* 1992). *In vitro*, 3TC has demonstrated activity against HIV-1 with a 50% inhibitory concentration (IC_{50}) ranging from 0.002 to 0.87 μM in various cell lines (Soudeyns *et al* 1991, Coates *et al* 1992b). Most nucleoside analogs currently available for clinical use, including AZT, ddI, ddC, and D4T, have clinically limiting toxicities such as peripheral neuropathy, myopathy, pancreatitis and hematological toxicity (Parker and Cheng 1994). AZT and ddI are possibly related to liver toxicity as well (Parker and Cheng 1994). These toxicities of 3TC are believed to be due to the inhibition of mitochondrial DNA replication by the 5'-triphosphates of the nucleoside analogues (Chen and Cheng 1992, Chen *et al* 1991, Parker and Cheng 1994). Though the phosphorylation steps of 3TC are

similar to ddC, it differs from ddC in that it does not inhibit mammalian mitochondrial DNA replication in intact cells. 3TC-TP is formed intracellularly, however, it is not transported into the mitochondria (Gray *et al* 1995). Therefore, 3TC is unlikely to induce clinically important hematological and hepatic adverse events, neuropathy or myopathy (Parker and Cheng 1994). The more favorable safety profile of 3TC compared to other available anti-HIV compounds has been confirmed by *in vitro* studies, animal toxicology studies and in clinical trials (Lisignoli *et al* 1992, Sommadossi *et al* 1992, Tyrrell *et al* 1993, Lai *et al* 1998). In a long-term placebo-controlled study involving 358 patients, there is no serious drug-related adverse events observed (Lai *et al* 1998) with the administration of 3TC. Another advantage to applying 3TC to antiviral therapy is its synergic effect with other antiviral compounds. Mutant strains resistant to 3TC arise rather rapidly during monotherapy through the acquisition of a Methionine-to-Valine (M184V) or a Methionine-to-Isoleucine (M184I) mutation at codon 184 at the YMDD motif of HIV-1 RT (Schinazi *et al* 1993). This mutation is associated with a delay in the emergence of AZT-resistant mutants in AZT-naïve patients, restoration of AZT sensitivity in patients already possessing AZT-associated resistance mutations, impairment of HIV-1 replication capacity, and a reduced ability of the HIV-1 *env* gene to mutate (Larder *et al* 1995). This provides the rationale for combination therapy using 3TC and other anti-HIV compounds. The combination of 3TC, AZT and an HIV protease inhibitor has showed a remarkable effect in decreasing HIV levels and improving CD4+ cell counts of AIDs patients (Torres and Barr 1997). The development of 3TC resistance in the immuno- and neuropathogenic molecular clone of FIV, FIV-pPPR, is similar with the 3TC resistance of HIV-1 developed in 3TC-treated HIV-1-

infected patients (McCrackin Stevenson and McBroom 2001). The similarity between the development of the resistance to 3TC in FIV and HIV-1 makes it reasonable to consider 3TC, which has been used in combination chemotherapy in HIV-1 infection, in combination chemotherapy for FIV-infected cats.

The pharmacokinetics of 3TC have been well characterized in different species. Following IV administration, 3TC is distributed throughout the body with the volume of distribution 1.3 L/kg in humans (Hussey *et al* 1994). The total clearance of 3TC in humans is 0.32 L/h/kg. 3TC is primarily eliminated as unchanged drug through renal clearance (Rajagopalan *et al* 1996). The renal clearance of 3TC is greater than the glomerular filtration rate (GFR) indicating active renal tubular secretion is involved in the elimination (van Leeuwen *et al* 1992). The half-life of 3TC is 2.8 h (Hussey *et al* 1994). 3TC is well absorbed following oral administration with a bioavailability of approximately 87% in humans (van Leeuwen *et al* 1992, Hussey *et al* 1994).

Hepatitis B virus (HBV)

As a major public health threat worldwide, hepatitis B virus (HBV) infection is strongly associated with acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Approximately 5% of the world's population (400 million people) are chronic carriers of HBV. Forty percent (40%) of these patients will die from HBV-related diseases, thus making HBV the ninth leading cause of death throughout the world (Maynard *et al* 1988, Ayoola *et al* 1988, Mahoney 1999, Mast *et al* 1999, Lok 2000). Interferon (IFN)-α was the only approved treatment for chronic hepatitis B infection until

1999 (Farrell 2000, Shaw and Locarnini 2000) thought IFN-α is only effective in 30-50% of the patients, and various side effects, relatively high expense and administration by injection limit its use (Saracco and Rizzetto 1997, Farrell 2000). Recently, some nucleoside analogues originally synthesized for chemotherapy of HIV or herpesvirus were found to have potent anti-HBV activity, this discovery opened a new door for investigating effective anti-HBV chemotherapy (Hong *et al* 1998, Kinchington 1999, Shaw and Locarnini 2000, Farrell, 2000). One of these nucleoside analogues is 3TC, which has been approved to treat HBV. 3TC is well tolerated and effectively inhibits HBV replication during the course of treatment. However, the rebound of viral replication during prolonged therapy and the development of drug resistance to lamivudine have been a problem (Berenguer and Wright 1998, Marzano *et al* 2001). Therefore, the search for additional safe and effective oral therapies for chronic HBV infection remains a task of health professionals.

(-)-b**-D-2,6-Diaminopurine dioxolane (DAPD)**

As more nucleoside analogues have been investigated for anti-HBV activity, (-)-β-D-2,6-Diaminopurine dioxolane (DAPD) appears to be one of the most promising anti-HBV agents due to it potent activity and low toxicity (Kim *et al* 1993, Schinazi *et al* 1994, Furman *et al* 2001). DAPD is a potent inhibitor of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), HBV, and Simian Immunodeficiency Virus (SIV) *in vitro*. The EC_{50} (effective concentration) of DAPD is 0.09 μM against HBV DNA replication intermediates or HBV virion synthesis inhibition in transfected HepG2 2.2.15 cells. DAPD is converted into (-)-β-D-dioxolane guanosine (DXG) *in vivo* by adenosine

deaminase (Figure 1.4.) (Chen *et al* 1999, Rajagopalan *et al* 1996, Painter *et al* 1999). DXG also shows potent anti-HBV activity (Schinazi *et al* 1994, Kim *et al* 1993). In transfected HepG-2 cells, the EC_{50} for DXG against HBV DNA replication intermediates and HBV virion synthesis inhibition was approximately 1 μM. No marked toxicity was observed up to 100 μM DXG and up to 300 μM DAPD in transfected HepG2 2.2.15 cells (Schinazi *et al* 1994, Kim *et al* 1993). DAPD, considered as a water-soluble and bioavailable prodrug of DXG, is currently being investigated as an anti-HIV and anti-HBV agent by Triangle Pharmaceuticals (Durham, NC) (Corbett and Rublein 2001) due to its favorable therapeutic index and pharmacokinetic profiles *in vitro* and in animals (Furman *et al* 2001, Chen *et al* 1999, Rajagopalan *et al* 1994, Rajagopalan *et al* 1996, Schinazi *et al* 1994, Kim *et al* 1993). DAPD is the only member of its chemical series in development for antiviral activity. DAPD is effective toward HBV strains resistant to lamivudine and /or famciclovir (Chin *et al* 2001), and HIV strains resistant to AZT, 3TC, adefovir and multidrug resistance (Bazmi *et al* 2000, Gu *et al* 1999). Thereby, it is synergistic with a number of current antiviral treatments. DAPD provides new options for anti-HIV and anti-HBV chemotherapy.

Duck hepatitis B virus (DHBV)

HBV belongs to the family of *hepadraviridae* which comprises human HBV, woodchuck hepatitis virus (WHV) (Summers *et al* 1978), ground squirrel hepatitis virus (GSHV) (Marion *et al* 1980), and duck hepatitis B virus (DHBV) (Mason *et al* 1980). The *hepadraviridae* viruses have been shown to cause persistent chronic infections in their natural hosts and share common biological features including the virion

ultrastructure, genomeic structure, and unique mechanism of replication. DHBV was first discovered in 1979 in serum samples taken from ducks in the People's Republic of China (Mason *et al* 1980). DHBV was detected in 10% of Peking ducks in commercial flocks in the United States (Mason *et al* 1980). Hepatitis caused by DHBV accounts for great losses among ducklings two to three weeks old (Raethel 1988). Due to the "striking resemblance" to HBV, DHBV has been used as an animal model to HBV. Part of this dissertation is to investigate the pharmacokinetics of DAPD and DXG in ducks in order to provide information for the further evaluation of DAPD and DXG as potential anti-HIV and anti-HBV agent *in vivo.*

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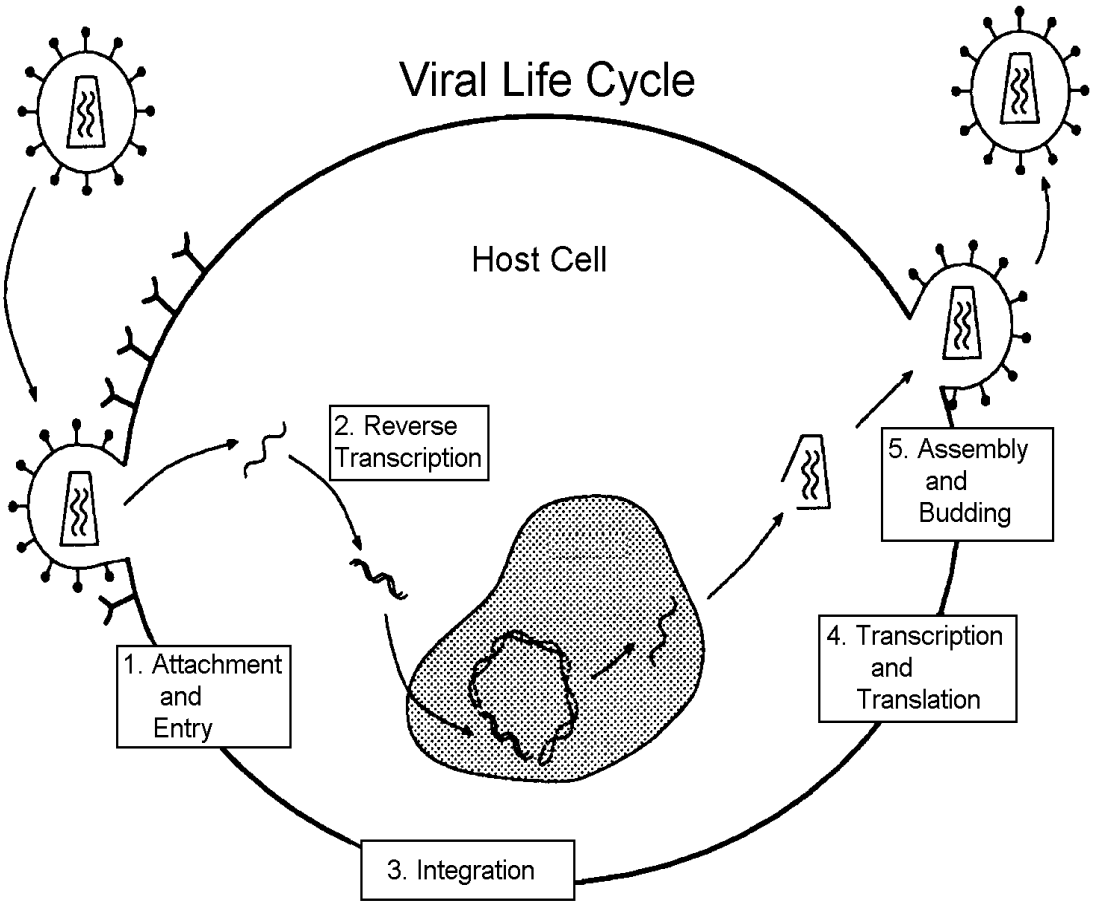


Figure 1.1. Viral life cycle of HIV and FIV.

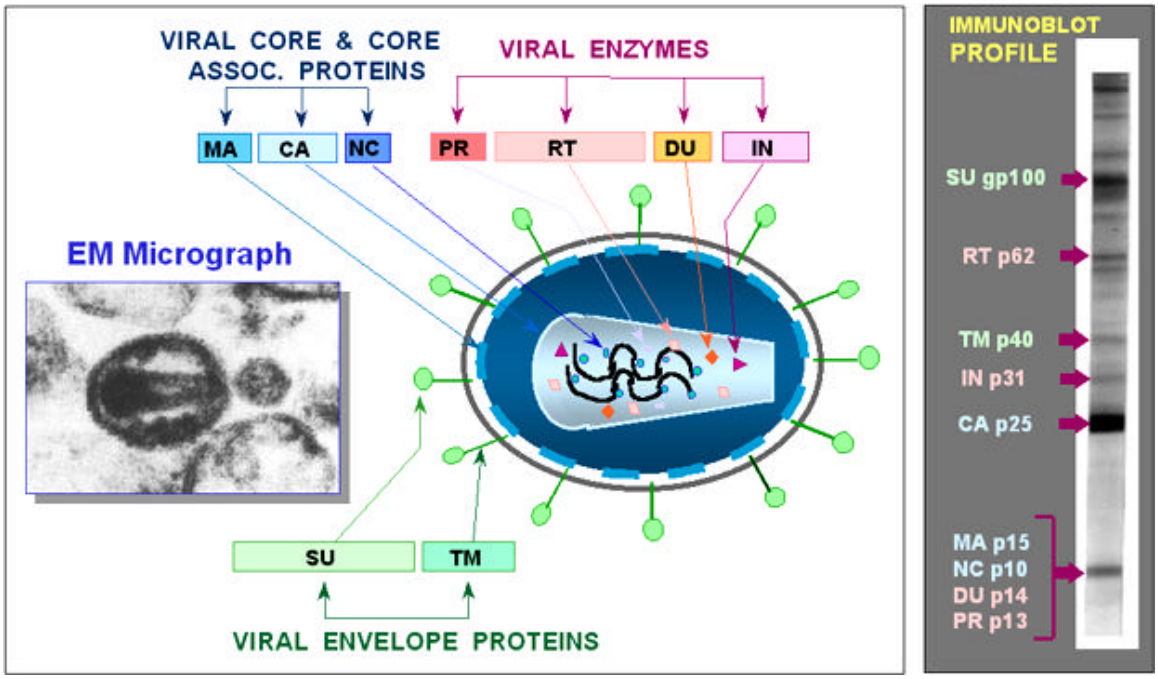


Figure 1.2. Structure of FIV.

http://www.vetmed.ufl.edu/path/teach/vem5110b/diagnostics/diagnostics.htm

Veterinary Immunology, University of Florida College of Veterinary Medicine

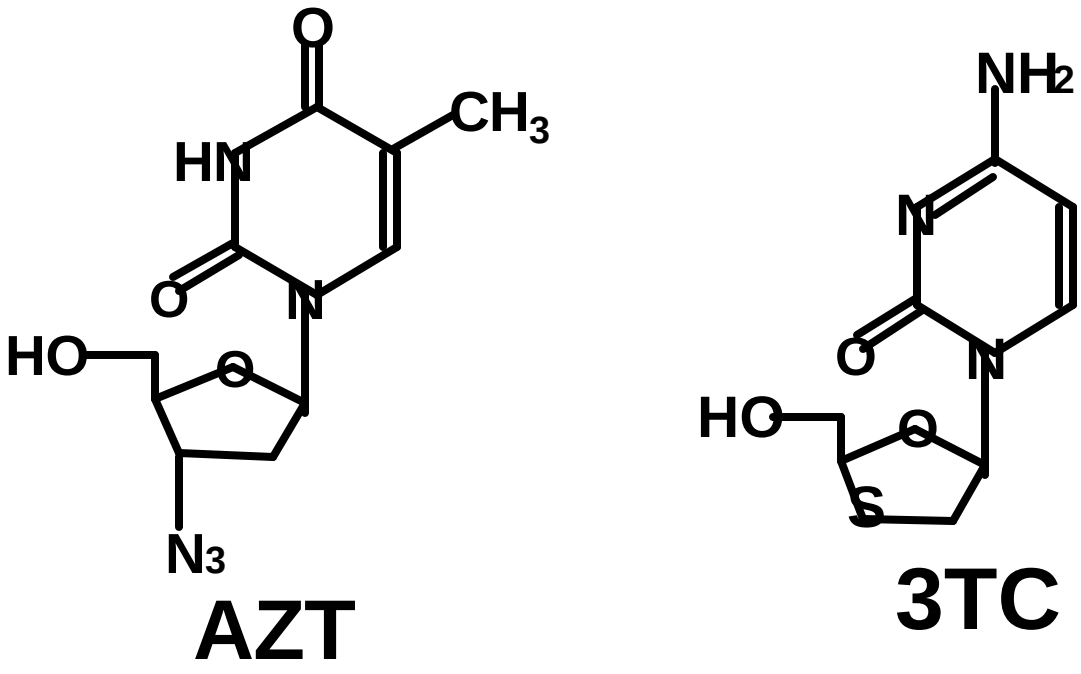
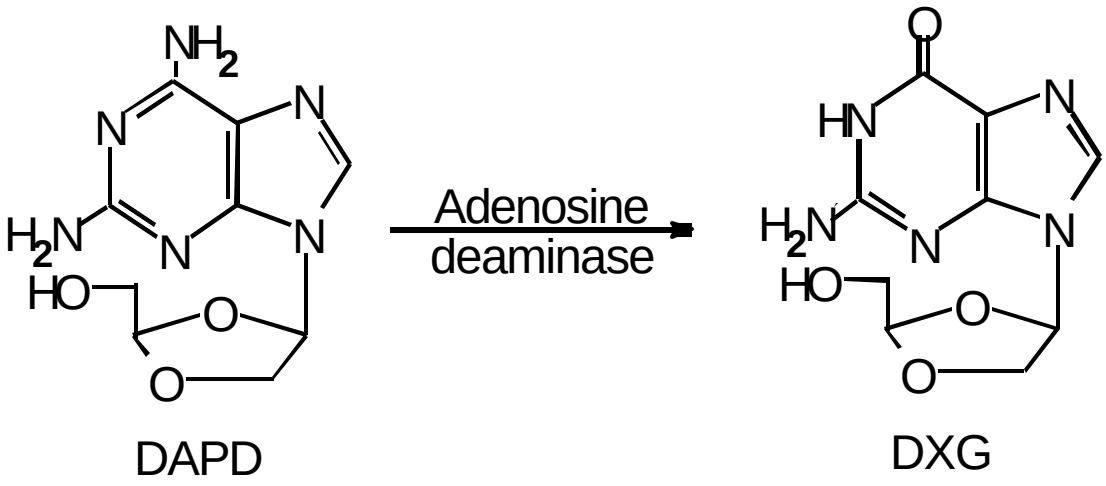


Figure 1.3. Structure of AZT and 3TC.



DAPD DXG

Figure 1.4. Structures of DAPD and DXG.

CHAPTER 2

PHARMACOKINETICS OF ZIDOVUDINE IN CATS [1]

[1] Zhang W, Mauldin JK, Schmiedt CW, Brockus CW, Boudinot FD, McCrackin Stevenson MA. To be submitted to *American Journal of Veterinary Research*

PHARMACOKINETICS OF ZIDOVUDINE IN CATS

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Objective—To characterize the pharmacokinetics of zidovudine (3'-azido-3'-deoxythymidine, AZT) in cats.

Animals—Six healthy intact 8-month-old barrier-reared domestic short hair cats (3 male, 3 female).

Procedure—Six cats, randomly divided into three groups, were administered 25 mg/kg AZT intravenously (IV), intragastrically (IG), and orally (PO) in a three-way cross over study design. The intragastric administration was achieved through skin-level low-profile gastrostomy ports. A two-week washout period was allowed between studies. Plasma samples were collected and AZT concentrations were determined by HPLC. Bone marrow aspirates were examined for cytologic evidence of acute bone marrow suppression. Pharmacokinetic parameters were obtained by area-moment analysis. ANOVA was conducted using SAS (*$p<0.05$).

Results—Plasma concentrations of AZT declined rapidly with a terminal half-life of 1.4 ± 0.19, 1.4 ± 0.16 and 1.6 ± 0.26 hr following IV, IG and PO administration, respectively. Total body clearance and steady-state volume of distribution were 0.41 ± 0.10 L/hr/kg and 0.82 ± 0.15 L/kg, respectively. The area under the curve (AUC) following IV, IG, and PO administration was 64.7 ± 16.6, 42.5 ± 9.41 and 60.5 ± 17.0 mg×h/L. AZT was well absorbed after IG and PO administration with bioavailability values of 0.70 ± 0.24 and 0.95 ± 0.23. Cytology of bone marrow aspirates was normal. The disposition of AZT in cats was similar to that in other species.

Conclusions—At a dose of 25 mg/kg, plasma concentrations of AZT were maintained above the minimum effective concentration (0.03 µM for wild-type FIV) for at least 12 h following IV, IG or PO administration.

The abbreviations used are: 3TC, lamivudine, L-2'-deoxy-3'-thiacytidine; ABC, abacavir, (-)-6-cyclopropylamino-2',3'-dihydro-2',3'-dideoxyguanosine; AIDs, acquired immune deficiency syndrome; AZT, zidovudine, 3'-azido-3'-deoxythymidine; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; RT, reverse transcriptase; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; DDI, 2',3'-dideoxyinosine; DDC, 2',3'-dideoxycytidine; D4T, 2',3'-didehydro-2',3'-didexoythymidine; PMEA, adefovir, 9-(2-phosphonylmethoxyethyl) adenine; CS-85, 3'-azido-2',3'-dideoxy-5-ethyluridine; (R)-PMPDAP, (R)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine; FTC, 2',3'-dideoxy-5-fluoro-3'-thiacytidine;

Key words: 3'-azido-3'-deoxythymidine (AZT), cats, pharmacokinetics, feline immunodeficiency virus (FIV).

Feline immunodeficiency virus (FIV) was first isolated in the United States in 1986, and cats with FIV infection show similar symptoms to humans with acquired immunodeficiency syndrome (AIDS).[1] Similar to human immunodeficiency virus (HIV), FIV belongs to the lentivirus sub family of *Retroviridae*. The pattern of early cellular and humoral responses, period of latency, and subsequent development of clinical disease are paralleled in cats and humans since FIV is similar to HIV in terms of morphologic, physical and biochemical characteristics.[1,2] Like HIV, the genetic material of FIV is composed of RNA; the production of a DNA copy of the viral RNA is an essential step in

the replication of FIV. This step requires the enzyme reverse transcriptase (RT). Most current available anti-HIV agents target RT to inhibit the replication of HIV. Although FIV has been used as an HIV model, veterinary medicine has benefited little from these investigations. Most FIV-infected cats with clinical illness are treated with empiric and supportive care as a safe and effective antiviral drug is not available for FIV treatment. [3,4] 3'-Azido-3'-deoxythymidine (zidovudine or AZT) was the first drug approved by Food and Drug Administration (FDA) for the treatment of HIV infection in humans. AZT is phosphorylated to AZT-triphosphate intracellularly. This triphosphate metabolite inhibits reverse transcriptase (RT). Because of the similarity of the RT in HIV and FIV, the antiretroviral efficacy of AZT against FIV was demonstrated as early as in 1989. [2] Hartmann and Egberink reported the efficacy of AZT against FIV *in vitro* and in *vivo* . [4-6] In feline thymocytes, the EC_{50} (50% effective concentration) was reported to be 0.05-0.07 μM , [4,7,8] and the CD_{50} (50% cytotoxic concentration, the concentration of AZT reducing the number of viable cells by 50%) was 100-120 μM . [4,7,8] These results indicated that AZT is a potent and selective inhibitor of FIV *in vitro*. In FIV-infected cats AZT improved the immuno status by raising the CD4/CD8 ratio which subsequently led to an improvement in FIV-related stomatitis, conjunctivitis, and alopecia . [5,6] The enhancement in the cats' overall clinical condition increased the quality of life, and prolonged life expectancy. The toxicity of AZT in cats includes anemia, and neutropenia.[9] This toxicity is found to be dose-dependent and reversible. The pharmacokinetics of AZT have been well documented in mice, [10] rats, [11] and monkeys; [12] however, few studies have attempted to characterize the pharmacokinetics of AZT in cats. The purpose of this study was to characterize the pharmacokinetics of AZT in cats

by three administration routes: intravenous (IV), oral (PO) and intragastric (IG). The IG route was investigated as an alternative administration route for long term application of AZT in cats in clinical practice.

Materials and Methods

Cats

Six healthy intact 8-month-old barrier-reared[a] domestic short hair cats (3 male, 3 female) weighing 2.56 to 4.04 kg (mean ± SD, 3.21 ± 0.73) were used in the study. Cats were housed at the University Veterinary Medical Animal Care Facility. Animal studies were approved by the University of Georgia Animal Care and Use Committee and were conducted in accordance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Complete blood counts (CBC), serum biochemistry panels, and urinalyses were done on all cats within 3 days of their arrival. All cats had skin-level gastrostomy ports placed 2 weeks before the start of the study.[13]

Study design

The study was done in accordance with a randomized 3-way crossover design, with 3 dosing periods and a 2-week washout period between doses. Doses were administered orally (PO), intragastrically (IG), and intravenously (IV). Twelve to 16 hours before drug administration, cats were anesthetized with intramuscular (IM) xylazine[b] (1.0 mg/kg) and ketamine[c] (12.5-15.0 mg/kg) for aseptic placement of 18.5-

gauge jugular catheters[d]. A 20-gauge catheter[e] was also placed in the cephalic vein of cats scheduled for IV drug administration. Jugular catheters were flushed with 1 ml, and cephalic catheters with 0.5 ml, of 0.9% saline containing 5 units/ml sodium heparin.

Food was withheld for 24 hours before and 5 hours after drug administration. Water was withheld for 12 to 16 hours prior to drug administration but was freely available after drug was given. AZT[f] was purchased in capsule and IV formulations and was dosed at 25 mg/kg for each cat, based on allometric interspecies scaling techniques.[14] Oral AZT, compounded for each cat, was put in gelatin capsules. For IG administration, a suspension of AZT in 5 ml water was given through the gastrostomy tube and flushed with 5 ml water. AZT solution (10 mg/ml) was diluted to 4 mg/ml with 5% dextrose in water as recommended by the manufacturer and injected in the cephalic catheter as a bolus over 1 to 2 minutes.

Blood samples were collected from the jugular catheter immediately before drug administration and 0.08, 0.25, 0.50, 0.75, 1, 1.5, 2, 4, 6, 8, and 12 hr afterwards. Sampling was a 4-step procedure. A 2.5-ml blood sample was aspirated from the jugular catheter into a sterile 3-ml syringe. Next, a 0.3-ml sample for drug analysis was collected into a tuberculin syringe and transferred into a heparinized polypropylene microcentrifuge tube. The initial 2.5-ml blood sample was then returned to the cat through the jugular catheter. Finally, the catheter was flushed with 1 ml heparinized saline. Heparinized blood samples were immediately centrifuged at 3000 g, and the harvested plasma was stored at -20^0C until analysis. Twenty-four hours after drug administration, a blood sample for CBC was collected from the jugular catheter before it was removed.

Two weeks after the third dose of AZT, cats were sedated with diazepam (0.12-0.2 mg/kg) and ketamine (2.5-4.0 mg/kg) IV and anesthetized with xylazine (1.0 mg/kg) and ketamine (12.5-15.0 mg/kg) IM for a concurrent study. Bone marrow aspirates were obtained from the proximal left femur to evaluate cytology for bone marrow suppression.

Determination of AZT concentration

Concentrations of AZT in plasma samples were determined by an established high performance liquid chromatographic (HPLC) method.[15] Briefly, 100 µl of plasma sample, 50 µl of internal standard (5 µg/ml 3'-azido-2',3'-dideoxy-5-ethyluridine, CS-85) and 50 µl of 2 M perchloric acid were added to 1.7 ml polypropylene microcentrifuge tubes. After vigorously mixing, the tubes were centrifuged at 9000 g for 10 min. The supernatant was directly injected onto HPLC. Chromatography was performed using an Alliance HPLC system with 996 Photodiode Array Detector (PDA) from Waters Associates[g]. Chromatographic separation of the compounds was accomplished with the use of a hypersil ODS C18 column[h] (5 µm, 4.6 × 150 mm) and a 12 % acetonitrile in 40 mM sodium acetate (pH 7.0) mobile phase at a flow of 2.0 ml/min. The UV detection wavelength was set at 263 nm. Standard curves were prepared by adding known amounts of AZT to blank cat serum and subjecting them to the same sample preparation procedure as described above. Standard curves were linear over a range of 0.1 µg/ml to 100 µg/ml.

Pharmacokinetic analysis

Plasma AZT concentration versus time data was analyzed by non-compartmental methods using Winnonlin[i]. The area under the plasma concentration-time curve (AUC)

from time zero to the last observed concentration was determined by the linear trapezoidal rule with extrapolation to time infinity by dividing the last concentration by the terminal elimination rate constant (λz). The terminal half-life ($t_{1/2}$) was calculated by $0.693/\lambda z$. The bioavailability after PO or IG administration was calculated by the ratio of AUC following PO or IG administration to AUC following IV administration. Statistical analysis was performed by using two-way ANOVA.

Results

Plasma concentrations of AZT following IV administration of 25 mg/kg AZT to cats are shown in Figure 2.1-a. Following IV administration, plasma concentrations of AZT declined rapidly with a terminal half-life of 1.4 ± 0.19 hr. Plasma AZT concentration-time profiles after PO and IG administrations are illustrated in Figure 2.1-b and 2.1-c, respectively. Rapid absorption of AZT after PO and IG administration was observed. The peak plasma AZT concentrations were achieved within 15 min after both PO and IG administration. The terminal half-life after PO and IG administrations were 1.4 ± 0.16 and 1.6 ± 0.26 hr, respectively.

The pharmacokinetic parameters after IV, PO and IG administration analyzed by the non-compartmental method are listed in Table 2.1. Total clearance averaged 0.41 ± 0.10 L/hr/kg and steady-state volume of distribution was 0.82 ± 0.15 L/kg. AZT was well absorbed after IG and PO administration with AUC values of 42.5 ± 9.41 mg×hr/L and 60.5 ± 17.0 mg×h/L. Interestingly, the bioavailability of AZT following IG administration (F = 0.70 ± 0.24) was less than that following PO administration (F = 0.95

± 0.23). This may be related to the location of the skin-level gastrostomy port through which the IG administration was given. The spleen was located cranial to the skin-level gastrostomy port in 3 cats and caudal to the gastrostomy port (the more desirable location) in 3 cats. Further, the solubility of in the relatively high concentrations of AZT as it entered the acid environment of stomach by IG bolus administration may also account for the lower bioavailability.

Transient hemolysis occurred after IV administration of AZT in cats weighing $\geq$ 3.5 kg. Gross hemolysis resolved within 2 hours. Two cats appeared anxious and restless during the injection of the last 20% of the drug dose volume. There was no evidence of bone marrow suppression detected with cytology.

Discussion

Though FIV was discovered in 1986, there is evidence suggesting that it has existed in cats for a long time. FIV has been found in cat populations worldwide, but the prevalence of infection depends on geographic location, and ranges between 2-12% among 'healthy' cats.[16-22] The percentage of cats infected can reach 48% in a sick cat population,[16-22] and even 83% among wild cat species in South Africa, East Africa, and North America.[23] As a significant health threat to the feline population, there is neither a cure nor a vaccine for FIV. Most FIV-infected cats are treated for the secondary infections by antibacterial and antifungal medications.[3,4] However as FIV progresses the effect of these treatments to the infections decreases with time. The search for effective antiviral drugs for FIV has been a task since this virus was isolated. Due to the similarity between FIV and HIV, the effective anti-HIV drugs are considered to be good candidates

for anti-FIV activity. Three types of anti-HIV agents are commercially available: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Most PIs used against HIV will not work for cats.[24] Almost all available NRTIs, including AZT, (-)-2'-deoxy-3'-thiacytidine (3TC), 2',3'-dideoxyinosine (DDI), 2',3'-dideoxycytidine (DDC), 2',3'-didehydro-2',3'-didexoythymidine (D4T), (-)-6-cyclopropylamino-2',3'-dihydro-2',3'-dideoxyguanosine (ABC) and 9-(2-phosphonylmethoxyethyl) adenine (PMEA), and one compound from NNRTIs (nevirapine) have been applied to treat FIV-infection by individual veterinary medicine practice, either alone or in combination with others (animalhealthchannel.com, www.specent.com/artg). However, there are few scientific studies to support these applications. Two of these compounds, AZT and PMEA were tested against FIV *in vitro* and *in vivo.* In a series of studies reported by Hartmann and Egberink, AZT and PMEA were shown to be potent and selective inhibitors of FIV in feline thymocytes with EC_{50} values of 0.05 and 0.60 μM, CD_{50} values of 120 and 80 μM.[4] Both compounds (2.5 and 5 mg/kg /12hr of PMEA, 5 and 10 mg/kg/12hr of AZT) were found to have significant therapeutic effect as indicated by improving clinical signs and raising the CD4/CD8 ratio in naturally infected cats.[5,6] However, there are still controversies related to the effectiveness and toxicity of AZT and PMEA in cats.[8,9,25-27] Meanwhile, the development of AZT-resistant virus has drawn some attention during the application of this drug. Smyth et al. reported that giving AZT 0.2, 1.0, 5, 25, or 50 mg/kg/day for 29 days to experimentally infected cats was much less effective than expected.[27] There are several other groups that showed similar results.[25,26] The discrepancy between these studies may be partly due to the susceptibility of the various stages of FIV infection.

Vahlenkamp et al. studied the effectiveness of AZT, PMEA, and (R)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine ((R)-PMPDAP, a derivative of PMEA) against FIV in primary feline thymocytes isolated from specific-pathogen-free cats and in Crandell feline kidney (CrFK) cells.[8] They found the EC_{50} and CD_{50} were dramatically different in these two kinds of cells. The EC_{50}s were 0.05, 0.5, 0.07 μM in thymocytes, and were 4.0, 1.5, 0.1 μM in CrFK cells. They suggested (R)-PMPDAP was a more potent and less toxic inhibitor of FIV *in vitro* and *in vivo.* These studies indicated that FIV-infection is a complicated disease and that more research needs to be done to find an effective therapeutic strategy.

In order to search for an effective treatment for FIV infection, activities of AZT, 3TC, (-)-2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC), and DDC were assessed in feline peripheral blood mononuclear cells for a clinical isolate of FIV, and a pathogenic molecular clone, FIV-pPPR.[7] AZT was the most effective agent among the tested compounds. The EC_{50}s of AZT for the clinical isolate and for FIV-pPPR were 0.07 ± 0.02 μM and 0.03 ± 0.02 μM, respectively.

This study serves as a part of a series of efforts looking for an effective anti-FIV compound for clinical veterinary practice. Currently in clinical veterinary practice, an oral or subcutaneous dose of 5 mg/kg of AZT twice daily has been recommended for maximum therapeutic effects and minimum toxic effects for long term application.[5,6] Previously, daily doses of 0.2 mg/kg to 50 mg/kg have been used for clinical studies in cats.[27] A single dose of 25 mg/kg was selected for this short term pharmacokinetic study based on interspecies scale up technique.[14] No bone marrow suppression was observed two weeks after this study.

The total plasma clearance of AZT after IV administration of a single-dose of 25 mg/kg to cats was 0.41 L/hr/kg smaller as compared to 1.6 L/hr/kg in rhesus monkeys, [12] 1.4 L/hr/kg in mice, [10] 2.0 L/hr/kg in rats, [11] and 3 L/hr/kg in humans.[28] The half-life of AZT in cats of 1.4 hr is longer than that in rhesus monkeys (0.5 hr), [12] rats (0.5 hr), [10] mice (1.1 hr) [10] and humans (1.0 hr).[29] This half-life is consistent with the previous reported half-life range of 1.3-1.9 hr for AZT in cats. [6] The lower clearance and prolonged half-life can be explained by the low activity of UDP-glucuronyltransferase in cat liver.[30] 5'-Glucuronyl-AZT (GAZT) is the most important metabolite of AZT accounting for 50-80% of total AZT elimination in humans. [29] In feline liver, glucuronidation is less efficient than in the human liver. [30] The volume of distribution at steady state, 0.82 L/kg, was comparable with that of other species; 1.1 L/kg in rhesus monkeys, [12] 1.0L/kg in rats, [11] 0.7 L/kg in mice, [10] but smaller than 1.6 L/kg in human. [28] Similar to the other species, AZT was rapidly absorbed after PO and IG administration. The bioavailability after PO and IG administration was greater than 70%.

In summary, administration of 25 mg/kg AZT to healthy cats resulted in plasma concentrations of AZT greater than EC_{50} for 12 hours following IV, IG, and PO administration. Further studies are needed to evaluate the clinical effectiveness of AZT in FIV-infected cats.

Footnotes

[a] Harlan, Indianapolis, IN, 46229.

[b] Rompun, Bayer Corp., Shawnee Mission, KS, 66201.

[c] Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, 50501.

[d] I-Cath, Charter Medical, Ltd., Lakewood, NJ, 08701.

[e] Sovereign, Sherwood Medical Industries, Tullamore, Ireland.

[f] Retrovir®, GlaxoWellcome Inc., Research Triangle Park, NC, 27709.

[g] Waters, Milford, Massachusetts 01757

[h] Alltech Associates, Deerfield, IL 60015

[i] Pharsight, Mountain View, California 94040

Table 2.1. Pharmacokinetic parameters of AZT following IV, IG, or PO 25 mg/kg single dose administration to healthy cats

Pharmacokinetic Parameters Mean ± SD	Administration Route IV	IG	PO
AUC (mg×h/L)	64.7 ± 16.6	42.5 ± 9.41*	60.5 ± 17.0
$t_{1/2}$ (hr)	1.4 ± 0.19	1.4 ± 0.16	1.6 ± 0.26
MRT (hr)	2.1 ± 0.33	2.1 ± 0.27	2.6 ± 0.60*
CL (L/h/kg)	0.41 ± 0.10		
Vss (L/kg)	0.82 ± 0.15		
F		0.70 ± 0.24*	0.95 ± 0.23

* : $p < 0.05$

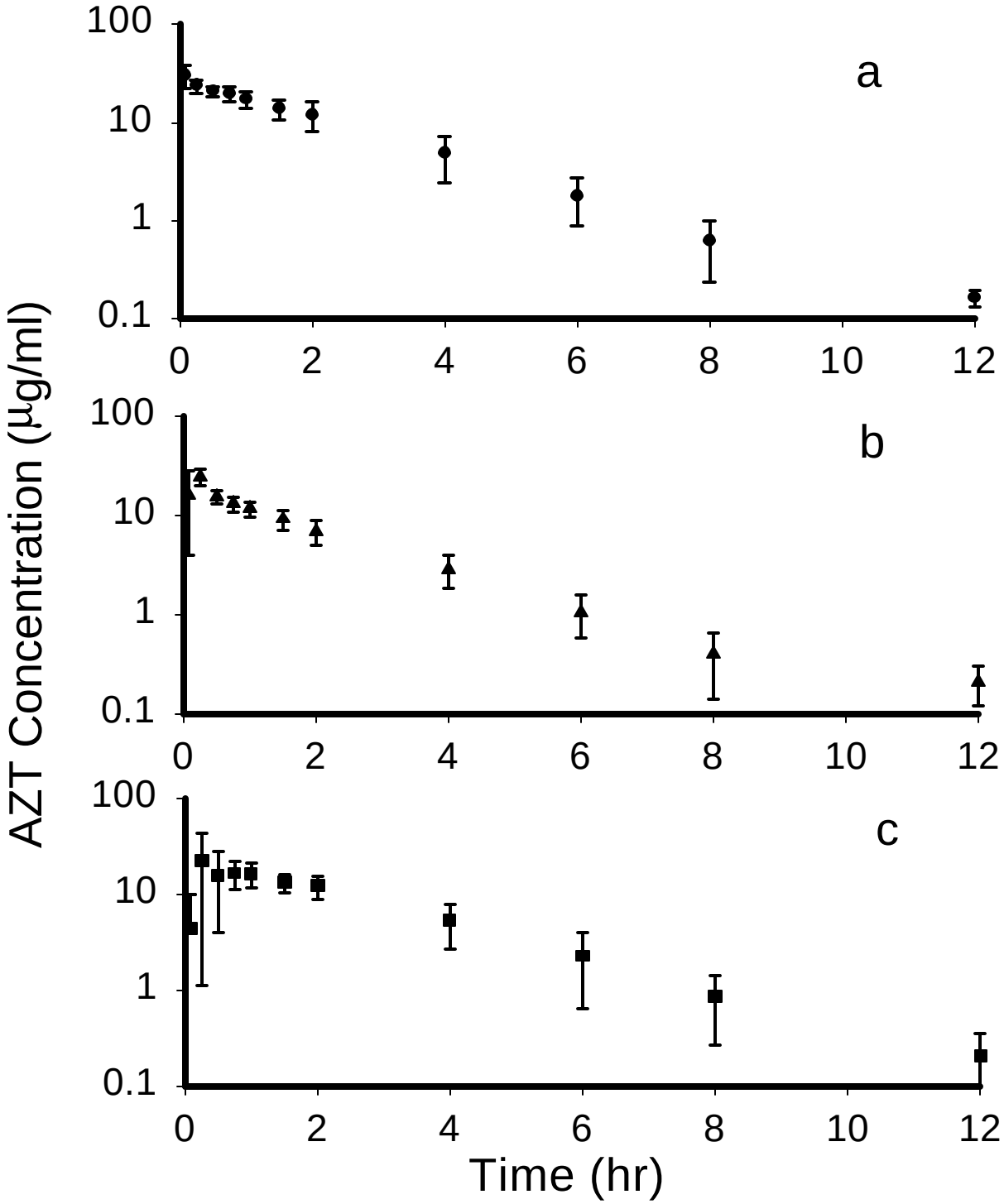

Figure 2.1. Concentration of AZT in cat plasma following administration of 25 mg/kg AZT by a: IV administration (filled circle), b: IG administration (filled triangle), and c: PO administration (filled square). Bars indicate the standard deviation.

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CHAPTER 3

PHARMACOKINETICS OF LAMIVUDINE IN CATS[1]

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PHARMACOKINETICS OF LAMIVUDINE IN CATS

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Abstract

Lamivudine ((-)- 2', 3'-dideoxy-3'-thiacytidine, 3TC), a nucleoside analogue applied in combination for the treatment of human immunodeficiency virus (HIV) infection and applied as monotherapy to treat hepatitis B virus (HBV) infection, may also be effective as a treatment for feline immunodeficiency virus (FIV) infection in cats. The purpose of this study was to characterize the pharmacokinetics of 3TC in normal cats. Six healthy cats (2.67 – 3.99 kg), randomly divided into three groups, were administered 25 mg/kg 3TC intravenously (IV), intragastrically (IG), and orally (PO) in a three-way cross over study design that included two-week washout periods between studies. The intragastric administration was achieved through skin-level low-profile gastrostomy ports. Plasma samples were collected for 12 hr and 3TC concentrations were determined by high performance liquid chromatography (HPLC). Bone marrow aspirates were examined for cytologic evidence of acute bone marrow suppression. Pharmacokinetic parameters were obtained by area-moment analysis. ANOVA was conducted using SAS ($p < 0.05$). Plasma concentrations of 3TC declined rapidly with a terminal half-life of 1.9 ± 0.21, 2.6 ± 0.66 and 2.7 ± 1.5 hr following IV, IG and PO administration to cats, respectively. Total body clearance and steady-state volume of distribution were 0.22 ± 0.09 L/hr/kg and 0.60 ± 0.22 L/kg, respectively. The area under the plasma 3TC concentration versus time curve (AUC) following IV, IG, and PO administration was 130 ± 55.2, 115 ± 97.5 and 106 ± 94.9 mg×hr/L. 3TC was well absorbed after IG and PO administration with bioavailability values of 0.88 ± 0.45 and 0.80 ± 0.52. Cytology of bone marrow aspirates was normal. The pharmacokinetics of 3TC in cats was similar to that in other species. At a dose of 25 mg/kg of 3TC, plasma concentrations of the

nucleoside analogue were maintained above the 50% inhibition concentration (0.14 µM for wild-type FIV) for at least 12 hr following IV, IG or PO administration.

The abbreviations used are: 3TC, lamivudine, (-)-2',3'-dideoxy-3'-thiacytidine; ABC, abacavir, (-)-6-cyclopropylamino-2',3'-dihydro-2',3'-dideoxyguanosine; AIDs, acquired immune deficiency syndrome; AUC, area under the curve; AZT, zidovudine, 3'-azido-3'-deoxythymidine; D4T, stavudine, 2',3'-didehydro-2',3'-dideoxythymidine; ddA, 2',3'-dideoxyadenosine; ddC, zalcitabine, 2',3'-dideoxycytidine; ddI, didanosine, 2',3'-dideoxyinosine; ddU, 2',3'-dideoxyuridine; FIV, feline immunodeficiency virus; FTC, 2',3'-dideoxy-5-fluoro-3'-thiacytidine; HBV, hepatitis B virus; HIV, human immunodeficiency virus; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; PMEA, adefovir, 9-(2-phosphonylmethoxyethyl) adenine; RT, reverse transcriptase.

Key words: (-)-2',3'-dideoxy-3'-thiacytidine (lamivudine, 3TC), cats, pharmacokinetics, feline immunodeficiency virus (FIV).

Introduction

(-)- 2',3'-Dideoxy-3'-thiacytidine (lamivudine, 3TC) is a dideoxynucleoside analogue with antiviral activities against human immunodeficiency virus (HIV) and hepatitis B virus (HBV). 3TC is approved for use in combination with other antiviral agents for the treatment of HIV infection and as monotherapy for the treatment of HBV infection. 3TC undergoes anabolic phosphorylation intracellularly to form lamivudine-

5'-triphosphate (3TC-TP) (Cammack *et al* 1992). The anabolic phosphorylation is a stepwise intracellular process catalyzed by a series of enzymes including deoxycytidine kinase, cytidine monophosphate kinase and deoxycytidine monophosphate kinase, and pyrimidine nucleoside diphosphate kinase (Cammack *et al* 1992). 3TC-TP prevents HIV-1 and HBV replication by competitively inhibiting viral reverse transcriptase (RT) and terminating proviral DNA chain extension.

Compared to other available nucleoside reverse transcriptase inhibitors (NRTIs), 3TC has potent activity against HIV-1, HIV-2, and HBV with low toxicity but synergistic activity with other anti-HIV compounds (Soudeyns *et al* 1991, Coates *et al* 1992, Coates *et al* 1992, Chang *et al* 1992). *In vitro*, 3TC has demonstrated activity against HIV-1 with a 50% inhibitory concentration (IC_{50}) ranging from 0.002 to 0.87 μM in various cell lines (Soudeyns *et al* 1991, Coates *et al* 1992). Most nucleoside analogs currently available for clinical use, including 3'-azido-3'-deoxythymidine (zidovudine, AZT), 2',3'-dideoxyinosine (ddI), 2',3'-dideoxycytidine (ddC), and 2',3'-didehydro-2',3'-dideoxythymidine (D4T), have clinically limiting toxicities such as peripheral neuropathy, myopathy, pancreatitis and hematologic toxicity (Parker and Cheng 1994). AZT and ddI are possibly related to liver toxicity as well (Parker and Cheng 1994). These toxicities are believed to be due to the inhibition of mitochondrial DNA replication by the 5'-triphosphates of the nucleoside analogues (Chen and Cheng 1992, Chen *et al* 1991, Parker and Cheng 1994). Though the phosphorylation steps of 3TC are similar to ddC, 3TC differs from ddC in that it does not inhibit mammalian mitochondrial DNA replication in intact cells. 3TC-TP is formed intracellularly; however, it is not transported into the mitochondria (Gray *et al* 1995). Therefore, 3TC is unlikely to induce

clinically important hematologic and hepatic adverse events, neuropathy or myopathy (Parker and Cheng 1994). The more favorable safety profile of 3TC than other available anti-HIV compounds has been confirmed by *in vitro* studies, animal toxicology studies and clinical trials (Lisignoli *et al* 1992, Sommadossi *et al* 1992, Tyrrell *et al* 1993, Lai *et al* 1998). In a long-term placebo-controlled study involving 358 patients, there were no serious drug-related adverse events observed with the administration of 3TC (Lai *et al* 1998). Another advantage to applying 3TC to antiviral therapy is its synergistic effects with other antiviral compounds. Mutant strains resistant to 3TC arise rather rapidly during monotherapy through the acquisition of a methionine-to-valine (M184V) or a methionine-to-isoleucine (M184I) mutation at codon 184 of the YMDD motif of HIV-1 RT (Schinazi *et al* 1993). This mutation is associated with a delay in the emergence of AZT-resistant mutants in AZT-naïve patients, restoration of AZT sensitivity in patients already possessing AZT-associated resistance mutations, impairment of HIV-1 replication capacity, and a reduced ability of the HIV-1 *env* gene to mutate (Larder *et al* 1995). This provides the rationale for combination therapy using 3TC and other anti-HIV compounds. The combination of 3TC, AZT and an HIV protease inhibitor has shown remarkable results in decreasing plasma viral loads and improving CD4+ cell counts of acquired immunodeficiency syndrome (AIDs) patients (Torres and Barr 1997).

Feline immunodeficiency virus (FIV) is the causative agent of immune deficiency symptoms similar to human AIDs in the feline population (Pedersen *et al* 1987). As FIV and HIV belong to the same subfamily of *Retroviridae*, the lentivirus family, FIV shares similar morphologic, physical and biochemical characteristics to HIV-1 (Pedersen *et al* 1987, North *et al* 1989). FIV RT is similar to RT from HIV-1 in

physical properties, catalytic activities, template specificity, Mg^{2+} requirement, and sensitivities to active forms of several RT-targeted antiretroviral compounds (North *et al* 1990a, North *et al* 1990b). These features not only make FIV an attractive *in vitro* and *in vivo* animal model for studies of HIV-1 pathogenesis and chemotherapy, but they also provide the rationale for considering currently available anti-HIV chemotherapeutic agents in veterinary medicine for treating FIV infection. In a study conducted by Smyth *et al*., 18 antiviral agents including 3TC, AZT, ddC, ddI, D4T, 2',3'-dideoxyuridine (ddU), and 2',3'-dideoxyadenosine (ddA) were tested for their activities against FIV in primary feline lymphocyte cell lines (Smyth *et al* 1994). Among these agents, 3TC was the most effective analogue with an IC_{50} value of 0.17 μM, and it was one of the drugs which demonstrated the least toxicity. The purpose of the current study was to characterize the pharmacokinetics of 3TC in cats following intravenous (IV), intragastric (IG), and oral (PO) administration. The IG route was investigated as an alternative for long term administration of 3TC to cats in clinical veterinary practice.

Materials and Methods

Cats

Six healthy intact 9-month-old barrier-reared[a] domestic short hair cats (3 male, 3 female) weighing 2.67 to 3.99 kg (mean ± SD, 3.30 ± 0.59) were used in the study. Cats were housed at the University of Georgia Veterinary Medical Animal Care Facility. Animal studies were approved by the University of Georgia Animal Care and Use Committee and were conducted in accordance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Complete blood

counts (CBC), serum biochemistry panels, and urinalyses were done on all cats within 3 days of their arrival. All cats had skin-level gastrostomy ports (Stevenson *et al* 2000) placed 2 weeks before the start of the study.

Study design

The study was done in accordance with a randomized 3-way crossover design, with 3 dosing periods and a 2-week washout period between doses. Doses were administered IV, IG, and PO. Twelve to 16 hours before drug administration, cats were anesthetized with intramuscular xylazine[b] (1.0 mg/kg) and ketamine[c] (12.5-15.0 mg/kg) for aseptic placement of 18.5-gauge jugular catheters[d]. A 20-gauge catheter[e] was also placed in the cephalic vein of cats scheduled for IV drug administration. Jugular catheters were flushed with 1 ml, and cephalic catheters with 0.5 ml, of 0.9% saline containing 5 units/ml sodium heparin.

Food was withheld for 24 hours before and 5 hours after drug administration. Water was withheld for 12 to 16 hours prior to drug administration but was freely available after drug was given. 3TC was purchased as tablets[f] and was dosed at 25 mg/kg for each cat, based on allometric interspecies scaling techniques. Oral 3TC was compounded for each cat and put in a gelatin capsule. For IG administration, a suspension of 3TC in 5 ml water was given through the gastrostomy tube and flushed with 10 ml water. 3TC for IV administration[g] was dissolved in 0.9% saline (10 mg/ml) and injected in the cephalic catheter as a bolus over 1 to 2 minutes.

Blood samples were collected from the jugular catheter immediately before and 0.08, 0.25, 0.50, 0.75, 1, 1.5, 2, 4, 6, 8, and 12 hr after drug administration. Sampling

was a 4-step procedure. Blood (2.5 – 3.0-ml) was aspirated from the jugular catheter into a sterile 3-ml syringe. A 0.3-ml sample for drug analysis was then collected into a tuberculin syringe and transferred into a heparinized polypropylene microcentrifuge tube. The initial 2.5 – 3.0-ml blood sample was returned to the cat through the jugular catheter, and the catheter was flushed with 1 ml heparinized saline. Heparinized blood samples were immediately centrifuged at 3000 g, and the harvested plasma was stored at –20^{o}C until analysis. Twenty-four hours after drug administration, a blood sample for CBC was collected from the jugular catheter before the catheter was removed.

Plasma samples were analyzed for 3TC concentrations by an established high performance liquid chromatographic (HPLC) method (Rajagopalan *et al* 1996). Briefly, 50 µl of plasma sample, 100 µl of internal standard (1 µg/ml 2',3'-didehydro-2',3'-dideoxythymidine, D4T) and 50 µl of 2 M perchloric acid were added to 1.5-ml polypropylene microcentrifuge tubes. After vigorously mixing, the tubes were centrifuged at 9500 g for 10 min. The supernatant was injected onto the HPLC column. The chromatography was performed using an Alliance HPLC system with 996 Photodiode Array Detector (PDA) from Waters Associates[h]. Chromatographic separation of the compounds was accomplished with the use of a Hypersil ODS C18 column[i] (5 µm, 4.6 × 250 mm) and a mobile phase of 4% acetonitrile in monobasic potassium phosphate buffer (pH 6.95) with 2 mM triethylamine at a flow of 1.5 ml/min. The absorbency was detected at 270 nm. Standard curves were prepared by adding known amounts of 3TC to blank cat serum and subjecting them to the same sample preparation procedure as described above. Retention times of 3TC and D4T were 7.8 and 12.9 minutes, respectively. Standard curves were linear over a range of 0.25 µg/ml to 25 µg/ml.

Plasma 3TC concentration versus time data was analyzed by non-compartmental methods using Winnonlin[j]. The area under the plasma concentration-time curve (AUC) from time zero to the last observed concentration was determined by the linear trapezoidal rule with extrapolation to time infinity by dividing the last concentration by the terminal elimination rate constant (λz). The terminal half-life ($t_{1/2}$) was calculated by $0.693/\lambda z$. The bioavailability of 3TC following PO and IG administration was calculated by the ratio of AUC after PO or IG administration to the AUC after IV administration. Statistical analysis was performed by two-way ANOVA.

Two weeks after the third dose of 3TC, cats were sedated with diazepam (0.12-0.2 mg/kg) and ketamine (2.5-4.0 mg/kg) IV and anesthetized with xylazine (1.0 mg/kg) and ketamine (12.5-15.0 mg/kg) IM. Bone marrow aspirates were obtained from the proximal left femur to evaluate cytology for bone marrow suppression.

Results

Plasma concentrations of 3TC following IV administration of 25 mg/kg to cats are shown in Figure 3.1-a. Following IV administration, concentrations of 3TC in plasma declined rapidly with a terminal half-life of 1.9 ± 0.21 hr. Plasma 3TC concentration-time profiles after IG and PO administrations are illustrated in Figure 3.1-b and 3.1-c, respectively. Rapid absorption after IG and PO administration was observed with peak plasma 3TC concentrations achieved at 30 minutes and 1 hr after IG and PO administration. The maximum plasma concentrations (C_{max}) following IG and PO administration of 3TC were 50.0 and 35.6 mg/L, respectively. The terminal half-lives of 3TC after IG and PO administration were 2.6 ± 0.66 hr and 2.7 ± 1.5 hr.

The pharmacokinetic parameters for 3TC following IV, IG and PO administration are listed in Table 3.1. Total clearance averaged 0.22 ± 0.09 L/hr/kg and steady-state volume of distribution was 0.60 ± 0.22 L/kg. 3TC was well absorbed after IG and PO administration with AUC values of 115 ± 97.5 mg×hr/L and 106 ± 94.9 mg×hr/L. There was no significant difference between the bioavailability of 3TC after IG (F = 0.88 ± 0.45) and PO (F = 0.80 ± 0.52) administration.

There was no evidence of bone marrow suppression detected with cytology.

Discussion

FIV has been identified worldwide in domestic and wild feline species (Pedersen *et al* 1987, Olmsted *et al* 1992). In the United States, FIV affects 1-4 % of healthy cats, and 13-15% of sick cats (Yamamoto *et al* 1989, O'Connor *et al* 1991). Neurologic, hematologic and immunologic abnormalities have been reported in FIV-infected cats (Pedersen and Barlough 1991, Bennett and Smyth 1992, Davidson *et al* 1993, Dua *et al* 1994, Phillips *et al* 1996). Along with these abnormalities, weight loss and a number of infections have been reported in FIV-infected cats, which leads to AIDs-like illness of cats within approximately 4 – 7 years of infection (Dua *et al* 1994). Cats with AIDs-like illness usually survive only 1 to 6 months (Pedersen and Barlough 1991). Most FIV-infected cats with clinical illness are treated symptomatically with antibacterial and antifungal medications for secondary infections. These treatments lose their effectiveness as FIV infection progresses (Pedersen and Barlough 1991, Egberink *et al* 1991, Bennett and Smyth 1992). FIV infection has been considered a useful model for investigating various aspects of HIV infection. While more anti-HIV chemotherapy

agents become available to AIDs patients, cats with FIV infection still suffer without many options for effective treatment.

FIV represents a significant health threat to the feline population; however, there has not been a safe and effective antiviral drug available for FIV treatment (Pedersen and Barlough 1991, Egberink *et al* 1991, Bennett and Smyth 1992). AZT and 9-(2-phosphonylmethoxyethyl) adenine (adefovir, PMEA) have been shown to inhibit FIV *in vitro* (Egberink *et al* 1991) and improve clinical signs in FIV-infected cats (Hartmann *et al* 1992,Hartmann *et al* 1995). However, controversy concerning the effectiveness and toxic side effects of AZT and PMEA in cats remains (Gregory *et al* 1997, Hayes *et al* 1993, Meers *et al* 1993, Smyth *et al* 1994, Vahlenkamp *et al* 1995). Further, the development of AZT-resistant virus has drawn concern with the clinical use of this drug.

3TC is one of the most potent nucleoside analogs against HIV and HBV (Soudeyns *et al* 1991, Coates *et al* 1992, Coates *et al* 1992, Chang *et al* 1992), and its activity against FIV in primary blood mononuclear cells (PBMC) has also been shown *in vitro* (Smyth *et al* 1994, McCrackin Stevenson and McBroom 2001). 3TC resistance in the immuno- and neuropathogenic molecular clone of FIV, FIV-pPPR, is similar to the 3TC resistance of HIV-1 that develops in 3TC-treated HIV-1-infected patients (McCrackin Stevenson and McBroom 2001). The similarity between the resistance to 3TC in FIV and HIV-1 makes it reasonable to consider 3TC, which has been used in combination chemotherapy in HIV-1 infection, in combination chemotherapy in FIV-infected cats. Recently, McCrackin Stevenson *et al*. studied the effects of AZT, 3TC, 2',3'-dideoxy-5-fluoro-3'-thiacytidine (FTC), and ddC on a natural isolate of FIV (FIV-Maxam), FIV-pPPR, and the 3TC-resistant mutant FIV-pPPR-M183V (McCrackin

Stevenson and McBroom 2001). The IC_{50} value of 3TC was 0.14 μM for the natural isolate, FIV-Maxam, and 0.46 μM for FIV-pPPR. The M183V mutation decreased the susceptibility to 3TC almost 100 times (IC_{50}, 38 μM). However, the M183V mutation (IC_{50} FIV-pPPR-M-183-V, 0.03 μM) did not change the susceptibility of FIV-pPPR to AZT (IC_{50} FIV-pPPR, 0.04μM). Preliminary *in vitro* studies showed that combination chemotherapy using AZT, 3TC, and an FIV-specific protease inhibitor was more effective in inhibiting FIV infection than AZT monotherapy in PBMC (Arai *et al* 1998). In a very recent *in vitro* study, the combination of AZT, 3TC, and abacavir, (-)-6-cyclopropylamino-2',3'-dihydro-2',3'-dideoxyguanosine (abacavir, ABC or 1592U89) effectively inhibited FIV replication (Bisset *et al* 2002). Limited information using AZT and 3TC in FIV-infected cats also suggested effectiveness against FIV-infection when applied together with bone marrow transplantation (BMT) (Yamamoto *et al* 1998). Further knowledge concerning the pharmacokinetics of 3TC in cats will provide valuable information for designing 3TC mono- or combination- chemotherapeutic dosage regimens for the treatment of FIV infection.

In general, the pharmacokinetics of 3TC in cats was comparable to that of other species, including humans. The volume of distribution of 3TC in cats (0.60 L/kg) is somewhat lower than that reported for humans (1.3 L/kg) (Hussey *et al* 1994). The half-life (1.9 hr) of 3TC following IV administration is shorter than that in humans (2.8 hr) (Hussey *et al* 1994). The total clearance of 3TC following IV administration to cats (0.22 L/hr/kg) is slightly lower than in humans (0.32 L/hr/kg). 3TC is primarily eliminated as unchanged drug through renal clearance (Rajagopalan *et al* 1996). The renal clearance of 3TC is greater than the glomerular filtration rate (GFR) indicating active renal tubular

secretion is involved in the elimination (van Leeuwen *et al* 1992). The creatine clearance for cats (2.7-2.8 ml/min/kg) (Brown *et al* 1996) is larger compared to that in humans (1.8 ml/min/kg). This may indicate the efficiency of the corresponding transporters present in cats is not as efficient as in humans. 3TC was well absorbed following both IG and PO administration with a bioavailability similar to the bioavailability following oral administration in humans (van Leeuwen *et al* 1992, Hussey *et al* 1994).

Overall, administration of 25 mg/kg 3TC to healthy cats maintained 3TC plasma concentrations greater than the 50% effective concentration (EC_{50}) for 12 hours after dosing. Further studies will be necessary to evaluate the long term effectiveness and toxicity of 3TC used in combination therapy for treating FIV infection in cats.

Footnotes

[a] Harlan, Indianapolis, IN, 46229.

[b] Rompun, Bayer Corp., Shawnee Mission, KS, 66201.

[c] Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, 50501.

[d] I-Cath, Charter Medical, Ltd., Lakewood, NJ, 08701.

[e] Sovereign, Sherwood Medical Industries, Tullamore, Ireland.

[f] Epivir®, GlaxoWellcome Inc., Research Triangle Park, NC, 27709.

[g] 3TC, purified powder, was a kind gift of GlaxoWellcome Inc., Research Triangle Park, NC, 27709

[h] Waters, Milford, Massachusetts 01757

[i] Alltech Associates, Deerfield, IL 60015

[j] Pharsight, Mountain View, California 94040

Table 3.1. Pharmacokinetic parameters of 3TC (25 mg/kg) following IV, IG, or PO administration to healthy cats

Pharmacokinetic Parameters Mean ± SD	Administration Route		
	IV	IG	PO
AUC (mg×h/L)	130 ± 55.2	115 ± 97.5	106 ± 94.9
$t_{1/2}$ (h)	1.9 ± 0.21	2.6 ± 0.66	2.7 ± 1.5
MRT (h)	2.2 ± 0.39	3.1 ± 0.57	3.9 ± 1.8
CL (L/h/kg)	0.22 ± 0.09		
Vss (L/kg)	0.60 ± 0.22		
F		0.88 ± 0.45	0.80 ± 0.52

* : $p < 0.05$

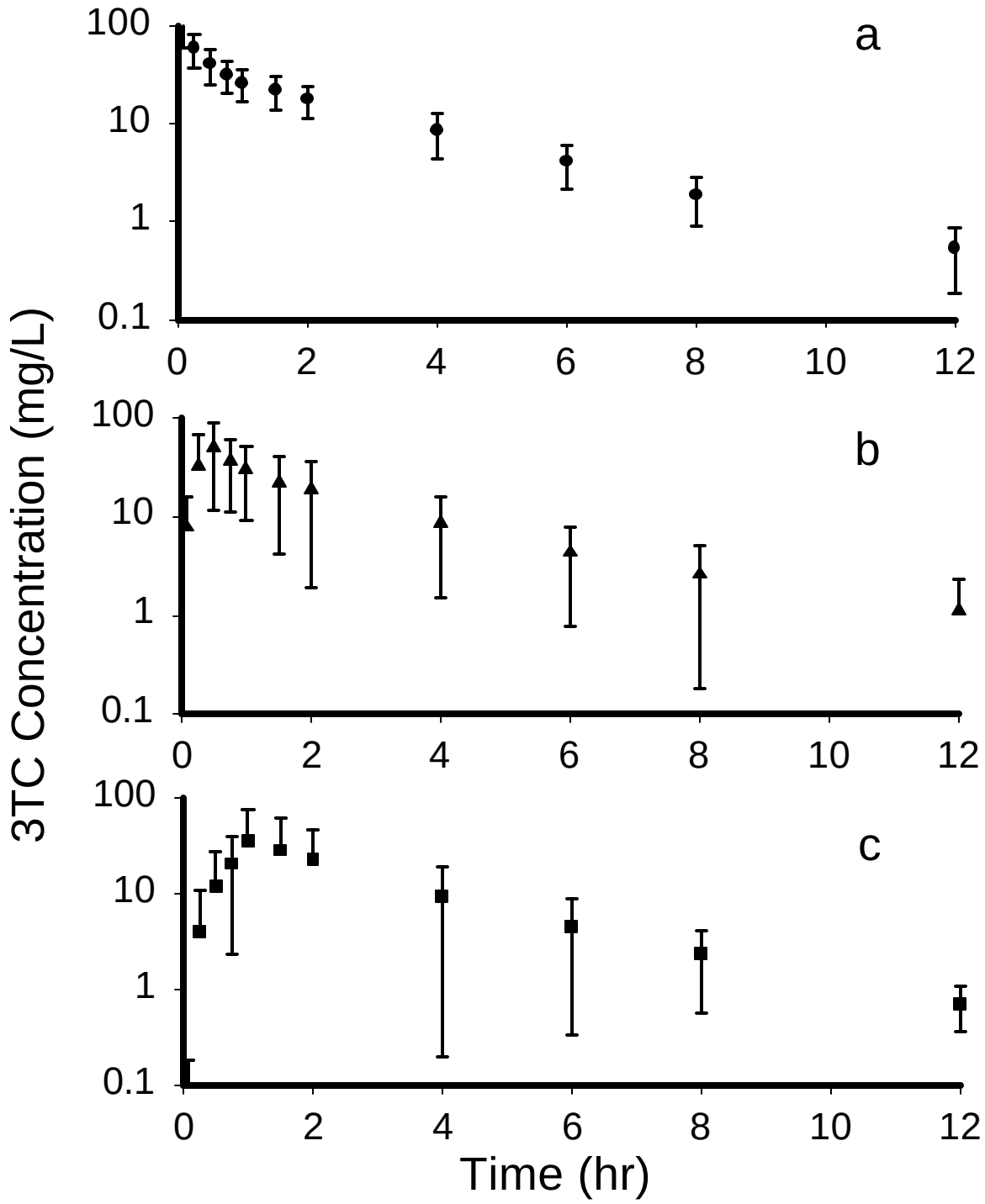

Figure 3.1. Concentration of 3TC in cat plasma following administration of 25 mg/kg 3TC by a: IV administration (filled circle), b: IG administration (filled triangle), and c: PO administration (filled square). Bars indicate standard deviation.

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CHAPTER 4

DISTRIBUTION OF ZIDOVUDINE AND LAMIVUDINE IN CAT BRAIN[1]

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DISTRIBUTION OF ZIDOVUDINE AND LAMIVUDINE IN CAT BRAIN

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Objective—To determine the brain distribution of zidovudine (3'-azido-3'-deoxythymidine, AZT) and lamivudine ((-)-2',3'-dideoxy-3'-thiacytidine, 3TC) in cats at steady state.

Animals—14 healthy intact 10-month-old barrier-reared[a] domestic short hair cats.

Procedure—Three cats were given 15 micron microspheres radio-labeled with 250 µCi of Strontium-85 to study the blood flow into specific cat brain area. The cats were sacrificed and the brains were taken immediately, and counted for the radioactivity in different region of brain. Five cats were received 7.0 mg/kg AZT loading dose, followed by three-hour infusion of AZT at 14.5 mg/kg/h through Intracath catheters. Six cats were given 7.0 mg/kg 3TC loading dose, followed by three-hour infusion of 3TC at 5.5 mg/kg/hr. Blood samples were collected from the jugular catheter immediately before drug administration and 1, 2, 3 hr afterwards. Cerebrospinal fluid (CSF) was collected at 3 hr during infusion and the cats were then euthanatized with pentobarbital. Brain samples were harvested immediately.

Results—Mean (± STD) plasma, CSF, and brain concentrations of AZT at steady state were 26.3 ± 4.21, 8.27 ± 1.21, 6.37 ± 0.61 mg/L, respectively. No significant difference of AZT concentrations was detected in different cat brain section. 3TC concentrations in plasma and CSF at steady states were 17.09 ± 5.26, and 1.73 ± 0.56 mg/L. Brain concentrations were undetectable for 3TC. The CSF/plasma ratios for AZT and 3TC were 0.32 ± 0.05 and 0.098 ± 0.01, respectively. The brain/plasma ratio of AZT, which was 0.25 ± 0.04, was similar to its value of CSF/plasma.

Conclusions—Both AZT and 3TC were able to penetrate into cat CSF, however the extent of AZT penetration was greater than that of 3TC. Significant amount of AZT also

distributed across cat blood brain barrier. In comparison, no detectable amount of 3TC was present in cat brain. AZT may be more effective to inhibit FIV infection in the central nervous system as it can penetrate the blood-CSF barrier and blood brain barrier to a significant extent.

The abbreviations used are: 3TC, lamivudine, (-)-2',3'-dideoxy-3'-thiacytidine; ABC, abacavir, (-)-6-cyclopropylamino-2',3'-dihydro-2',3'-dideoxyguanosine; AIDs, acquired immune deficiency syndrome; AZT, zidovudine, 3'-azido-3'-deoxythymidine; CSF, cerebrospinal fluid; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; RT, reverse transcriptase.

Key words: (-)-2',3'-dideoxy-3'-thiacytidine (3TC), 3'-azido-3'-deoxythymidine (AZT), brain, cats, cerebrospinal fluid (CSF), feline immunodeficiency virus (FIV), pharmacokinetics.

Introduction

Feline immunodeficiency virus (FIV) was isolated from cats with similar symptoms to humans with acquired immunodeficiency syndrome (AIDS) (Pedersen *et al* 1987). FIV belongs to the same lentivirus sub family of *Retroviridae* as human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). Cats with FIV-infection show a similar pattern of early cellular and humoral responses, period of

latency, and subsequent development of clinical disease as HIV-positive humans since FIV is similar to HIV in terms of morphologic, physical and biochemical characteristics. Although FIV has been used as an HIV model, veterinary medicine has benefited little from these investigations. A safe and effective antiviral drug is not available for FIV infection. Most FIV-infected cats with clinical illness are treated with empiric and supportive antibiotics. Due to the similarity between HIV and FIV, compounds with anti-HIV activity are good candidates in consideration for treatment of FIV-infection. Currently, there are three classes of anti-HIV compounds available: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). These compounds target two enzymes, reverse transcriptase and protease of HIV. Inhibition of one of these two enzymes will terminate the replication of HIV. Although FIV and HIV protease inhibitors share the identical catalytic mechanism and superimposable active site structure (Wlodawer *et al* 1995), FIV protease has a more restricted binding subsite that resembles many drug-resistant HIV proteases (Lee *et al* 1998). This property makes FIV protease a perfect model of drug-resistant phenotype of HIV protease to develop new inhibitors without resistance. However, it also means current PIs of HIV are not effective toward FIV infections. The genetic material of FIV and HIV is composed of RNA; the production of a DNA copy of the viral RNA is an essential step in the replication of FIV and HIV. The enzyme reverse transcriptase (RT) plays crucial role in this step. FIV reverse transcriptase (RT) is similar to RT from HIV-1 in physical properties, catalytic activities, template specificity, requirement of Mg^{2+}, and sensitivities to active forms of several RT-targeted antiretroviral compounds (North *et al* 1990a, North *et al* 1990b). Currently available

reverse transcriptase inhibitors with anti-HIV activities are considered for veterinary medicine in FIV infection.

Both 3'-azido-3'-deoxythymidine (zidovudine or AZT) and (-)- 2', 3'-dideoxy-3'-thiacytidine (lamivudine, 3TC) belong to NRTIs. AZT was the first commercially available drug for the treatment of HIV infection in humans. 3TC is a dideoxynucloside analogue with antiviral activities against human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV). The intracellular phosphorylation products of AZT and 3TC, AZT-triphosphate (AZT-TP) and 3TC 5'-triphosphate (3TC-TP), prevent HIV-1 replication by competitively inhibiting viral reverse transcriptase (RT) and terminating proviral DNA chain extension, which will finally terminate the replication of HIV. Favorable pharmacokinetic profiles of AZT and 3TC in cats have been demonstrated in previous studies in our lab.

Similar to HIV infection in humans, both central and peripheral neurologic diseases have been observed in FIV infections. One third of FIV-infected cats showed neurologic dysfunction in a three-year study. Most common neurologic abnormalities were altered behavior (Dow *et al* 1992). Symptoms may be slight or severe as obvious central and peripheral neurologic disease, such as seizures, striking behavior changes, anisocoria, or paresis. Therefore, the penetration of anti-virus compound to the nervous system, especially the central nervous system (CNS), will be a critical factor to consider while investigating the effective treatment of HIV or FIV. In this study, the distribution of AZT and 3TC in cat cerebrospinal fluid (CSF) and brain tissue brain after intravenous infusion was studied at steady state as part of efforts to develop these compounds for use in the treatment of FIV-infection in cats.

Materials and Methods

Cats

Fourteen healthy intact 10-month-old barrier-reared[a] domestic short hair cats (7 male, 7 female) weighing 2.44 to 4.35 kg (mean ± SD, 3.45 ± 0.76) were used in the study. Cats were housed at the University Veterinary Medical Animal Care Facility. Animal studies were approved by the University of Georgia Animal Care and Use Committee and were conducted in accordance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Cats complete blood counts (CBC), serum biochemistry panels, and urinalyses were done on all cats..

Study design

Three cats (one male, two female) were injected with radiolabelled 15 micro microspheres[b] to study the cerebral blood distribution. Microspheres labeled with 250 microCuries (μCi) of Strontium (Sr) 85 in 0.9% saline and 0.01% Tween were injected into the left atrium. The brain tissue was soon harvested in 10-15 minutes. The brain was then sliced into 23 slices (numbered 1 through 23) and on each even slice, two to six small spots (labeled A, B, C, D, E, F, respectively) were punched out to be samples as indicated in Figure 4.1 while frozen. For example, sample number 16F means sixth spot on cat brain slice 16. The brain spots were then placed in vials and counted using an ICN Isomedic Gamma Counter[c] for their radioactivity. The radioactivity in each spots was then compared to estimate whether the volume of blood was different among different regions of cat brain.

The rest of the cats were randomly divided into two groups for AZT (five cats) and 3TC (six cats) study. An 18.5-gauge jugular catheter[d] was placed in the aseptic vein for collecting blood samples under anesthetization with intramuscular xylazine[e] (1.0 mg/kg) and ketamine[f] (12.5-15.0 mg/kg). A 22-gauge Intracath catheter[g] was also placed by surgical cut-down into the medial saphenous vein of a rear leg for drug administration, in which the tip of the catheter is located in the caudal vena cava. Jugular catheters were flushed with 1 ml, and Intracath catheters with 0.5 ml, of 0.9% saline containing 5 units/ml sodium heparin.

Dosing

Food was withheld for 24 hrs before and after drug administration. Water was withheld for 12 to 16 hrs prior to drug administration but was freely available after drug was given. AZT[h] solution (10 mg/ml) in IV formulations was diluted to 6.67 mg/ml with 5% dextrose in water as recommended by the manufacturer and injected in the cephalic catheter as a bolus (7.0 mg/kg AZT) over 1 to 3 min as a loading dose. This was followed by an intravenous infusion of the same diluted AZT solution for 3 hrs at a rate of 14.5 mg/kg/hr. The loading dose of 3TC[i] (7.0 mg/kg) was diluted to 10.0 mg/ml with 0.9% saline and injected in the cephalic catheter as a bolus over 1 to 3 minutes. The infusion of 5.0 mg/ml 3TC in 0.9% saline was given for 3 hrs at rate 5.5mg/kg/hr.

Sample collection

Blood samples were collected from the jugular catheter immediately before drug administration and 1, 2, 3 hr afterwards. Sampling was a 4-step procedure. A 2.5-ml

blood sample was aspirated from the jugular catheter into a sterile 3-ml syringe. Next, a 0.3-ml sample for drug analysis was collected into a tuberculin syringe and transferred into a heparinized polypropylene microcentrifuge tube. The initial 2.5-ml blood sample was then returned to the cat through the jugular catheter. Finally, the catheter was flushed with 1 ml heparinized saline. Heparinized blood samples were immediately centrifuged at 3000 g, and the harvested plasma was stored at -20^0C until analysis. Cats were sedated with diazepam (0.12-0.2 mg/kg) and ketamine (2.5-4.0 mg/kg) IV and anesthetized with xylazine (1.0 mg/kg) and ketamine (12.5-15.0 mg/kg) IM at end of infusion. The cerebrospinal fluid (CSF), and brain were immediately (within 10 to 15 minutes) harvested and stored at -80^0C. The brain spots were then made by the same procedure as used in radiolabelled microspheres study.

Sample preparation and analysis

Brain tissue was homogenized with two volumes (1:2, g:ml) of ice cold isotonic phosphate buffer, pH 7.4 in a 1.5 ml polypropylene microcentrifuge tubes. Pellet Pestle Motor[j] and Pestle Pellet Blue Polyprop[j] were used to homogenize the brain.

Concentrations of AZT in plasma and CSF samples were determined by an established high performance liquid chromatographic (HPLC) method (Manouilov *et al* 1995). Briefly 100 µl of plasma sample, 50 µl of internal standard (5 µg/ml 3'-azido-2',3'-dideoxy-5-ethyluridine, CS-85) and 50 µl of 2 M perchloric acid were added to 1.7 ml polypropylene microcentrifuge tubes. After vigorously mixing, the tubes were centrifuged at 9000 g for 10 min. The supernatant was directed injected onto HPLC. The chromatography was performed using an Alliance HPLC system with 996 Photodiode

Array Detector (PDA) from Waters Associates[k]. Chromatographic separation of the compounds was accomplished with the use of a hypersil ODS C18 column[l] (5 um, 4.6 × 150 mm) and a 12 % acetonitrile in 40 mM sodium acetate (pH 7.0) mobile phase at a flow of 2.0 ml/min. The UV detection wavelength was set at 263 nm. Standard curves were prepared by adding known amounts of AZT to blank cat serum and subjecting them to the same sample preparation procedure as described above. Standard curves were linear over a range of 0.1 μg/ml to 100 μg/ml.

AZT concentrations in brain tissues were determined by the method of Doshi *et al* (Doshi *et al* 1989). Briefly, the 100 μl of brain homogenate together with 10 μl internal standard (40 μg/ml CS-85) and 50 μl of 2 M perchloric acid were votexed for about 30 sec. The tubes were then centrifuged at 9000 g for 10 min, and 80 μl of the supernatant was directly injected onto the HPLC system. Standard curves were prepared by mixing cat plasma with a known amount of AZT subjected with the same procedure as above. The extraction difference between plasma and brain tissue was corrected by adding a known amount of AZT to blank brain tissue homogenate, which went through the extraction procedure and HPLC analysis. The ratio of the measured value and the true value was used to correct the cat brain concentration of AZT from the HPLC analysis.

3TC plasma and CSF samples were analyzed by an established HPLC method for 3TC concentrations (Rajagopalan *et al* 1996). Briefly 50 μl of plasma sample, 100 μl of internal standard (1 μg/ml D4T) and 50 μl of 2 M perchloric acid were added to 1.5 ml polypropylene microcentrifuge tubes. After vigorously mixing, the tubes were centrifuged at 9500 g for 10 min. The supernatant was directly injected onto the HPLC column. The chromatography was performed using an Alliance HPLC system with 996

Photodiode Array Detector (PDA) from Waters[k]. Chromatographic separation of the compounds was accomplished with the use of a hypersil ODS C18 column[l] (5 um, 4.6 × 250 mm) and a mobile phase of 4 % acetonitrile in monobasic potassium phosphate buffer (pH 6.95) with 2 mM triethylamine at a flow of 1.5 ml/min. The detection wavelength was at 270 nm. Standard curves were prepared by adding known amounts of 3TC to blank cat serum and subjecting them to the same sample preparation procedure as described above. Retention times for 3TC and D4T were 7.8 and 12.9 respectively. The standard curves were linear over a range of 0.25 μg/ml to 25 μg/ml.

Concentrations of 3TC in brain tissue were determined by a slight modification of the same method. Briefly, 10 μl internal standard (40 μg/ml D4T) and 50 μl of 2 M perchloric acid were added to 100 μl of brain homogenate in 1.7 ml polypropylene microcentrifuge tubes, the tubes were then vortexed about 30 sec and centrifuged at 9000 g for 10 min. 80 μl of the supernatant was directly injected onto the HPLC system. Standard curves were prepared by cat plasma with known amount of 3TC subjected to the same procedure as above.

Results

Radio-labeled microspheres study showed no significant difference of radioactivity in various region of cat brain (data not shown), indicating roughly even distribution of blood flow to cat brain. However, a relatively high level of inter-individual difference has been observed.

The distribution of AZT in cat brain at steady state is presented in Table 4.1. Significant levels of AZT have been detected in cat all brain spots with a total average

value of 6.37 ± 0.61 (mean ± SD). No significant difference was detected among brain spots. Similar to radiolabeled microspheres, inter-individual variance was relatively high. The 3TC was undetectable in all cat brain spots, possibly due to the low amount present and the limited assay sensitivity.

The average concentrations of AZT and 3TC present in plasma, CSF and brain spot at steady state, as well as ratios of CSF/plasma and brain/plasma are shown in Table 4.2. The concentrations of AZT in plasma and CSF were 26.3 ± 4.21 and 17.09 ± 5.26 mg/L, respectively. The corresponding concentrations of 3TC were 17.09 ± 5.26 and 1.73 ± 0.56 mg/L. The CSF/plasma ratios of AZT and 3TC were dramatically different with values 0.32 ± 0.05 and 0.098 ± 0.01. The brain/plasma ratio of AZT (0.25 ± 0.04) is close to its CSF/plasma ratio.

Discussion

As a significant health threat to feline population, FIV has been identified worldwide in domestic and wild feline species (Grindem *et al* 1989, Hopper *et al* 1991, Hosie *et al* 1989, Ishida *et al* 1988, O'Connor Jr. *et al* 1991, Shelton *et al* 1989, Yamamoto *et al* 1989). Though the prevalence of FIV is different by geography location, the general infected rate is around 1-12% throughout the world. The infection rates are significantly higher in wild and sick feline populations. FIV has been considered a useful model for HIV since its discovery, however, there is no effective treatment for FIV infection as fourteen compounds are available for HIV infection. The cats with FIV will die from a terminal AIDS-like illness though supportive antibacterial and antifungal medications are applied to treat secondary infection. Some anti-HIV compounds have

been applied to FIV infected cats by individual practice, however, there is no systemic study to support the application (animalhealthchannel.com, www.specent.com/artg).

AZT, the first anti-HIV agent, is a potent and selective inhibitor of FIV *in vitro*. The 50% effective concentrations (EC_{50}) of AZT for FIV was 0.07 μM in feline peripheral blood mononuclear cells (McCrackin Stevenson *et al* 2001), 0.05 μM in feline thymocytes (Egberink *et al* 1991, Vahlenkamp *et al* 1995), 4.0 μM in Crandell feline kidney (CrFK) cells (Vahlenkamp *et al* 1995). The CD_{50} (50% cytotoxic concentration, the concentration of compound reducing the number of viable cells by 50%) was reported 110-120 μM in thymocytes (Egberink *et al* 1991, Vahlenkamp *et al* 1995) and greater than 200 μM in CrFK cells (Vahlenkamp *et al* 1995). Hartmann *et al* also showed the therapeutic effect of AZT (5 and 10 mg/kg/12 hr) in naturally infected cats (Hartmann *et al* 1992, Hartmann *et al* 1995). However, there are still controversies about the effectiveness and toxicity of AZT to treat FIV-infected cats (Gregory *et al* 1997, Hayes *et al* 1993, Meers *et al* 1993, Smyth *et al* 1994a, Vahlenkamp *et al* 1995, Hayes *et al* 1995, Hayes *et al* 2000). Though the toxicity and emergence of drug resistant strains to AZT limited its application in HIV infection, AZT remain one of most often used anti-HIV compounds, especially in combination therapy.

3TC is one of most potent nucleoside analogs against HIV and HBV, meanwhile its less toxicity and synergic action with other antiviral compounds make it a more valuable antiviral agent. 3TC has demonstrated activity against HIV-1 with EC_{50} ranging from 0.002 to 0.87 μM/L in various cell lines (Soudeyns *et al* 1991, Coates *et al* 1992). Smyth *et al* reported 3TC as the most effective antiviral agent against FIV among the eighteen agents tested in primary feline lymphocyte cell lines with the lowest EC_{50} (0.17

μM) (Smyth *et al* 1994b). This value was consistent with a later report of 0.14 μM EC_{50} in feline peripheral blood mononuclear cells (McCrackin Stevenson *et al* 2001). Different from most nucleoside analogs, 3TC does not inhibit mammalian mitochondrial DNA replication; thereby 3TC is unlikely to induce marked toxicity in clinical application. The development of 3TC resistant strains of FIV is similar to that of HIV, in which 3TC has been approved for its therapeutic effect by combining with other antiviral agents. The emergence of 3TC resistant strains of HIV has remained the susceptibility of HIV to other antiviral agents. Similar results were demonstrated for FIV in feline peripheral blood mononuclear cells, implying combination chemotherapy may be more effective than monotherapy (McCrackin Stevenson *et al* 2001). Recent *in vitro* study demonstrated that combination of AZT, 3TC, and abacavir (ABC) was more effective in suppressing FIV replication than monotherapy (Bisset *et al* 2002).

Similar to most lentivirus, FIV is a neurotropic virus and has been associated with some neurologic malfunctions, which often present as behavioral changes (Dow *et al* 1990, Pedersen *et al* 1989, Yamamoto *et al* 1989). The clinical signs include dementia, twitching movements of the face and tongue, psychotic behavior (hiding, rage, aggression), loss of toilet training, and compulsive roaming, or as severe as seizures (Pedersen *et al* 1989). This abnormality is believed to be directly related to cortical involvement as pathology studies have shown the most lesions present in the cerebral cortex in FIV-infected cats (Pedersen *et al* 1989). The presence of FIV in CSF and brain tissue of FIV-infected cats further confirmed the penetration of FIV through blood brain barrier (BBB) and blood-CSF barrier into central nervous system (Dow *et al* 1990, Yamamoto *et al* 1989). Thereby, distribution of antiviral agents into the central nervous

system (CNS) is an important requirement. The results from the current study were consistent with a previous report that the extent of penetration of nucleoside analogs into CSF depends on the nucleobase structure rather than the lipid solubility (Collins *et al* 1988). The CSF/plasma ratio for AZT was significantly higher than that of 3TC, which corresponded to the fact that thymidine derivatives penetrate CSF much better than cytidine derivatives. The CSF/plasma ratio of AZT in cats (0.32) is comparable with values of 0.21 in rhesus monkeys (Collins *et al* 1988) and 0.24 in human (Balis *et al* 1989). The brain/plasma ratio of AZT in cats (0.25) was consistent with the previous reported value in mice (0.28) (Doshi *et al* 1989). The CSF/plasma ratio of 3TC in cats (0.098) was also similar with the value demonstrated in rhesus monkeys (0.079) (Blaney *et al* 1995). The level of 3TC in cat brain was undetectable using the current assay, however, the compound may be present in the tissue at concentrations below the limit of quantitation of the assay.

The distribution of antiviral compounds to CSF and brain tissue is pivotal to eradicate the virus. The concentrations of antiviral compounds present in CSF and brain tissue reflect the net effect between influx and efflux through blood-CSF barrier and BBB. Though the mechanism of antiviral nucleoside analogs into CNS is unclear, studies demonstrated that both passive diffusion and active transport were involved in the process. Unlike that of the endogenous nucleoside, passive diffusion has been indicated to be the major entry route of antiviral nucleoside analogs to CNS (Sawchuk and Yang 1999). The CSF/plasma ratios are independent of octanol/buffer ratios of antiviral nucleoside analogs, indicating the involvement of active transport systems (Collins *et al* 1988). The exact transport system is yet to be determined, but it appears that multi-drug

resistance protein (MRP)-like transporters may be involved in the CNS (Sawchuk and Yang 1999). MPR1 and MPR2 have been identified to transport organic anions (Keppler *et al* 1998). The fact that probenecid, a known inhibitor of MRPs, increased the distribution of some nucleoside analogs further demonstrated the existence of active efflux transport systems in the blood-CSF barrier and BBB (Wong *et al* 1992). The expression of MRP1 in cerebral endothelial cell lines has been confirmed by two independent studies (Han *et al* 1998, Kusuhara *et al* 1998). The possible role of MRP1 in the distribution of AZT in CNS has been investigated by a preliminary study (Miller and Maier 1995). The various CSF/plasma ratios among different nucleoside analogs imply that more than one transporter is possibly involved in the efflux of these compounds into CSF.

In summary, following intravenous infusion, AZT and 3TC penetrated into cat CSF yielding CSF/plasma ratios of 0.32 and 0.098, respectively. Significant levels of AZT were also present in different areas of cat brain tissue. This study will assist in designing an appropriate chemotherapy strategy to treat FIV-infected cats.

Footnotes

[a] Harlan, Indianapolis, IN, 46229.

[b] New England Nuclear Life Science Products, Inc. Boston, MA 02118

[c] ICN Biomedicals Inc. Costa Mesa, CA

[d] I-Cath, Charter Medical, Ltd., Lakewood, NJ, 08701

[e] Rompun, Bayer Corp., Shawnee Mission, KS, 66201.

[f] Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, 50501.

[g] Sovereign, Sherwood Medical Industries, Tullamore, Ireland.

[h] Retrovir®, GlaxoWellcome Inc., Research Triangle Park, NC, 27709.

[i] Epivir®, GlaxoWellcome Inc., Research Triangle Park, NC, 27709.

[ji] Kontes Glass Company, Vineland, New Jersey 08360

[k] Waters, Milford, Massachusetts 01757

[l] Alltech Associates, Deerfield, IL 60015

Figure 4.1. Sampling of cat brain spot (A, B, C, D, E, F) from even number of cat brain slices.

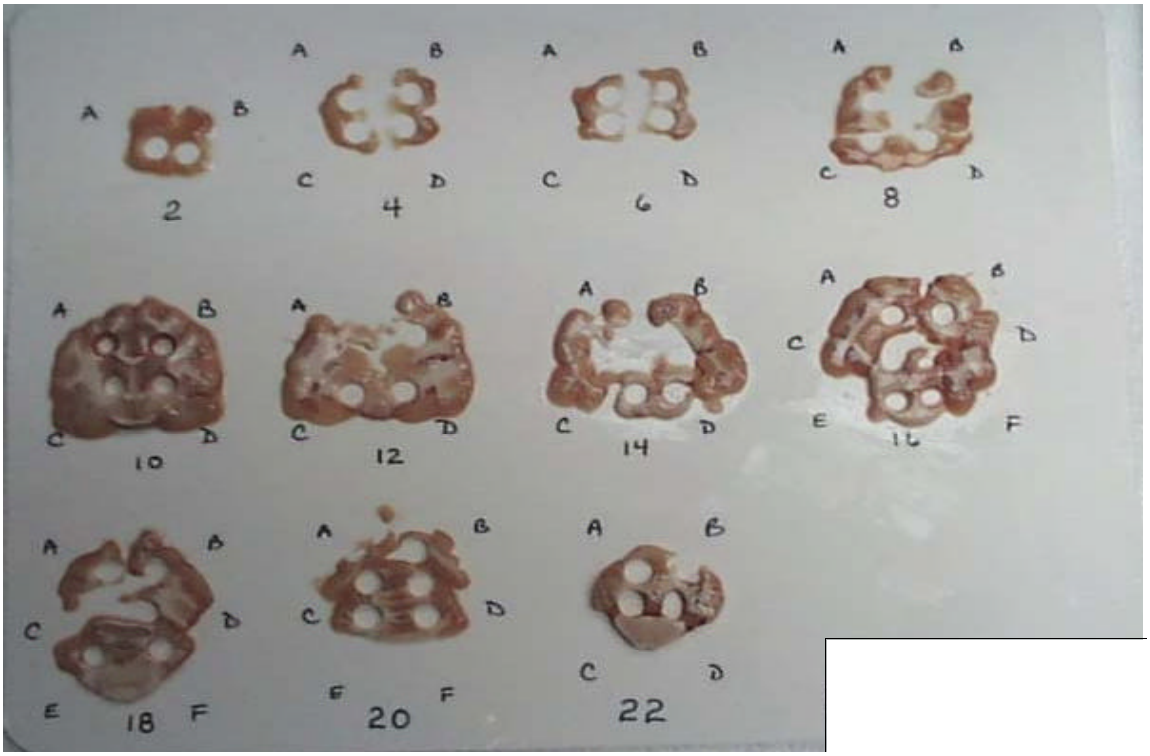

Table 4.1. AZT concentrations in different brain spots of cats. (n=5)

Brain Spot	Mean (mg/g)	SD		Brain Spot	Mean (mg/g)	SD
2A	7.81	1.52		14C	7.59	2.18
2B	7.07	1.06		14D	6.66	1.32
4A	5.36	0.93		16A	6.64	1.71
4B	5.62	1.19		16B	5.78	1.14
4C	4.81	1.60		16C	6.97	0.77
4D	5.17	1.89		16D	6.82	0.67
6A	5.03	1.14		16E	6.41	1.11
6B	5.37	1.32		16F	5.72	0.55
6C	4.98	1.67		18A	6.52	1.34
6D	6.25	3.19		18B	5.92	0.76
8A	4.69	1.76		18C	7.35	1.04
8B	5.12	1.03		18D	7.45	1.54
8C	5.57	1.23		18E	6.44	0.65
8D	6.22	1.06		18F	6.59	1.50
10A	6.91	1.50		20A	7.32	1.59
10B	6.93	1.01		20B	7.45	1.06
10C	6.41	1.42		20C	6.73	1.96
10D	5.40	0.83		20D	6.73	1.70
12A	5.57	0.79		20E	6.75	0.90
12B	5.85	0.82		20F	6.54	0.88
12C	5.93	0.52		22A	6.68	1.46
12D	6.04	1.13		22B	6.75	1.43
14A	7.25	1.57		22C	7.67	0.91
14B	7.63	1.70		22D	7.40	0.95

Table 4.2. Plasma, CSF and brain concentrations of AZT and 3TC after intravenous infusion to cats, and the ratios of CSF/plasma, brain/plasma.

Compound Mean ± SD	Concentrations (Mean ± SD) (mg/L or mg/g)				
	Plasma	CSF	Brain	CSF/plasma	Brain/plasma
AZT[a]	26.3 ± 4.21	8.27 ± 1.21	6.37 ± 0.61	0.32 ± 0.05	0.25 ± 0.04
3TC [b]	17.09 ± 5.26	1.73 ± 0.56	ND	0.098 ± 0.01	ND

[a] : n = 5; [b] : n = 6;

ND: Concentrations of 3TC undetectable in cat brain.

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CHAPTER 5

PHARMACOKINETICS OF (-)-β-D-2,6-DIAMINOPURINE DIOXOLANE AND DIOXOLANE GUANOSINE IN DUCKS[1]

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PHARMACOKINETICS OF (-)-β-D-2,6-DIAMINOPURINE DIOXOLANE AND DIOXOLANE GUANOSINE IN DUCKS

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ABSTRACT

(-)-β-D-2,6-Diaminopurine dioxolane (DAPD) and its active metabolite (-)-β-D-dioxolane guanosine (DXG) are promising antiviral compounds. The pharmacokinetics of DAPD and DXG have been previously characterized in rats, rhesus monkeys, and woodchucks. The purpose of this study was to evaluate the pharmacokinetics of DAPD and its metabolite, DXG, in ducks. Four adolescent ducks (34 days of age) were used in the studies. Two ducks received 50 mg/kg DAPD intravenously and two ducks were given the same dose orally. Blood samples were collected prior to DAPD administration

and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hr following dosing. Plasma concentrations of DAPD and DXG were determined by HPLC assay. Plasma concentration-time data were analyzed using noncompartmental analysis. DAPD concentrations were below the limit of quantitation except for the first two samples from the intravenously dosed ducks. The area under the curve (AUC) of metabolite DXG was 17.08 and 13.86 mg·hr/L following intravenous administration of DAPD and 18.26 and 23.11 mg·hr/L following oral administration. The C_{max} of DXG was achieved at 0.50 and 0.25 hr after intravenous DAPD and 0.50 hr in both ducks receiving DAPD orally. The half-life of DXG was 0.33 and 0.56 hr after intravenous DAPD and 0.85 and 0.86 hr following oral dosing of DAPD. DAPD was rapidly metabolized to the active metabolite DXG followed by both intravenous and oral administration to ducks. The higher AUC and longer half-life of DXG following oral administration are likely due to first pass metabolism and slower absorption of DAPD.

The abbreviations used are: 3TC, lamivudine, L-2'-deoxy-3'-thiacytidine; ADA, Adenosine deaminase; AUC, area under curve; D4T, 2',3'-didehydro-2',3'-didexoythymidine; DAPD, (-)-β-D-2,6-Diaminopurine dioxolane; DHBV, duck hepatitis B virus; DXG, (-)-β-D-dioxolane guanosine; HBV, hepatitis B virus; HIV, human immunodeficiency virus.

Key words: (-)-β-D-2,6-Diaminopurine dioxolane (DAPD), duck hepatitis B virus (DHBV), ducks, (-)-β-D-dioxolane guanosine (DXG), pharmacokinetics.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health problem worldwide. Approximately 5% of the world's population (400 million people) are chronic carriers of HBV, which is strongly associated with acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Maynard et al., 1988; Ayoola et al., 1988; Mahoney, 1999; Mast et al., 1999; Lok, 2000). Among the chronic carriers, 40% of these patients will die from HBV-related diseases, thus making HBV the ninth leading cause of death throughout the world. Though it had been realized for more than 30 years that HBV is a cause of viral hepatitis, until 1999 interferon (IFN)-α was the only Food and Drug Administration (FDA) approved treatment for chronic hepatitis B infection (Farrell, 2000; Shaw and Locarnini, 2000). However, IFN-α is only effective in 30-50% of the patients, and various side effects, relatively high expense and administration by injection limit its use (Saracco and Rizzetto, 1997; Farrell, 2000). A better understanding of HBV replication during the past decade has enhanced efforts to discover more effective chemotherapy for chronic HBV infection (Nassal and Schaller, 1996). The many nucleoside analogues recently synthesized for potential chemotherapy for human immunodeficiency virus (HIV) and herpesvirus infections provides an abundant compound resource to develop successful chemotherapy for the treatment of HBV infection (Hong et al., 1998; Kinchington, 1999; Shaw and Locarnini, 2000; Farrell, 2000). Though some of compounds have shown anti-HBV activity, additional drugs will still be necessary to effectively treat HBV infection. For example, (-)-2',3'-dideoxy-3'-thiacytidine (lamivudine, 3TC) is well tolerated and effectively inhibits HBV replication during the course of treatment, but the rebound of viral replication during prolonged

therapy and the development of drug resistance to lamivudine have been a problem (Berenguer and Wright, 1998; Marzano et al., 2001). Therefore, the search for additional safe and effective oral therapies for chronic HBV infection, either mono- or coactive-therapy, remains a task of health professionals. As more nucleoside analogues have been investigated for anti-HBV activity, (-)-β-D-2,6-diaminopurine dioxolane (DAPD) appears to be one of the most promising anti-HBV agents due to it potent activity and low toxicity (Kim et al., 1993; Schinazi et al., 1994; Furman et al., 2001). DAPD is an effective inhibitor of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), HBV, and Simian Immunodeficiency Virus (SIV) *in vitro*. The EC_{50} (effective concentration) of DAPD is 0.09 μM against HBV DNA replication intermediates or HBV virion synthesis inhibition in transfected HepG2 2.2.15 cells. No marked toxicity was noted when tested up to 300 μM in transfected HepG2 2.2.15 cells (Schinazi et al., 1994; Kim et al., 1993). It is believed that DAPD is converted into (-)-β-D-dioxolane guanosine (DXG) *in vivo* by adenosine deaminase (Figure 5.1) (Chen et al., 1999; Rajagopalan et al., 1996; Painter et al., 1999). DXG also shows potent anti-HBV activity (Schinazi et al., 1994; Kim et al., 1993). In transfected HepG-2 cells, the EC_{50} for DXG against HBV DNA replication intermediates and HBV virion synthesis inhibition was approximately 1 μM. No significant toxicity was observed up to 100 μM DXG. Therefore, both DXG and its prodrug, DAPD have favorable therapeutic indexes towards HBV.

HBV belongs to the family of *hepadraviridae* which comprises human HBV, woodchuck hepatitis virus (WHV) (Summers et al., 1978), ground squirrel hepatitis virus (GSHV) (Marion et al., 1980), and duck hepatitis B virus (DHBV) (Mason and Summers,

1980). The *hepadraviridae* viruses have been shown to cause persistent subacute infections in their natural hosts and share common biological features including the virion ultrastructure, genomeic structure, and unique mechanism of replication. DHBV was first discovered in 1979 in serum samples taken from ducks in the People's Republic of China (Mason and Summers, 1980). DHBV was detected in 10% of Peking ducks in commercial flocks in the United States (Mason and Summers, 1980). Hepatitis caused by DHBV accounts for great losses among ducklings two to three weeks old (Raethel, 1988). Due to the "striking resemblance" to HBV, DHBV has been used as an animal model to HBV. The purpose of this study was to investigate the pharmacokinetics of DAPD and DXG in ducks.

MATERIALS AND METHODS

DAPD and DXG were synthesized as previously described (Kim et al., 1993; Schinazi et al., 1994). The internal standard, 2'3'-didehydro-2'-deoxythymidine (D4T), was provided by the Developmental Therapeutic Branch, AIDS Program, National Institutes of Health (Rockville, MD). The chemical purity of each compound was greater than 98%. HPLC grade Acetonitrile and all other chemicals (analytical grade) were obtained from J. T. Baker (Phillipsburg, NJ).

Four adolescent Peking ducks (2 male, 2 female; 34 days of age) were used in this study. Ducks were maintained in a 12 hr light/dark cycle with free access to food and water. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). The animal study was approved by Stanford University Animal Care and Use Committees and conducted in accordance with

guidelines established by the Animal Welfare Act and *Guide for the Care and Use of Laboratory Animals* from the National Research Council (Institute of Laboratory Animal Resources, 1996). Two ducks (1 male, 1 female) received 50mg/kg DAPD intravenously, and two ducks (1 male, 1 female) were given 50 mg/kg DAPD orally. Blood samples were collected at 0 (pre-dose) and at 0.25, 0.5, 1, 1.5, 2 3, 4, 6, and 8 hours after dosing. The blood samples were placed into chilled tubes containing EDTA, immediately centrifuged, and the plasma samples were stored at -70°C until analysis.

Plasma concentrations of DAPD and DXG were determined by HPLC (Rajagopalan et al., 1994, 1996). Briefly, 100 µl of the internal standard (D4T, 2 µg/ml) was mixed well with 50 µl plasma, followed by the addition of 50 µl of 2 M perchloric acid. After centrifugation, the supernatant was injected onto a C18 HPLC column (4.6mm ID x 25 cm, 5 µ). The mobile phase, which contained 4% acetonitrile and 5 mM triethylamine in 5 mM potassium phosphate, was delivered at a flow rate of 1.5 mL min^{-1}. The absorbency of eluants was measured by an UV detector at 270 nm. The retention times were 5.7, 8.1, and 12.1 min for DXG, DAPD, and D4T, respectively. The limits of quantitation were 125 ng/ml and 100ng/ml for DAPD and DXG, respectively.

Individual plasma concentration-time data were used to calculate pharmacokinetic parameters using noncompartmental methods (Metzler et al., 1974) with WinNonlin (version I, Pharsight Corp., Mountain View, CA). The maximum concentration (C_{max}), time of maximum concentration (T_{max}) and final quantifiable sampling time (t_f) were the observed values. The elimination phase rate constant (k) was calculated by linear regression analysis of the terminal log-linear portion of the plasma concentration-time curve. The terminal phase half-life ($t_{1/2}$) was calculated as 0.693/k. The AUC value from

time zero to tf (AUC_{0-tf}) was calculated using the linear trapezoidal method with extrapolation to infinity by dividing the last measured concentration by k ($AUC_{0-\infty}$).

RESULTS AND DISCUSSION

Plasma concentrations of DXG following intravenous administration of 50 mg/kg of DAPD to ducks are illustrated in Figure 5.2. Corresponding pharmacokinetic parameters are presented in Table 5.1. Plasma DAPD concentrations were quantifiable only after intravenous administration at the 0.25 and 0.5 hr time points. The lack of DAPD at later time points after intravenous administration is likely due to the rapid conversion of DAPD to DXG by adenosine deaminase as indicated by the early T_{max} (0.25-0.5 hr) and relative high C_{max} (26.07 and 22.98 mg/L) of DXG. DXG was detected for up to 4 hr after both intravenous and oral administration of DAPD. Following intravenous administration of DAPD, plasma concentrations of DXG declined rapidly with an estimated $t_{1/2}$ of 0.33 hr for the female and 0.56 hr for the male ducks. This value is comparable with the half-life of DXG after intravenous administration of 25 mg/kg DAPD to rats, which is 0.62 hr, but lower than the 1.6 hr in monkeys (Chen et al., 1999). The $t_{1/2}$ of DXG was 0.86 hr (female) and 0.85 hr (male) in the ducks receiving DAPD orally. Interestingly, the AUC of DXG following DAPD oral administration (18.26 mg·hr/L and 23.11 mg·hr/L) was higher than intravenous administration (17.08 mg·hr/L and 13.86 mg·hr/L) while the C_{max} after oral administration (10.20 mg/l and 12.51 mg/l) was much lower than intravenous administration (26.07 mg/l and 22.98 mg/L). Meanwhile, the $t_{1/2}$ of DXG following oral dosage was longer than the $t_{1/2}$ of DXG after intravenous dosage. The likely explanation is the slower absorption and more extensive

first pass metabolism of DAPD following oral administration. The contribution of intestinal and/or hepatic adenosine deaminase responsible for this metabolism remains unknown. Although higher adenosine deaminase activity in the intestinal tract than in the liver has been reported in rodents (Mohamedali et al., 1993) and humans (Daddona, 1981), little information is available about the distribution of this enzyme in ducks, making it difficult to predict the exact location of DAPD metabolism.

Adenosine deaminase (ADA) is a key enzyme in purine metabolism (Conway and Cooke, 1939; Wiginton et al., 1981). It is responsible for the deamination of adenosine, deoxyadenosine, and a number of other modified adenine nucleosides which are produced endogenously or exogenously (Conway and Cooke, 1939; Wiginton et al., 1981; Cristalli et al., 2001). ADA has been found in plants, bacteria, invertebrates, vertebrates, and mammals, including humans (Brady and O'Donovan, 1965; Daddona, 1981; Cristalli et al., 2001). Its amino acid sequence is highly conserved from bacteria to humans. This enzyme is present in virtually all animal and human tissues, but the highest levels in human are in the lymphoid system such as lymph nodes, spleen, thymus, and circulation peripheral blood lymphocytes (Daddona, 1981). In rat, guinea-pig, mouse, cat, rabbit, dog and some calf, highest ADA activity was found in spleen and duodenum, followed by lung, ovary and placenta; activity is low in the blood, brain, muscle, pancreas and testis (Brady and O'Donovan, 1965). Though the functions of ADA have not been fully elucidated, it is believed that ADA plays a critical role in the regulation of adenine nucleotide levels. The deficiency of ADA causes severe combined immunodeficiency disease (ADA-SCID) (Cristalli et al., 2001). The available information of ADA present in duckling (*Anas domesticus*) demonstrated similar activity

in erythrocyte homogenate as it in human (Yamada and Sherman, 1981). This may indicate similar biotransformation of DAPD to DXG in human and ducks.

DAPD, considered as a water-soluble and bioavailable prodrug of DXG, is currently being investigated as an anti-HIV and anti-HBV agent by Triangle Pharmaceuticals (Durham, NC) (Corbett and Rublein, 2001) due to its favorable efficacy, toxicology and pharmacokinetic profiles *in vitro* and in animals (Furman et al., 2001; Chen et al., 1999; Rajagopalan et al., 1994, 1996; Schinazi et al., 1994; Kim et al., 1993). DAPD is the only member of its chemical series in development for antiviral activity. It is synergistic with a number of current antiviral treatments. DAPD is effective toward HBV strains resistant to lamivudine and /or famciclovir (Chin et al., 2001), and HIV strains resistant to zidovudine, lamivudine, adefovir and multidrug resistance (Bazmi et al., 2000; Gu et al., 1999). DAPD provides new options for anti-HIV and anti-HBV chemotherapy. In this study, following intravenous administration of 50 mg/kg of DAPD to ducks, its metabolite DXG concentration remained above EC_{50} about 2 hr, and DXG concentrations were above EC_{50} at least 3 hr following oral administration. This study provides information for the further evaluation of DAPD and DXG as potential anti-HIV and anti-HBV agent *in vivo.*

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Table 5.1. Pharmacokinetic parameters of DXG following 50mg/kg DAPD administration to ducks

DXG Pharmacokinetic Parameters	**Male**	**Female**
Intravenous Administration		
C_{max} (mg/L)	22.98	26.07
T_{max} (hr)	0.25	0.50
$AUC_{0-\infty}$ (mg·hr/L)	13.86	17.08
$t_{1/2}$ (hr)	0.56	0.33
Oral Administration		
C_{max} (mg/L)	12.51	10.20
T_{max} (hr)	0.50	0.50
$AUC_{0-\infty}$ (mg·hr/L)	23.11	18.26
$t_{1/2}$ (hr)	0.85	0.86

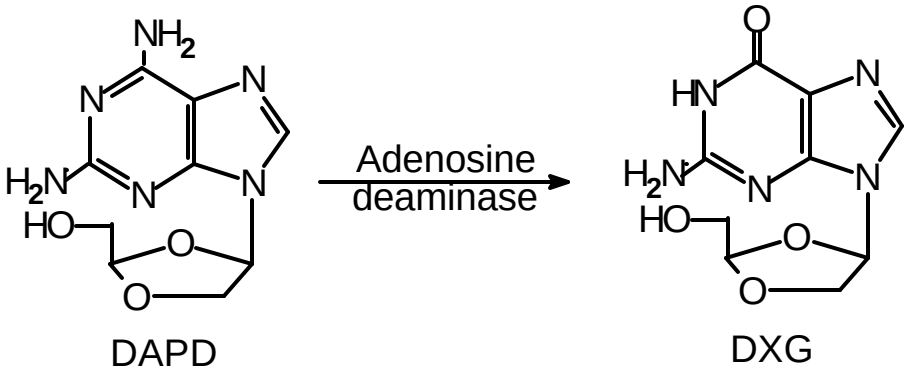

Figure 5.1. Chemical structures of DAPD and DXG

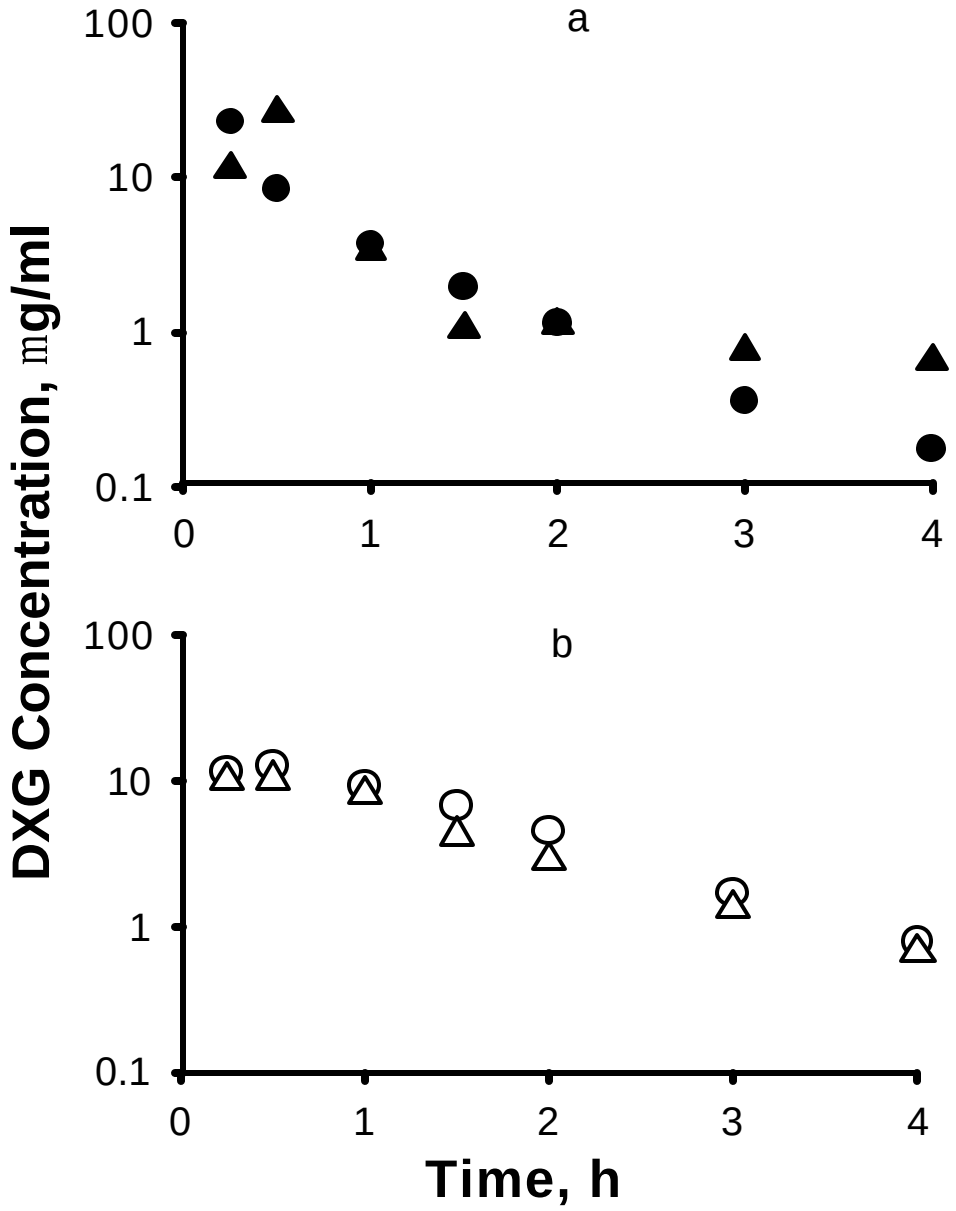

Figure 5.2. Concentrations of DXG in plasma following administration of 50 mg/kg DAPD to ducks by (a) intravenous administration (filled triangle-female, filled circle-male) and (b) oral administration (open triangle-female, open circle-male).

CHAPTER 6

CONCLUSIONS

Feline immunodeficiency virus (FIV), a significant health threat to the feline population, has been identified worldwide. However, there are not many options for effective treatment of FIV, neither a cure nor a vaccine. Due to the similarity between human immunodeficiency virus (HIV) and FIV, current anti-HIV compounds zidovudine (3'-azido-3'-deoxythymidine, AZT) and lamivudine ((-)- 2', 3'-dideoxy-3'-thiacytidine, 3TC) were investigated as a part of an effort to search for an effective treatment of FIV infection. AZT was the first commercially available drug for the treatment of HIV infection in humans. 3TC is one of most potent nucleoside analogues against HIV and HBV, its low toxicity and synergic action with other antiviral compounds make it a more valuable antiviral agent. In current studies, pharmacokinetics and brain distribution of AZT and 3TC were characterized in healthy cats.

Hepatitis B virus (HBV) infection is a major public health problem, which is the ninth leading cause of death throughout the world. (-)-β-D-2,6-diaminopurine dioxolane (DAPD) appears to be one of the most promising anti-HBV agents due to it potent activity and low toxicity. The pharmacokinetics of DAPD and its active metabolite, (-)-β-D-dioxolane guanosine (DXG), was investigated in ducks.

Pharmacokinetics of zidovudine in cats

The pharmacokinetics of AZT following intravenous (IV), intragastrical (IG), and oral (PO) administration of 25 mg/kg of AZT were evaluated by a three-way cross over study in six healthy cats. Plasma concentrations of AZT declined rapidly with a terminal half-life of 1.4 ± 0.19, 1.4 ± 0.16 and 1.6 ± 0.26 hr following IV, IG and PO administration, respectively. Total body clearance and steady-state volume of

distribution were 0.41 ± 0.10 L/hr/kg and 0.82 ± 0.15 L/kg, respectively. The area under the curve (AUC) following IV, IG, and PO administration was 64.7 ± 16.6, 42.5 ± 9.41 and 60.5 ± 17.0 mg×h/L. AZT was well absorbed after IG and PO administration with bioavailability values of 0.70 ± 0.24 and 0.95 ± 0.23. The disposition of AZT in cats was similar to that in other species. At a dose of 25 mg/kg, plasma concentrations of AZT were maintained above the minimum effective concentration (0.03 µM for wild-type FIV) for at least 12 h following IV, IG or PO administration.

Pharmacokinetics of lamivudine in cats

The pharmacokinetics of 3TC following intravenous (IV), intragastrical (IG), and oral (PO) administration of 25 mg/kg were evaluated by a three-way cross over study in six healthy cats. Plasma concentrations of 3TC declined rapidly with a terminal half-life of 1.9 ± 0.21, 2.6 ± 0.66 and 2.7 ± 1.5 hr following IV, IG and PO administration to cats, respectively. Total body clearance and steady-state volume of distribution were 0.22 ± 0.09 L/hr/kg and 0.60 ± 0.22 L/kg, respectively. The area under the plasma 3TC concentration versus time curve (AUC) following IV, IG, and PO administration was 130 ± 55.2, 115 ± 97.5 and 106 ± 94.9 mg×hr/L. 3TC was well absorbed after IG and PO administration with bioavailability values of 0.88 ± 0.45 and 0.80 ± 0.52. The pharmacokinetics of 3TC in cats was similar to that in other species. At a dose of 25 mg/kg of 3TC, plasma concentrations of the nucleoside analogue were maintained above the 50% inhibition concentration (0.14 µM for wild-type FIV) for at least 12 hr following IV, IG or PO administration.

Distribution of zidovudine and lamivudine in cat brain

Five cats were received 7.0 mg/kg AZT loading dose, followed by three-hour infusion of AZT at 14.5 mg/kg/h through Intracath catheters. The plasma, cerebrospinal fluid (CSF), and brain samples were then obtained to determine the distribution of AZT to CSF and brain. Mean (± STD) plasma, CSF, and brain concentrations of AZT at steady state were 26.3 ± 4.21, 8.27 ± 1.21, 6.37 ± 0.61 mg/L, respectively. No significant difference of AZT concentrations was detected in different cat brain sections. The distribution of 3TC to cat brain was investigated in six cats after 7.0 mg/kg 3TC loading dose, followed by three-hour infusion of 3TC at 5.5 mg/kg/hr. 3TC concentrations in plasma and CSF at steady states were 17.09 ± 5.26, and 1.73 ± 0.56 mg/L. Brain concentrations were undetectable for 3TC. The CSF/plasma ratios for AZT and 3TC were 0.32 ± 0.05 and 0.098 ± 0.01, respectively. The brain/plasma ratio of AZT, which was 0.25 ± 0.04, was similar to its value of CSF/plasma.

Both AZT and 3TC were able to penetrate into cat CSF, however the extent of AZT penetration was greater than that of 3TC. A significant amount of AZT was also distributed across cat blood brain barrier. In comparison, no detectable amount of 3TC was present in cat brain. AZT may be more effective to inhibit the FIV infection in central nervous system as it can penetrate into blood-CSF barrier and blood brain barrier in significant extent.

Pharmacokinetics of (-)-β-D-2,6-diaminopurine dioxolane and dioxolane guanosine in ducks

Pharmacokinetics of DAPD and DXG were estimated following administration of 50 mg/kg DAPD intravenously or orally to four adolescent ducks (34 days of age). DAPD concentrations were below the limit of quantitation except for the first two samples from the intravenously dosed ducks. The area under the curve (AUC) of metabolite DXG was 17.08 and 13.86 mg·hr/L following intravenous administration of DAPD and 18.26 and 23.11 mg·hr/L following oral administration. The C_{max} of DXG was achieved at 0.50 and 0.25 hr after intravenous DAPD and 0.50 hr in both ducks receiving DAPD orally. The half-life of DXG was 0.33 and 0.56 hr after intravenous DAPD and 0.85 and 0.86 hr following oral dosing of DAPD. DAPD was rapidly metabolized to the active metabolite DXG followed by both intravenous and oral administration to ducks. The higher AUC and longer half-life of DXG following oral administration are likely due to first pass metabolism and slower absorption of DAPD. In this study, following intravenous administration of 50 mg/kg of DAPD to ducks, its metabolite DXG concentration remained above EC_{50} (50% effective concentration) about 2 hr, and DXG concentrations were above EC_{50} at least 3 hr following oral administration.

Summary

Nucleoside analogues represent one group of most effective antiviral agents to treat various virus infections, including HIV, HBV, FIV, etc. In the current studies, favorable pharmacokinetic profiles were observed following IV, IG, and PO

administration of AZT and 3TC to healthy cats. 3TC was able to penetrate into cat CSF, AZT can distribute to both CSF and brain tissue. The pharmacokinetics of DAPD and DXG provide valuable information for the further evaluation of DAPD and DXG as potential anti-HIV and anti-HBV agent *in vivo*. The current studies provide valuable information for further investigating the application of AZT, 3TC and DAPD in veterinary medicine.