BIOANALYSIS AND PHARMACOKINETICS-PHARMACODYNAMICS

MODELING OF THE ENDOGENOUS 2’DEOXYNUCLEOSIDE TRIPHOSPHATE POOL IN INDIVIDUALS RECEIVING TENOFOVIR/EMTRICITABINE

by

XINHUI CHEN

B.M., Central South University, China, 2011

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This thesis for the Doctor of Philosophy degree by

Xinhui Chen

has been approved for the

Toxicology Program

By

Jennifer J. Kiser, Chair

Peter L. Anderson, Advisor

Melanie S. Joy

Michael F. Wempe

Samantha MaWhinney

Jose M. Castillo-Mancilla

Date: 12/16/16
ABSTRACT

Human immunodeficiency virus (HIV) uses the endogenous nucleoside triphosphates (dNTP)—which consists of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and thymidine triphosphate (TTP)—as the natural substrates of reverse transcriptase (RT) to synthesize viral DNA. Tenofovir (TFV) and emtricitabine (FTC) are nucleos(t)ide analogs (NA) that act against this process. In cells, their active anabolites: TFV-diphosphate (TFV-DP) and FTC-triphosphate (FTC-TP), compete with dNTPs at the active site of HIV RT, slowing down and terminating the viral DNA biosynthesis. As NAs, TFV and FTC have the potential to disturb the dNTP pool, which could augment or reduce their efficacies via altering the analog:dNTP ratio. This work focused on the development of a quantitative method for intracellular dNTPs, the characterization of the change of dNTP pool in vivo, and pharmacokinetics-pharmacodynamics (PKPD) modeling to evaluate the interaction between TFV/FTC and dNTPs. A sensitive and reliable liquid chromatography-tandem mass spectrometry (LC/MS/MS) method was developed and validated for dNTPs in cell lysates with an analytical range of 50 to 2500 fmol/sample. A phase 4, observational, intensive PKPD study was performed in 21 HIV-negative and 19 HIV-positive treatment naïve individuals. The dNTPs were reduced by 14% to 37% relative to baseline within 3 days, in both HIV-negative and HIV-positive individuals (p ≤ 0.003). An indirect response model described the interaction between TFV/FTC and
dNTPs. The $EC_{50}$ (interindividual variability, (%CV)) of TFV-DP on the inhibition of dATP formation was 1022 fmol/10^6 cells (130%). A transient effect that waned over time ($E_{\text{max}}/(1+\text{time}^{0.9})$) was observed on dGTP, with an $EC_{50}$ of 54 fmol/10^6 cells. In addition, the $EC_{50}$ of FTC-TP on the inhibition of dCTP and TTP formation were 44 pmol/10^6 cells (82.5%) and 19 pmol/10^6 cells (101%). This study was limited by the small sample size ($n=40$), however, the model successfully characterized dNTPs reductions and interactions between TFV/FTC and dNTPs. This model enabled PKPD simulations to help understand clinical observations.

The form and content of this abstract are approved. I recommend publication.

Approved: Peter L. Anderson
DEDICATION

I would like to dedicate this work to my grandfather, a former physics professor, rocket/missile scientist, and dean of the basic research division of our local University. He taught me so many things. I am always grateful for what he did for my family and me. He is a courageous son, who was born in a farmer family, left his hometown, and became the first man that finished college with very limited financial support from his family. From him, I have learned to be independent. He is an outstanding scientist, who traveled far and did his research in Beijing and Shanghai. He has gifted me the ambition to become a good scientist. He is a reliable father, who brought my dad, my uncle, and my grandmother from an impoverished rural area to a well-developed city in our province, and provided them with better lives. His pursuit of happiness inspired me to come to the United States. He is a considerate husband, who loves my grandmother, gave up positions in renowned research institutes and went back to his family. He demonstrates to me a husband should always put his family first. His hard work and achievement have also inspired many young people in his hometown to study hard and through education to change their destiny. He also encouraged me to contribute to society. It is these values he brings to my family that leads me to where I am, and will keep carrying me on the coming adventures.
ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Peter Anderson for his mentorship, patience, and support. He sees my potential and encourages me to do my best. I am grateful for the opportunity to work in his lab and for this wonderful journey in the last five years. I would like to thank my thesis committee for their helpful advice: Dr. Jennifer Kiser for her instructions on using NONMEM for population pharmacokinetics-pharmacodynamics analysis; The incisive points on pharmacology that Dr. Melanie Joy brought up in every thesis committee meeting; Dr. Michael Wempe for his advice on analytical method development and his viewpoints on scientific writing in English for Asians, in which he shares his experiences working with Japanese scientists; Dr. Sam MaWhinney for her interesting discussions on biostatistical analysis and her recommendation on coursework that really helped me grasp the theory and application of mixed model analysis; Dr. Jose Castillo-Mancilla for his tutoring from the clinic, advice from the medical standpoint, his caring for patients, and encouragement as an first-generation immigrant. I wish to also thank the pharmacometricians, Dr. David Bourne and Dr. Serge Guzy, for interesting discussions on model development. I wish to thank the manager of the Colorado Antiviral Pharmacology Lab, Lane Bushman, for his passionate teaching and attention to details on analytical chemistry. I admire the way he manages people and enables them to perform their best. I would like to thank analytical chemists: Kevin McAllister, Jia-Hua Zheng, Brandon Klein, and Michelle Ray, for their help and advice during analytical method development, and Anthony Guida for his spiritual guidance during my most discouraging time during model development. I also thank Dr. Joseph Rower, for his help on initiating the model development using NONMEM for a plasma model of tenofovir; and Dr. Sharon Seifert for her help with the “Cell–PrEP” clinical study data and
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<td>3GPK</td>
<td>3'-phosphoglycerate kinase</td>
</tr>
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<td>3TC</td>
<td>lamivudine</td>
</tr>
<tr>
<td>5'-DNT</td>
<td>5'-deoxynucleotidase</td>
</tr>
<tr>
<td>5'-NT</td>
<td>5'-nucleotidase</td>
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<tr>
<td>ABC</td>
<td>abacavir</td>
</tr>
<tr>
<td>ACTG</td>
<td>AIDS clinical trial group</td>
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<tr>
<td>ADA</td>
<td>adenosine deaminase</td>
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<tr>
<td>AdeDA</td>
<td>adenine deaminase</td>
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<td>ADK</td>
<td>adenosine kinase</td>
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<td>ADPase</td>
<td>adenosine diphosphatase</td>
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<tr>
<td>ADSL</td>
<td>adenylosuccinate lyase</td>
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<tr>
<td>ADSS</td>
<td>adenylosuccinate synthase</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AMPDA</td>
<td>adenosine monophosphate deaminase</td>
</tr>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APRT</td>
<td>adenosine phosphoribosyltransferase</td>
</tr>
<tr>
<td>ART</td>
<td>antiretroviral therapy</td>
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<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>ATPase</td>
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<td>ATV</td>
<td>atazanavir</td>
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ATV/r  atazanavir boosted with ritonavir
AUC  area under the concentration-time curve
AZT  zidovudine
BLK-BLK  blank sample
BLK-IS  blank sample with internal standard
BMD  bone mineral density
BMI  body mass index
BSM  binary solvent manager
BW/S(CR)  weight/serum creatinine ratio
CD  cluster of differentiation
CDA  cytidine deaminase
CDC  center for disease control
CI  confidence interval
CK  creatine kinase
CL  clearance
CLCR  creatinine clearance
CNS  central nervous system
CsDA  cytosine deaminase
CSF  cerebrospinal fluid
CTPS  cytidine triphosphate synthase
CV  coefficient of variation
dAdK  deoxyadenosine kinase
dAK  deoxyadenylate kinase
dATP  2’-deoxyadenosine triphosphate
dCDA  deoxycytidylate deaminase
dCK  deoxycytidine kinase
dCTP: 2'-deoxycytidine triphosphate

dCTPase: deoxycytidine-triphosphate pyrophosphatase

dCTPDA: deoxycytidine triphosphate deaminase

ddl: didanosine

DDIs: drug-drug interactions

dGK: deoxyguanosine kinase

dGTP: 2'-deoxyguanosine triphosphate

dGTPase: deoxyguanosine triphosphate triphosphohydrolase

DHGK: dedhydrogluconokinase

DNA: deoxyribonucleic acid

dNTPs: deoxynucleoside triphosphates

DP: diphosphate

dUTPase: deoxyuridine-triphosphatase

DV: dependent variables

EC: enteric-coated

ED50: effective dose that leads to 50% response rate

EFV: efavirenz

eGFR: estimated glomerular filtration rate

FDA: food and drug administration

FDTS: flavin dependent thymidylate synthase

FTC: emtricitabine

FTC-TP: emtricitabine triphosphate

GDA: guanine deaminase

GK: guanylate kinase

GMPR: guanosine monophosphate reductase

GMPS: guanosine monophosphate synthase
GP  guanosine phosphorylase  
HBV  hepatitis B virus  
HGPRT  hypoxanthine-guanosine phosphoribosyltransferase  
HIV  human immunodeficiency virus  
HSV  herpes simplex virus  
IC  intracellular  
IC50  concentration that leads to 50% inhibition rate  
IDP  inosine diphosphate phosphatase  
IIV  interindividual variability  
IK  inosine kinase  
IL  interleukin  
IMP  Inosine monophosphate  
IMPDH  inosine monophosphate dehydrogenase  
IN  inosinate nucleosidase  
InSTIs  integrase inhibitors  
IQR  interquartile range  
IRB  institutional review board  
IS  internal standard  
ISR  incurred sample reanalysis  
LC/MS/MS  liquid chromatography-tandem mass spectrometry  
LFTs  liver function tests.  
LLOQ  lower limit of quantitation  
LRT  likelihood ratio test  
MDRD  eGFR by Cockcroft-Gault or modification of diet in renal disease  
ME  matrix effect  
MP  monophosphate
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<td>MRP</td>
<td>multidrug-resistant protein</td>
</tr>
<tr>
<td>MSM</td>
<td>men who have sex with men</td>
</tr>
<tr>
<td>NA</td>
<td>nucleos(t)ide analogs</td>
</tr>
<tr>
<td>NAMs</td>
<td>nucleoside analog mutations</td>
</tr>
<tr>
<td>NDP</td>
<td>nucleoside diphosphate phosphatase</td>
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<tr>
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<td>NDRT</td>
<td>nucleoside deoxyribosyltransferase</td>
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<td>NIH</td>
<td>national institute of health</td>
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<td>NNRTI</td>
<td>non-nucleoside reverse transcriptase inhibitors</td>
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<td>organic anion transporter</td>
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<td>organic cation transporter</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
</tr>
<tr>
<td>PE</td>
<td>processing efficiency</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>protease inhibitors</td>
</tr>
<tr>
<td>PK</td>
<td>pharmacokinetics</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PKLR</td>
<td>pyruvate kinase isozymes from liver and red blood cells</td>
</tr>
<tr>
<td>PKM</td>
<td>pyruvate kinase isozymes from muscle</td>
</tr>
<tr>
<td>PN</td>
<td>purine nucleosidase</td>
</tr>
<tr>
<td>PNP</td>
<td>purine-nucleoside phosphorylase</td>
</tr>
<tr>
<td>PRED</td>
<td>predicted values</td>
</tr>
<tr>
<td>PrEP</td>
<td>preexposure prophylaxis</td>
</tr>
<tr>
<td>PRPP</td>
<td>phosphoribosylpyrophosphate</td>
</tr>
<tr>
<td>Py-NP</td>
<td>pyrimidine-nucleoside phosphorylase</td>
</tr>
<tr>
<td>Pyr5N</td>
<td>pyrimidine-5’-nucleotide nucleosidase</td>
</tr>
<tr>
<td>QA</td>
<td>quality assurance</td>
</tr>
<tr>
<td>QC</td>
<td>quality control</td>
</tr>
<tr>
<td>QH</td>
<td>higher level of quality control sample</td>
</tr>
<tr>
<td>QL</td>
<td>lower level of quality control sample</td>
</tr>
<tr>
<td>QM</td>
<td>medium level of quality control sample</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>RE</td>
<td>recovery</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleoside diphosphate reductase</td>
</tr>
<tr>
<td>RNTR</td>
<td>ribonucleoside triphosphate reductase</td>
</tr>
<tr>
<td>RPyN</td>
<td>ribosylpyrimidine nucleosidase</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RTPR</td>
<td>ribonucleoside triphosphate reductase</td>
</tr>
<tr>
<td>RV</td>
<td>residual variability</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SM</td>
<td>sample manager</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>TAD</td>
<td>time after dose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TAF</td>
<td>tenofovir alafenamide fumarate</td>
</tr>
<tr>
<td>TDF</td>
<td>tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td>TFV</td>
<td>tenofovir</td>
</tr>
<tr>
<td>TFV-DP</td>
<td>tenofovir diphosphate</td>
</tr>
<tr>
<td>TFV-MP</td>
<td>tenofovir monophosphate</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TK1</td>
<td>thymidine kinase 1</td>
</tr>
<tr>
<td>TK2</td>
<td>thymidine kinase 2</td>
</tr>
<tr>
<td>Tmax</td>
<td>maximum plasma level</td>
</tr>
<tr>
<td>TMPK</td>
<td>thymidylate kinase</td>
</tr>
<tr>
<td>TP</td>
<td>thymidine phosphorylase</td>
</tr>
<tr>
<td>TP</td>
<td>triphosphate</td>
</tr>
<tr>
<td>TS</td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td>TTP</td>
<td>thymidine triphosphate</td>
</tr>
<tr>
<td>TTPase</td>
<td>thymidine triphosphatase</td>
</tr>
<tr>
<td>UCMPK</td>
<td>uridine monophosphate-cytidine monophosphate kinase</td>
</tr>
<tr>
<td>UK</td>
<td>uridine kinase</td>
</tr>
<tr>
<td>UMP</td>
<td>uridine monophosphate</td>
</tr>
<tr>
<td>UMPK</td>
<td>uridine monophosphate kinase</td>
</tr>
<tr>
<td>UN</td>
<td>uridine nucleosidase</td>
</tr>
<tr>
<td>UP</td>
<td>uridine phosphorylase</td>
</tr>
<tr>
<td>UPLC</td>
<td>ultra-performance liquid chromatography</td>
</tr>
<tr>
<td>UPRT</td>
<td>uracil phosphoribosyltransferase</td>
</tr>
<tr>
<td>Vd</td>
<td>volume of distribution.</td>
</tr>
<tr>
<td>VPC</td>
<td>visual predictive checks</td>
</tr>
<tr>
<td>XD</td>
<td>xanthine dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>XdITDP</td>
<td>xanthine triphosphate / deoxyinosine triphosphate diphosphatase</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
</tr>
<tr>
<td>XPRT</td>
<td>xanthine phosphoribosyltransferase</td>
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</tbody>
</table>
1.1 Timeline of HIV/AIDS

In 1981, the Center of Disease Control (CDC) first described “immune system dysfunction” in 5 gay men from Los Angeles, California, all of whom died a couple months later. This marked the outbreak of the HIV (human immunodeficiency virus) pandemic. The top priority at that time was to find a treatment for the HIV infection, seemingly regardless of its toxicity. We did not have any antiretroviral agents until in 1987 when the United States Food and Drug Administration (US FDA) approved the first HIV drug, zidovudine (AZT), which was a nucleoside reverse transcriptase inhibitor (NRTI). In 1996, a new drug in a new class of HIV drugs, nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI), was approved by FDA. The optimization of HIV treatment efficacy gradually became the goal for researchers. By the 2000s, there were 23 antiretroviral agents available, consisting of more than 100 different combinations of treatments. Finally, HIV/AIDS (acquired immunodeficiency syndrome) had become a chronic disease. Over this time, there has been a strong demand to understand the clinical pharmacology and dose-response relationships of antiretroviral drugs to minimize adverse effects and optimize treatment efficacy.

With no available vaccine, combination antiretroviral therapy (ART) is the mainstream approach for the treatment and control of HIV pandemic. Unfortunately, the currently available treatment is not able to eradicate HIV. As a compromise, the current goal of HIV therapy is to optimize and prolong HIV suppression of viral load in plasma [1]. According to US AIDS fact sheet 2015, 15.8 million people are accessing antiretroviral therapy (June 2015), 36.9 million [34.3 million–41.4 million] people globally
are living with HIV (end 2014), 2 million [1.9 million–2.2 million] people became newly infected with HIV (end 2014), 1.2 million [980 000–1.6 million] people died from AIDS-related illnesses (end 2014) [2]. Thus, HIV continues to be a dynamic epidemic.

In 2010, the National Institute of Health (NIH) announced the iPrEx study results (the Colorado Antiviral Pharmacology Lab participated in this study), showing that a daily dose of tenofovir/emtricitabine (TFV/FTC) reduced HIV-infection rate in men who have sex with men (MSM) by at least 44%. This landmark study showed that pharmacology helped for interpreting the efficacy results. The scientific community is now particularly concerned with the pharmacology and risk-benefit balance of using antiretroviral drugs in HIV-negative people to prevent HIV infection. The understanding of the clinical pharmacology of antiretrovirals in HIV-positive individuals is also important for guiding TFV/FTC treatment [3]. This dissertation research focused on the clinical pharmacology of TFV/FTC in both HIV-positive and HIV-negative participants.

1.2 Pharmacokinetics and Pharmacodynamics

Pharmacokinetics (PK) is the quantitative analysis of the processes of drug absorption, distribution, and elimination that determine the time course of drug concentrations in the body. This dissertation research investigated the PK of TFV/FTC. Pharmacodynamics (PD) deals with the mechanism of drug action and treatment response. In this dissertation research, we chose changes in endogenous deoxynucleoside triphosphates as the biomarker to represent the PD of TFV/FTC.

PK and PD are the two major subdivisions of clinical pharmacology [4]. Clinical pharmacology can be defined as the study of drugs in humans, as opposed to in vitro or in animals. In fact, all facets of pharmacology must be studied in humans to ultimately
define the absorption, distribution and elimination of drug in man [5]. The development of clinical pharmacology took the advantage of modern chemistry, which provided the chemically pure pharmaceutical products for sensitive assays to study the relationships between drug dosage and biological effect. The population PKPD analysis in patients is based on the application of biostatistics, especially the concept of mixed models, which enables us to estimate interindividual variability and residual variability simultaneously [6]. This dissertation research used advanced biostatistical analysis methodologies such as linear and nonlinear regression to model the population PKPD of TFV/FTC. An example of nonlinear regression using mixed effect modeling is illustrated in figure 1.1.

1.3 Bioanalysis

Bioanalysis techniques are a necessity of PKPD studies, due to the quantitative nature of the analyses. The investigation of TFV/FTC PKPD has been enabled with the rise of the modern analytical chemistry. The biological matrix from humans contains many potentially interfering exogenous and endogenous compounds and are limited in availability. Usually, whole blood samples are taken from human participants due to better accessibility (as opposed to tissues or other fluids). Thus, sensitive analytical methods are required to study PKPD in humans. The bioanalysis in this dissertation research was powered by sample processing technology such as solid phase extraction (SPE) and sensitive instruments such as liquid chromatography-tandem mass spectrometry (LC/MS/MS). The principals of SPE and tandem mass spectrometry (MS/MS) are illustrated in figures 1.2 and 1.3. This dissertation research developed and validated both SPE and LC/MS/MS for the bioanalysis of endogenous deoxynucleoside triphosphates (dNTPs).
Figure 1.1 Graphical illustration of the basic statistical model used in NONMEM. The example shows a one-compartmental model with intravenous bolus administration of the drug. Log C: nature log transformation of drug concentrations. CL: clearance. Vd: volume of distribution. (Adapted from Vozeh S, et al. Eur J Clin Pharmacol 1982;23:445-51 [7].)
Figure 1.2 Graphical illustration of the basic procedures of solid phase extraction. (http://www.mlo-online.com/articles/201501/images/MLO201501-SpecFeat-Phenomenex-Fig1.pdf last accessed on July 21, 2016)
Figure 1.3 Illustration of the Principles of tandem mass spectrometry quantitative technology. SIM: Secondary ion mass. (http://www.alphabiolabs.co.uk/wp-content/files/2014/01/drug-test-process.png last accessed on July 21, 2016)
1.4 Cellular Pharmacology

The understanding of the clinical pharmacology of TFV/FTC requires the investigation of cellular pharmacology. This is because both TFV and FTC in plasma are inactive forms that require cellular uptake and successive intracellular phosphorylation for the formation of active triphosphate anabolites (which are produced by cellular machinery). The efficacy and toxicity of TFV/FTC are associated with the concentrations of these intracellular triphosphate anabolites [8]. However, pharmacological research is limited by the lack of a sensitive methods to measure the active anabolites of TFV/FTC in cells. In previous studies, our lab developed and validated sensitive and reliable analytical methodologies for both the plasma and the intracellular TFV/FTC [9]. In addition, this dissertation research developed and validated a sensitive, reliable, and novel quantitative methodology for dNTPs measurements in cell lysates. This was needed for determining changes in dNTP during TFV/FTC therapy, to quantify PD.

Peripheral blood mononuclear cells (PBMC), due to easy access and relatively high cell number [10], have always been the reference tissue for antiviral drug studies in vivo [11]. These cells contain the clinically relevant cell type: CD4 T-cells (CD: cluster of differentiation), which are infected by HIV. This dissertation research used isolated PBMC as well as plasma for the PKPD investigation of TFV/FTC.
CHAPTER II

REVIEW OF THE LITERATURE

2.1 The Endogenous Nucleoside Triphosphate Pool

2.1.1 Structure and Chemical Properties

The endogenous nucleoside triphosphate (dNTP) pool consists of 2'-deoxy-adenosine triphosphate (dATP), 2'-deoxycytidine triphosphate (dCTP), 2'-deoxyguanosine triphosphate (dGTP), and thymidine triphosphate (TTP). They are the building blocks of DNA (deoxyribonucleic acid). A constant and balanced supply of the dNTP pool is important for the synthesis and maintenance of both nuclear and mitochondrial genetic materials [12].

Generally, a deoxynucleoside triphosphate is made of a nucleobase (can be either purines or pyrimidines), a five-carbon sugar (2'-deoxyribose), and three phosphate groups. Purine compounds include adenine and guanine, while pyrimidines include cytosine, uracil, and thymine. These compounds use a glycosidic bond to bind a pentose at the 1’ position to form nucleosides. For the 2’ position of this five-carbon sugar, if it is a hydroxyl group, the whole structure is defined as a nucleoside. This addition would result in adenine and guanine being referred to as adenosine and guanosine, while cytosine, uracil, and thymine would be referred to as cytidine, uridine, and thymidine. The reduction of 2’-hydroxyl group transforms a nucleoside into a deoxynucleoside. The 5’ position on the pentose can further bind to 1-3 phosphate groups, and depending on the number of the phosphate groups, the structure is defined as monophosphate (1 phosphate group), diphosphate (2 phosphate groups), and triphosphate (3 phosphate groups). The dNTP pool consists of triphosphates only. Figures 2.1 and 2.2 detail the
Figure 2.1 General structure of deoxynucleoside phosphates. (www.wikipedia.com Last accessed on Aug 8, 2016)

Figure 2.2 Structures of dNTPs. dATP: deoxyadenosine triphosphate. dGTP: deoxyguanosine triphosphate. dCTP: deoxycytidine triphosphate. TTP: thymidine triphosphate. (www.pubchem.com Last accessed on Aug 8, 2016)
general structures of nucleoside triphosphates. The dNTP pool components have molecular weight ranges of 482-507 g/mol. Detailed compound chemistry properties are listed in table 2.1. Regarding bioanalysis perspectives, dNTPs are highly polar and similar in structure, which places challenges on chromatographic separation using traditional methods.

2.1.2 Anabolism and Metabolism

2.1.2.1 *De novo* pathways

The *de novo* pathway plays a major role in the dNTP pool biosynthesis in proliferating cells. The *de novo* pathway starts from alanine, aspartate, and glutamine, using multiple enzymes and multifunctional proteins in this process. Inosine monophosphate (IMP) plays the central role in the purine nucleotides pathway. For pyrimidines, uridine monophosphate (UMP) plays the central role. The *de novo* pathway is summarized in figure 2.3.

2.1.2.2 Salvage pathways

The salvage pathway dominates the biosynthesis of the dNTP pool in resting cells, which requires less energy than the *de novo* synthesis process. Different from *de novo* pathway, the salvage pathway recycles formerly synthesized purine and pyrimidine (base) or nucleosides (base plus sugar). The enzymes involved in this process either transfer a phosphoribosyl group to base, or phosphorylate a nucleoside into nucleotide (monophosphate), using individual enzymes such as adenine or hypoxanthine guanine phosphoribosyl transferase (APRT and HGPRT, respectively), thymidine kinase 1 (TK1), thymidine kinase 2 (TK2), deoxycytidine kinase (dCK) and/or deoxyguanosine kinase (dGK).
Table 2.1 Chemical Properties of dNTPs.

<table>
<thead>
<tr>
<th></th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>TTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>491.181626 g/mol</td>
<td>467.156926 g/mol</td>
<td>507.181026 g/mol</td>
<td>482.168266 g/mol</td>
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<tr>
<td>Molecular Formula</td>
<td>C_{10}H_{16}N_{5}O_{12}P_{3}</td>
<td>C_{9}H_{16}N_{5}O_{13}P_{3}</td>
<td>C_{10}H_{16}N_{5}O_{13}P_{3}</td>
<td>C_{10}H_{17}N_{2}O_{14}P_{3}</td>
</tr>
<tr>
<td>Water Solubility</td>
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<td>11.8 mg/mL</td>
<td>5.59 mg/mL</td>
<td>7.78 mg/mL</td>
</tr>
<tr>
<td>XLogP3</td>
<td>-4.4</td>
<td>-5.6</td>
<td>-5.1</td>
<td>-5</td>
</tr>
<tr>
<td>pKa (Strongest Acidic)</td>
<td>0.9</td>
<td>0.95</td>
<td>0.82</td>
<td>0.9</td>
</tr>
<tr>
<td>pKa (Strongest Basic)</td>
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<td>-0.05</td>
<td>1.61</td>
<td>-3.2</td>
</tr>
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<td>Hydrogen Bond Donor Count</td>
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<td>6</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Hydrogen Bond Acceptor Count</td>
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<td>13</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Rotatable Bond Count</td>
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<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Exact Mass</td>
<td>491.000831 g/mol</td>
<td>466.989597 g/mol</td>
<td>506.995745 g/mol</td>
<td>481.989263 g/mol</td>
</tr>
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<td>248 A^2</td>
<td>275 A^2</td>
<td>239 A^2</td>
</tr>
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<td>Heavy Atom Count</td>
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<td>31</td>
<td>29</td>
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<td>Formal Charge</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Complexity</td>
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<td>895</td>
<td>853</td>
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<td>0</td>
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<td>0</td>
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<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Defined Bond Stereocenter Count</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Covalently-Bonded Unit Count</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 2.3 De novo pathway of dNTP biosynthesis. dATP: deoxyadenosine triphosphate. dGTP: deoxyguanosine triphosphate. dCTP: deoxycytidine triphosphate. IMP: inosine monophosphate. UMP: uridine monophosphate. TTP: thymidine triphosphate.
The production of the deoxynucleotide also relies upon the conversion from ribonucleotides, through enzymes such as ribonucleoside diphosphate reductase (RNR) and ribonucleoside triphosphate reductase (RNTR). The anabolism of purine deoxynucleotides are derivations from IMP and eventually results in the production of dATP and dGTP. Purine nucleotides are eventually metabolized to urate. On the contrary, the upregulation of pyrimidine deoxynucleotides are derivations from UMP and ultimately leads to the biosynthesis of dCTP and TTP. Pyrimidine nucleotides are metabolized to urea, which is excreted from the body. Figures 2.4 and 2.5 detail the salvage pathways for purine nucleotides and pyrimidine nucleotides.

In both replicating and resting cells, both de novo pathways and salvage pathways are involved in the regulation of the dNTP pool. In replicating cells, mitochondrial and cytosolic kinases are involved in the upregulation of the dNTP pool. However, in resting cells, mitochondrial kinases dominate this process [12].

The homeostasis of the dNTP pool components is orchestrated by complicated pathways involving multiple enzymes. Thus, they are subjected to potential disturbance by exogenous compounds which might either induce or inhibit the activity of these enzymes. For example, nucleoside analogs (NA) have structural similarities to dNTPs, and thus have the potential to affect the equilibrium of the dNTP pool.

The dNTP pool components can also be affected by several factors, such as proliferation, immune activation, and cell type. For example, the T lymphoblastoid cell line–CEM is a widely studied T-cell line derived from a malignant human lymphoblastic T-cell lymphoma. CEM cells are maintained by very high proliferation rates, which make it easy to culture and investigate through in vitro methods.
Figure 2.4 Purine metabolism via the salvage pathway.

(Adapted from http://www.genome.jp/kegg/pathway/map/map00230.html Last accessed on Aug 8, 2016.)
Figure 2.5 Pyrimidine metabolism via the salvage pathway.

(Adapted from http://www.genome.jp/kegg/pathway/map/map00240.html Last accessed on Aug 8, 2016.)
The dNTP pool of CEM is much higher than in resting T-cells [13, 14]. Activated vs. resting primary T-cells also have a much higher dNTP pool level. *In vitro*, phytohemagglutinin (PHA) and interleukin (IL-2) are widely used to induce T cell activation [15]. The upregulation of mitosis will greatly increase the dNTP pool. By blocking the immune activation of T cells, Pauls et al observed a decrease in dNTPs in T cells [16]. In PBMC, macrophages have a lower dNTP pool level compared to activated CD4 T-cells, which can be as high as a 130-250 fold difference, reported by diamond et al [17]. These results suggest that the baseline of the dNTP pool components are determined by multiple factors and that changes in pool size can be dynamic. Thus, diseases such as HIV/AIDS and concomitant drugs could alter dNTPs.

2.1.3 Biosynthesis of DNA

The dNTP pool is ultimately incorporated into the elongating DNA chain during DNS replication. DNA polymerase is the enzyme that synthesizes DNA molecules from dNTPs. The chemical reaction can be summarized as: dNTP + DNA\(_n\) → diphosphate + DNA\(_{n+1}\). The HIV reverse transcriptase is a viral DNA polymerase, which utilizes dNTPs for the biosynthesis of the viral genetic materials. This will be further discussed in the next section.

2.2 HIV Treatment

2.2.1 A Brief History

Before 1996, few treatments for HIV existed, and the therapy for HIV-infection was largely management of AIDS-related illnesses and prevention of common opportunistic infections [18]. Mono- and dual-therapies were attempted until the early 1990s. However, these regimens were not potent enough to fully suppress replication...
and viral resistance was a common consequence. Groundbreaking advances in HIV treatment took place in the mid-1990s with the introduction of combination therapy with three antiviral agents from two drug classes including reverse transcriptase inhibitors (RTI) and protease inhibitors (PI). This therapy advance led to fully suppressive therapy and a reduction in the morbidity and mortality related to HIV-infection and AIDS, as shown by AIDS clinical trial group (ACTG) in 1996 [19-21]. As time went on, newer treatments for HIV controlled the viral load to an undetectable level (<50 RNA copies/mL), facilitated a restoration of the immune system (increased CD4 T-cell counts), and greatly increased the life expectancy of HIV-infected patients [22-24].

Today, clinicians possess an arsenal of 26 FDA-approved antivirals that target numerous key aspects of the HIV life cycle (see table 2.2). Nevertheless, current HIV treatment still cannot eradicate viral reservoirs which includes pro-virus incorporated in long-lived cells and other viral sanctuaries, and this necessitates life-long antiretroviral therapy. This creates a continuing need to understand the clinical pharmacology of antiretroviral drugs.

2.2.2 HIV Life Cycle and the Classification of HIV Treatment

The first step of the HIV replication cycle is fusion of the HIV envelope to the target cell membrane, mainly CD4 T-cells [25], which enables viral entry. Drugs that target this process are entry inhibitors. The gp120/gp41 on the HIV envelope initially binds to the CD4 receptor, with the help of additional co-receptors, including the CC chemokine receptor (CCR5) or the CXC chemokine receptor (CXCR4) [26-29]. These interactions pull the HIV envelope into contact with the cell membrane enabling the fusion process and delivery of the viral contents into the cell. The whole process is completed within an hour [18]. A drug that targets this process can either bind to the gp120/gp41 protein on HIV (enfuvirtide) or the CCR5 receptors on T cells (maraviroc).
<table>
<thead>
<tr>
<th>Type</th>
<th>Entry Inhibitor</th>
<th>RTI NRTI</th>
<th>NNRTI</th>
<th>InSTIs</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maraviroc</td>
<td>Abacavir</td>
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</tr>
<tr>
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<tr>
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RTI: reverse transcriptase inhibitor. NRTI: nucleoside reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. InSTIs: integrase inhibitors. PI: protease inhibitor. The dissertation research focused on bolded drugs. Drugs that used in this dissertation research are in red.
After viral entry, the uncoating of the viral core occurs, which gives the virus access to the dNTP pool for pro-viral DNA synthesis, which is catalyzed by HIV reverse transcription. Reverse transcriptase (RT) is an RNA-dependent DNA polymerase, which uses the HIV single stranded RNA for the biosynthesis of HIV double-stranded DNA [30]. This process consumes the endogenous dNTP pool. An RT inhibitor (RTI) targets this viral enzyme. RT inhibitors include two sub-types: nucleos(t)ide reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). NRTI have similar structures to endogenous nucleotides. NRTIs compete with the dNTP pool at the active site of RT, slowing the process down and, if incorporated by RT, terminate the biosynthesis of the HIV DNA chain. NNRTI do not bind to the active site of RT, instead, they bind to a noncatalytic allosteric pocket close to the active site that induces a conformational change of RT, suppressing the activity of RT. Detailed mechanisms of NRTI will be discussed in the following sections.

Following production of this pro-virus, the HIV DNA chain is transported to the nucleus where it is integrated into the host DNA by HIV integrase. This viral enzyme orchestrates the integration of the 3’ end of the viral DNA chain with cellular DNA [31]. After successful DNA integration, HIV expresses its mRNA and viral RNA using the host cellular transcription system. Antivirals that target this process are integrase inhibitors (InSTIs) [32, 33]. They inhibit the viral DNA strand transfer and block the integration of the viral DNA into the human genome.

HIV uses cellular machinery to synthesize long chain viral proteins. These proteins are further processed (cleaved) by HIV protease, which catalyzes the proteolysis of the viral polyprotein [34, 35]. This process is essential for the assembly of infectious HIV particles, a requisite final maturation of HIV enabling a mature virus for the next replication cycle. Drugs that target this process are protease inhibitors (PI).
To summarize, five distinct classes of drugs target four key processes of HIV life cycle, the entry inhibitors, NRTIs, NNRTIs, InSTIs, and PIs. Figure 2.6 illustrates the HIV life cycle and the anti-HIV drug targets.

### 2.3 Clinical Pharmacology of Tenofovir and Emtricitabine

#### 2.3.1 Mechanism of Action

NRTIs were the first class of antivirals approved by the FDA for HIV treatment [36]. All NRTIs are activated to triphosphate analogs by cellular kinases [37-40]. Due to the similar structure to endogenous dNTPs but with the lack of a 3’-hydroxyl group at the 2’-deoxyribosyl (sugar) moiety, they are incorporated by RT but cannot bind to the next dNTPs by forming a 3’5’-phosphodiester bond. Thus, in addition to competition for the RT active site, they also terminate HIV DNA chain elongation if incorporated. The mechanism of action of NRTI is summarized in figure 2.7. The clinical pharmacology of the two NRTIs that this dissertation research focused on, tenofovir (TFV) and emtricitabine (FTC), will be described below.

dATP is the corresponding dNTP that tenofovir diphosphate (TFV-DP) competes with and dCTP is the corresponding dNTP with which emtricitabine triphosphate (FTC-TP) competes at the active site of HIV reverse transcriptase. The dNTP pool is maintained by an intricate enzymatic network, making it very susceptible to possible influence by xenobiotics that are processed by similar pathways. Thus, TFV-DP/FTC-TP have the potential of disturbing the dNTP pool balance by affecting the enzymes involved in the metabolism and anabolism of dNTPs. The understanding of these pathways is very important to the study of TFV-DP/FTC-TP and the dNTP pool.
Figure 2.6 HIV life cycle. (https://aidsinfo.nih.gov/education-materials/fact-sheets/19/73/the-hiv-life-cycle)
HOW NRTIs WORK

1. HIV REVERSE TRANSCRIPTASE
The HIV reverse transcriptase enzyme uses the HIV RNA chain as a template to synthesize a DNA copy using nucleotides in the host T-cell.

2. NRTIs
NRTIs are small molecule drugs that are very similar to the host cell nucleotides, and reverse transcriptase incorporates them into the new HIV DNA chain as if they were the endogenous nucleotides.

![Natural state nucleotide diagram]

3. DNA CHAIN TERMINATION
The difference between NRTIs and the endogenous nucleotides is that the NRTIs do not possess the chemical group necessary to allow for continued synthesis of the DNA chain. Consequently, once the NRTI is inserted into the DNA chain it is impossible for the reverse transcriptase to add any further nucleotides, resulting in termination of the DNA chain and interruption of the HIV replication process.

Figure 2.7 Mechanism of NRTIs. (http://www.5wgraphics.com/img/gallery/5w-sample-030-hiv-nrti.jpg Last assessed on Aug 8, 2016.)
2.3.2 Tenofovir

Tenofovir [9-(R)-(2-phosphonomethoxypropyl)adenine, PMPA] (TFV) is given as the prodrug Tenofovir disoproxil fumarate (TDF; Viread®; Gilead Sciences, Inc.). TFV has potent activity against retroviruses and hepadnaviruses. In October 2004, TDF was approved by the FDA for the treatment of HIV in adults based on its efficacy and safety data [21, 41-43]. Tenofovir is a nucleotide analog similar to but safer than adefovir and cidofovir. In 2014, after more than 7.5 million person-years of global administration of tenofovir, TDF has demonstrated excellent safety and has been established as a fundamental component of HIV antiviral regimens [44]. TDF is now the first line treatment for HIV in most countries, replacing thymidine analogs such as stavudine and zidovudine, and is now used in preexposure prophylaxis of HIV infection [45]. TDF will soon become generic in many countries within the next several years, possibly increasing its use further.

2.3.2.1 Pharmacokinetics

2.3.2.1.1 Absorption and distribution

TDF is an orally bioavailable ester of TFV. Since TFV is an acyclic nucleotide phosphonate analog (dAMP analog) that carries two negative charges on the phosphonate moiety, the absorption of hydrophilic TFV is permeability limited [46]. The addition of two alkyl methyl carbonate esters (bis-ester) increases the lipophilicity of TFV, facilitating oral bioavailability [46, 47]. After oral administration, TDF undergoes ester hydrolysis, first yielding monoester TFV, then TFV. Esterases can be found in blood plasma, organs such as gut and liver, and other tissues throughout the body, thus, TDF is efficiently cleaved on first pass and TFV is the predominate form in the blood plasma [48]. TFV \( C_{\text{max}} \) (maximum concentration) and AUC (area under the plasma
concentration-time curve) are dose proportional, with doses of 75 mg to 600 mg [49, 50]. In plasma, less than 1% is bound, and in blood, less than 7.2% is bound.

2.3.2.1.2 Metabolism and elimination

TFV and TDF are not substrates for CYP enzymes, based on in vitro studies. TFV is excreted unchanged in urine, via a combination of filtration and tubular secretion [51]. TFV is also excreted in breast milk in a very small concentration [48], only 0.03% of the proposed oral infant dose [52]. The concentration decline in plasma is biphasic [53]. The plasma elimination half-life of TFV is 12-17 hours [48, 49, 54].

2.3.2.2 Cellular pharmacology

2.3.2.2.1 Intracellular pharmacokinetics

Tenofovir enters cells by a passive process and/or fluid-phase endocytosis. It is not a transporter facilitated process that can be saturated. TFV is a phosphonate that does not require initial phosphorylation, which is the rate-limiting process for other nucleoside analogs. Also, TFV can be phosphorylated in both resting and active cells, which is a unique feature and allows this drug to be anabolized in T cells, macrophages, and tissues such as rectal or cervical tissue [55, 56]. The typical TFV (300 mg TDF) AUC$_{0-24}$ in plasma is 2-4 ug*h/mL [49, 54], and the TFV-DP concentration in PBMC is 80-160 fmol/10$^6$ cells [54, 57-64]. The understanding of intracellular half-life of TFV-DP is controversial, ranging from 50-150 hours [57, 63, 65]

2.3.2.2.2 Intracellular formation

In PBMC, TFV is phosphorylated via the cellular enzymatic system for endogenous purine nucleotides. TFV is initially phosphorylated by adenylate kinase 2 (AK2) to the intermediate form of TFV monophosphate, then is further phosphorylated by creatine kinase, pyruvate kinase (PK) and nucleoside diphosphate kinase (NDPK) to its active form, TFV-DP [55]. Figure 2.8 briefly summarizes these processes. These
enzymes also participate in the production of dATP and dGTP (figures 2.4 and 2.5), indicating TFV’s potential for disturbing dNTPs hemostasis. The breakdown of intracellular TFV-DP could be facilitated by enzymes such as 5’-NT, NDPK, and purine nucleoside phosphorylase (PNP) [55, 66, 67]. However, research is needed to understand individual contributions from these enzymes as well as other possible elimination pathways.

2.3.2.2.3 Kinases and transporters

TFV-DP and TFV-MP are inhibitors of PNP, which is responsible for a drug-drug interaction with ddl (see section 2.3.2.3.4). In vitro, TFV was shown to increase 5'-ecto-nucleotidase (NT5E), inhibit mitochondrial nucleotidase (NT5M) gene expression, and increases 5' nucleotidase (5'-NT) activity. In additional in vitro studies, TFV stimulated the expression and secretion of IL-8 and increased the expression and secretion of MIP3α [68]. TFV or its anabolites competitively inhibit creatine kinase (CK), phosphoglycerate kinase (PGK), which participate in ATP production. [67, 69]. ATP is the phosphate donor for the biosynthesis of dNTPs. Other NRTIs such as zidovudine (AZT), a thymidine analog, have been shown to inhibit its phosphorylation enzyme thymidylic kinase 2 (TK2) [70, 71]. Therefore, it is possible that TFV can inhibit AK2 (or other enzymes) leading to decreased dATP production. However, in vitro results do not always translate in vivo, so human studies are needed for confirmation.

In HIV-infected patients, dATP increased during TDF/ABC (abacavir) dual therapy compared to TDF monotherapy [60], and decreased during didanosine (ddl)-containing therapy relative to TDF-containing therapy [72]. These findings are not predicted by the discussion above, demonstrating the need for more research in this area.
In terms of transporters, TDF is a substrate and inhibitor of P-gp [47], which is responsible for drug-drug interaction with PIs (see section 2.3.2.3.4). TFV is a substrate of BCRP, MRP2/4, OAT1/3, and inhibitor of MRP1/2/3, which may help explain the possible mechanism of the renal toxicity (see section 2.3.2.6.3) [73-75]. However, TFV has also been shown to have lower affinity to DNA polymerase-\( \gamma \) compared with other NRTIs [76, 77]. Thus, toxicity effects may result in cells with very high tenofovir exposure and/or dNTP depletion. This further underscores the need to study cellular pharmacology \textit{in vivo}.

\textbf{2.3.2.3 Clinical considerations}

\textbf{2.3.2.3.1 Food effect}

The oral bioavailability of TDF (as the TFV component) is 25\% in the fasted state [48]. The time required to reach maximum plasma level (\( T_{\text{max}} \)) is 0.25-2.3 hours [48]. TFV absorption is affected by a high-fat diet (700-1000 kcal, 40-50\% fat), manifesting a 39\% increase in bioavailability (40\% increase in AUC\(_{\infty}\) and 14\% higher \( C_{\text{max}} \)), and a slower absorption profile. However, TFV has a relatively wide therapeutic index, indicating that TDF can be administered without regard to meal, offering flexibility for patients [48].

From modeling development perspectives, the low bioavailability of TFV introduces interindividual and intraindividual variabilities in population PK data, as all plasma PK parameters are bioavailability adjusted (e.g. CL/F, V/F). For controlled studies, it important for us to use standardized meals or fasting conditions in the investigation on drugs with low bioavailability.

\textbf{2.3.2.3.2 Demographic variables}

TFV clearance was faster in children and young adolescents (less than 25 years) compared to patients age greater than 25 years [78]. Modeling also demonstrated statistically significant effect of eGFR and body weight/serum creatinine ratio
Figure 2.8 Summary of the conversion and anabolism of TFV-DP. TDF: tenofovir disoproxil fumarate. TFV: tenofovir. TFV-MP: tenofovir monophosphate. TFV-DP: tenofovir diphosphate. AK2: adenylate kinase 2. PKM: pyruvate kinase isozymes from muscle. PKLR: pyruvate kinase isozymes from liver and red blood cells. NDPK: nucleoside diphosphate kinase.
(BW/S(CR)) on the plasma clearance of TFV [79]. These findings are expected for renally eliminated drugs.

**2.3.2.3.3 Special populations**

HIV and HBV/HCV coinfection do not have significant effects on TFV plasma PK [48, 80]. No substantial alteration of TFV plasma PK was observed in individuals with hepatic impairment, likely due to the fact that TFV is not eliminated through hepatic clearance [48].

Since tenofovir is eliminated via renal filtration and secretion, subjects with moderate to severe renal impairment (CL$_{CR}$<50 mL/min) have significant increases in the TFV plasma exposure, manifesting an increase in C$_{max}$, AUC, and half-life that necessitates dose adjustments [48].

**2.3.2.3.4 Drug-drug interactions**

Currently, only didanosine (ddI) and atazanavir (ATV) have demonstrated clinically relevant DDIs with TDF when coadministered (ie requires dose adjustment).

TFV lacks relevant renal interactions with other anti-HIV medications that also undergo renal elimination, such as emtricitabine (FTC), lamivudine, and stavudine, indicating relevant DDIs between them are unlikely. No DDIs were observed between TDF and NNRTI such as efavirenz (EFV) and nevirapine [81].

TFV has been shown not to be a substrate or an inhibitor/inducer of cytochrome P450 (CYP) enzymes, suggesting a low potential for drug-drug interactions (DDIs) with drugs that are substrates or inhibitors/inducers of CYP enzymes [48, 82]. TDF has been shown to have no DDIs when coadministered with common concomitant medications: opioid receptor antagonists such as methadone [83], hormonal contraceptives such as
norgestimate-ethinyl estradiol [84], and tuberculosis medications such as rifampicin (rifampin) [85].

TDF PK is not affected by ddI, but when ddI is administered either as the buffered tablet or the enteric-coated (EC) formulation, the AUC of ddI is increased by 44% (buffered tablet) and 48% (EC). This interaction exacerbates ddI-related side effects such as pancreatitis and lactic acidosis [86-88]. It is suggested that a lower dose of ddI (250 mg, instead of 400 mg) be administered [89]. Possible mechanisms for the interaction between TDF and ddI might be the inhibition of purine nucleoside phosphorylase (PNP) by TFV monophosphate. PNP is the main enzyme responsible for the breakdown of intracellular ddI [66, 67]. Other possible mechanisms such as enhanced permeability in the gut or renal competition have been assessed and ruled out [48, 89].

When coadministered with ATV, TFV plasma AUC increased by 24% and significant decreases in the ATV concentration in plasma was observed. At steady state, AUC, $C_{\text{max}}$, and $C_{\text{min}}$ (minimum concentration) were decreased by 25%, 21%, and 40% [48]. The increase in TFV AUC might be explained by the inhibition of P-gp mediated efflux of TDF, and the inhibition of TDF hydrolysis in intestinal tissue. The decrease in ATV level had been explained by induction of CYP3A4 [90]. When coadministered with TDF, ATV is recommended to be given with a PK booster such as 100 mg ritonavir (ATV/r), no adjustment of TDF dosing is needed [91].

**2.3.2.4 Pharmacodynamics**

The pharmacodynamic effects of oral doses of TDF at 75, 150, 300, and 600 mg have been assessed. Compared with the placebo group, significant viral suppression was observed in all four dosage groups. No additional viral suppression increase was observed between 300 mg and 600 mg [49], suggesting 300 mg gives the maximum
viral suppression effect. The $ED_{50}$ (effective dose that leads to 50% response rate) for TDF was estimated as 115 mg, as the PK of TDF is dose proportional [49, 50]. After discontinuation of TDF treatment, the viral suppression persisted for up to a week, which can be explained by the prolonged half-life of intracellular TFV-DP [53].

2.3.2.5 Resistance

For TFV/FTC, K65R and M184V are of particular importance for drug resistance. These are well-known and common nucleoside analog mutations (NAMs) in the HIV RT gene, which confer susceptibility changes to TFV/FTC. The K65R mutation is an amino acid substitution of a lysine to arginine at position 65, which develops in response to TFV exposure [92]. The expression of K65R reduces the antiviral efficacy of TFV by increasing the $IC_{50}$ (concentration that leads to 50% inhibition rate) by 2-4 fold (reduction in susceptibility) [93]. The M184V mutation is an amino acid substitution of a valine to methionine at position 184. It is selected by lamivudine (3TC), FTC, and abacavir (ABC). It leads to extensive resistance to these compounds, but is a hypersensitivity mutation for TFV leading to a 0.7 fold change (decrease) of $IC_{50}$ [94]. Interestingly, the coexistence of K65R and M184V results in slight decrease ($IC_{50}$ increased by 2 fold) compared to a normal susceptibility profile of HIV to TFV [95].

2.3.2.6 Tolerability and toxicity

2.3.2.6.1 General

Overall, TDF is a very well tolerated therapy for HIV treatment [44]. The most frequently reported adverse effects from mild to moderate severity are asthenia (6%), headache (14%), pain (13%), diarrhea (11%), flatulence, nausea (8%), vomiting (5%), pharyngitis, and rash (18%). Depression is the most frequently observed grade 3-4 adverse effect during therapy with TDF, with an incidence of 6%. TDF does not appear to cause fetal abnormalities [96, 97] or increased cardiovascular risks [98, 99].
2.3.2.6.2 Cytotoxicity

Mitochondrial toxicity had been reported with most NRTIs, particularly ZDV, ddI, d4T, and ddC [100]. This toxicity appears to be related to an affinity of the drug triphosphate for DNA polymerase-\( \gamma \). This leads to similar pharmacology as described above for HIV RT, causing depletion and inhibition of mitochondrial DNA synthesis [76, 77]. Notably, compared with older NRTIs such as zidovudine, zalcitabine, didanosine, stavudine, TFV and FTC are weaker inhibitors of mitochondrial DNA polymerase-\( \gamma \), thus cause lower cytotoxicity [101-103]. Two clinically relevant TDF adverse effects potentially related to mitochondrial toxicity are nephrotoxicity and bone mineral density (BMD) loss. It may be that TFV accumulates to high concentrations in these cell types. For example, TFV is taken up by OAT1/3 in the renal proximal tubule, which may expose those cells to high exposures.

2.3.2.6.3 Nephrotoxicity

The most prominent and concerning adverse effect of TDF is renal toxicity, which can vary from benign plasma creatinine increases, with minor decreases in estimated glomerular filtration rate (eGFR) to significant renal tubular dysfunction, including Fanconi’s syndrome and renal failure [44]. This can manifest as a decline in eGFR after months of treatment, which is also associated with a decrease in TFV clearance leading to a progressively worsening scenario. However, the impairment of renal function is usually reversible after the discontinuation of TDF [104]. Severe tubular impairments such as Fanconi’s syndrome and renal failure are rare, and may be exacerbated by the coadministration of boosted PIs such as LPV/r, which reduce the renal clearance of TFV [90] [105]. A possible mechanism might be inhibition of the ATP-binding cassette transporter: multidrug-resistant protein 4 (MRP4) [74, 106-108]. MRP4 facilitates the active secretion of TFV, the inhibition of MRP4 leads to the accumulation of TFV in
proximal tubule cells, possibly exacerbating the mitochondrial associated nephrotoxicity. The uptake transporters OAT 1 and OAT 3, expressed in the renal proximal tubular cells also participate in the intracellular accumulation of TFV [109-112]. Close monitoring of renal function before and during treatment, especially the evaluation of early markers of proximal tubular dysfunction is recommended in patients [88, 113, 114]. This includes assessments of increased phosphaturia, normoglycemic glucosuria, and aminoaciduria as warranted [115]. In clinical practice, TDF should be avoided in patients with preexisting renal diseases.

2.3.2.6.4 Osteomalacia

Bone mineral density (BMD) loss is another major concern of TDF treatments, especially in HIV-infected patients, who are at an increased risk of developing osteoporosis and fracture compared with the general population [116-118]. TDF induces a slight (1%-5%) decrease in bone mineral density and an increase in bone turnover [119, 120]. The decrease of BMD usually happens within the first 48 weeks of treatment and then stabilizes [121]. TFV might be affecting osteoblasts and osteoclasts directly via mitochondrial toxicity, but the mechanism has not been elucidated. Also, the loss in BMD might also be explained by the increase in phosphate wasting and renal osteodystrophy (hypophosphatemic osteomalacia) [122-125].

2.3.3 Emtricitabine

Emtricitabine [the (–)-enantiomer of 2',3'-dideoxy-5-fluoro-3'-thiacytine [FTC]] is a nucleoside reverse transcriptase inhibitor (NRTI) that was approved by the FDA in July 2003 [126]. FTC has activity against HIV-1, HIV-2, and hepatitis B virus (HBV) [127-129]. It has a chemical structure and stereochemistry similar to lamivudine (3TC), with the exception of a fluorine on the cytidine base. FTC is similar to 3TC in terms of activity,
resistance, pharmacokinetics, convenience (daily single tablet), and safety properties [130].

2.3.3.1 Pharmacokinetics

2.3.3.1.1 Absorption and distribution

FTC is very well absorbed after oral administration with a bioavailability of 86%. FTC also demonstrates linear PK in dose from 100-1200 mg [53]. The $T_{\text{max}}$ (time to reach maximum concentration) for FTC is 1-3 hours [53]. In plasma, only 4% of the drug is bound to plasma proteins [131]. FTC has a low penetration to the central nervous system, with only 4% of the serum concentration found in the CSF (Cerebrospinal Fluid) [132]. In breast milk, only 2% of the proposed oral infant dose is delivered [52]. The typical FTC $AUC_{0-24}$ in plasma is 8-11 ug*h/mL [133].

2.3.3.1.3 Metabolism and elimination

FTC is excreted predominantly in the urine, via glomerular filtration and active secretion [134]. FTC is not a substrate or an inhibitor inducer of the hepatic CYP450 enzyme system [131, 135]. Only 9% of the dose undergoes oxidation to form 3'-sulfoxide diastereomers, and 4% of the dose is conjugated with a glucuronide to form 2'-O-glucuronide. No other metabolites have been identified [131]. FTC is presumed to have no effect on liver metabolism, nor is any dosage adjustment needed in patients with hepatic impairment [136]. The elimination half-life of FTC in plasma is 7-10 hours [131, 133].

2.3.3.2 Cellular pharmacology

2.3.3.2.1 Intracellular pharmacokinetics

FTC can be formed in multiple cell types, including PBMC, RBC and tissue samples such as rectal and female genital tissues [50]. FTC-TP concentration in PBMC
is 1000-4000 fmol/10^6 cells [133, 137]. And the intracellular FTC-TP half-life is 38-39 hours [133, 138].

2.3.3.2.2 Intracellular formation

FTC is activated like other NRTIs by phosphorylation to the active triphosphate form, emtricitabine triphosphate (FTC-TP), using intracellular host pathways [11, 139]. FTC is phosphorylated via deoxycytidine kinase (dCK), uridylate-cytidylate kinase (dCMK), and likely 3’-phosphoglycerate kinase (3GPK). The detailed metabolism pathway of FTC is illustrated in figure 2.9. These enzymatic catalytic processes are saturable. They also participate in the biosynthesis of dCTP and TTP (figures 2.4 and 2.5), indicating FTC’s potential of disturbing dNTPs hemostasis.

2.3.3.2.3 Kinases and transporters

The information on kinases that might be affected by FTC and its anabolites is limited. However, lamivudine (3TC) and FTC inhibit deoxycytidine kinase (dCK) [141, 216], indicating the potential of FTC to alter dCTP.

FTC is a substrate of CNT1 and MATE1 [10]. Recently, studies reported that FTC is not a substrate of OCT1, OCT2, P-gp, BCRP or MRP2 transporters [140], which demonstrates the controversy in the understanding transporter effects in vivo. FTC might be the inducer of P-gp, and inhibitor of P-gp, MRP1, OCT1, OCT2, OAT1, and OAT2 [75]. However, future study is required to fully understand the net effects of transporters on FTC cell and tissue distribution.
Figure 2.9 Intracellular metabolism of FTC. FTC: emtricitabine. FTC-MP: emtricitabine monophosphate. FTC-DP: emtricitabine diphosphate. FTC-TP: emtricitabine triphosphate. dCK: deoxycytidine kinase. dCMK: uridylate-cytidylate kinase. 3PGK: 3'-phosphoglycerate kinase.
2.3.3.3 Clinical considerations

The absorption of FTC is not affected by food intake. However, when administered with a high-fat meal, the $C_{\text{max}}$ was decreased by 23-29% and $T_{\text{max}}$ increased by 1.5 hours, but no effect on AUC and bioavailability was observed [50, 53]. No clinically relevant drug interactions have been reported in the literature. FTC does not have drug interactions with tenofovir or efavirenz [48, 126, 141]. However, it is possible that other drugs that undergo or inhibit active tubular secretion might have interaction potential with FTC [134]. Also, other drugs that go through the same intracellular phosphorylation process, such as 3TC, may antagonize the phosphorylation of FTC [142].

Since FTC is eliminated via the renal pathway, the impairment of renal function affects the plasma concentration of FTC. The dosing interval should be adjusted in those patients who have mild to moderate renal impairment ($\text{CL}_{\text{CR}}<50$ ml/min) [143]. Close monitoring of renal function during FTC therapy is also recommended.

2.3.3.4 Pharmacodynamics

FTC (50-400 mg) produces a significant reduction in HIV RNA levels in patients. The maximum reduction occurs with FTC $\geq$ 200 mg/day. The $IC_{50}$ of FTC is 12.7 mg/day [144]. The $IC_{50}$ of FTC in peripheral blood mononuclear cells (PBMC) in vitro was reported to be 0.002–0.0085 μmol/L [127, 145], in the same range as lamivudine ($IC_{50}$: 0.001–0.11 μmol/L) and AZT ($IC_{50}$: 0.003–0.0055 μmol/L) [131, 134].

2.3.3.5 Resistance

The development of resistance to FTC occurs by a similar pathway as observed with 3TC. The most clinically relevant resistance mutation is at position 184 of HIV RT gene and is a methionine replaced by valine or isoleucine (M184V/I) [146-148].
2.3.3.6 Tolerability and toxicity

2.3.3.6.1 General

FTC is overall very well tolerated [131]. It appears to have no adverse effects on reproduction, fertility, or teratogenesis based on in vitro mutagenesis analysis and cumulative data in vivo [132, 149, 150]. The most commonly observed adverse effects of FTC are headache (7.3%), dizziness, asthenia, gastrointestinal discomfort (nausea (9.8%), diarrhea (4.9%), abnormal pain), pharyngitis (4.9%), and skin rashes, from mild to moderate severity [134, 144]. Common laboratory abnormalities are elevations in creatine kinase (7%) and triglyceride (5%). There is also reported abnormalities in liver enzymes (2%): aspartate aminotransferase (AST), alanine aminotransferase (ALT) [151, 152].

2.3.3.6.2 Cytotoxicity

Like the other NRTIs, the major theoretical cytotoxicity of FTC is its potential of interaction with human mitochondrial DNA polymerase γ. However, FTC has weak affinity towards DNA polymerase α, β, γ, and ε. The interaction for other NRTIs has also been linked to myopathy and neuropathy and possibly hepatic steatosis and lacticacidosis syndrome [77, 153-155]. However, this cytotoxicity is unlikely for FTC based on in vitro studies, which showed that FTC-TP has a very low affinity toward mitochondrial DNA polymerase γ (24 times lower compared with 3TC) [126].

2.3.3.6.3 Skin discoloration

One relatively unique side effect of FTC treatment is skin discoloration, with an incidence of 3% [156]. The toxicity presents as hyperpigmentation on the palms and soles to a mild degree. The disorder also occurs disproportionately in persons of black race, affecting 13% of black individuals, compared to only 1% of white patients. Asian
and Hispanic patients have an incidence of 3-4%. The adverse effect is reversible after the discontinuation of FTC therapy, and is generally mild and asymptomatic [131]

2.3.4 Truvada and Preexposure Prophylaxis

2.3.4.1 Advantages of co-formulation

Truvada® (Gilead Sciences, Inc.) is a co-formulated single tablet of 200 mg emtricitabine (FTC) and 300 mg tenofovir disoproxil fumarate (TDF) (136 mg of tenofovir (TFV)). Co-formulation is an effective way to reduce pill burden [1], which is a major factor affecting adherence among patients [126]. Truvada® combines the benefits of TDF and FTC, which include their favorable PK and safety properties, as mentioned above, that not only support their once daily administration but also support a lack of interactions between them. It is also reported that TDF and FTC have synergistic antiviral effects \textit{in vivo} [43], which may be explained by synergistic intracellular phosphorylation (active metabolite formation) \textit{in vitro}, together supporting co-administration [148].

2.3.4.2 Clinical pharmacokinetic considerations

As with the individual components, Truvada® can be administered without regard to food. It is generally well tolerated and effective, and as a result of safety and efficacy studies, it obtained an accelerated approval from the FDA in August 2004 [126]. The clinically relevant adverse effects of Truvada® are inherited from TDF and FTC, which are nephrotoxicity, osteomalacia, and hyperpigmentation. The low toxicity profile supports the desirable risk-benefit balance for PrEP usage (described below) [157]. As mentioned, elimination of TFV/FTC is through glomerular filtration and active tubular secretion. Thus, it is necessary to closely monitor renal function in patients taking Truvada®, such as baseline and periodic determination of eGFR (Cockcroft-Gault or modification of diet in renal disease (MDRD)), blood urea nitrogen, and serum creatinine.
Patients with CL_cr < 60 mL/min/1.73m² should not be administered a full dose of Truvada®. Because Truvada® is not a substrate or inducer of hepatic elimination pathways (eg CYP450 enzymes), there is no need for dose adjustment in patients with liver impairment [126]. Clinical significant DDIs occurs with coadministration of atazanavir (ATV) and didanosine (ddI), as described above.

2.3.4.3 Preexposure prophylaxis (PrEP)

Preexposure Prophylaxis is a new approach for controlling the HIV epidemic. It is the prophylactic use of antiretroviral agents in HIV-negative individuals to prevent HIV infection when high-risk HIV exposure may take place [10]. In 2012, Truvada® was approved by the US FDA for preexposure prophylaxis (PrEP) of HIV, based on several pivotal studies, which demonstrated safety and the protective effect of Truvada® against HIV-infection in HIV-negative individuals, including men and transgender women who have sex with men, serodiscordant men and women, and injecting drug users. HIV acquisition was reduced by 44-86% compared with placebo [158-162]. A monthly supply of Truvada® as PrEP costs $1,258, but programs are available to help with access [163]. When screening patients for PrEP, it is necessary assess HIV infection every 3 months including risk behavior assessment and evidence of acute infection. Adherence should be assessed at every clinical visit and renal function should be monitored 3 months after initiation and annually afterward) [164]. Similar to Truvada® for HIV infection treatment, PrEP also demands long-term toxicity monitoring, particularly related to renal and bone health [165]. The clinical pharmacology of TFV/FTC is summarized in table 2.3.

2.3.4.4 Other considerations on PrEP

Although Truvada® has demonstrated the protective effect of safely reducing the risk of HIV infection, there are two major studies that reported the failure of protection [166, 167]. The major reason for this failure appears to be suboptimal medication
adherence, as 60% to 70% of participants did not have measurable TFV/FTC in their blood during the study [168]. Adherence as measured by drug concentrations has also been shown to be highly correlated with efficacy among demonstration trials [169], making the assessment of adherence imperative in PrEP usage.

A potential disadvantage of PrEP is the selection of drug resistance-associated mutations if HIV is acquired during use (e.g. TDF: K65R, FTC: M184V/I, as described above) [165]. However, there is only a 3% incidence of emergence of drug resistance virus, and these were mostly in PrEP users who inadvertently started PrEP during seroconversion [158-160, 166, 167, 170, 171]. Several mathematical models agree that the relative contribution of PrEP to HIV infection reduction outweighs the risk of resistance, as PrEP contributes little (<5%) to the burden of resistance [171-174]. As mentioned above, the major concern of PrEP-associated drug resistance is when “HIV-negative” individuals have undiagnosed HIV-infection at the time of starting PrEP [171, 175, 176]. This underscores the need to rule out acute HIV infection prior to starting PrEP[10].

An ongoing concern of PrEP usage is the potential association of increased risky sexual behavior among PrEP users, also known as “risk compensation”. However, that the major PrEP trials did not observe a significant increase in high-risk behavior [177-180]. Furthermore, research in public health recognized that increased sexual pleasure is an under-acknowledged advantage of PrEP, and should be treated as an benefit for individuals considering PrEP usage [181].
Table 2.3 Summary of the clinical pharmacology of tenofovir and emtricitabine.

<table>
<thead>
<tr>
<th></th>
<th>Tenofovir</th>
<th>Emtricitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemistry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₉H₁₄N₅O₄P</td>
<td>C₈H₁₀F₅N₃O₃S</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>287.21 g/mol</td>
<td>247.25 g/mol</td>
</tr>
<tr>
<td><strong>Structure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prodrug</td>
<td>Tenofovir disoproxil fumarate</td>
<td>none</td>
</tr>
<tr>
<td>dNTP to compete with</td>
<td>dATP</td>
<td>dCTP</td>
</tr>
<tr>
<td>Isomer</td>
<td>none</td>
<td>(-)-β-enantiomer</td>
</tr>
<tr>
<td>Dose in Truvada®</td>
<td>300 mg (TDF) or 136 mg (TFV)</td>
<td>200 mg FTC</td>
</tr>
<tr>
<td><strong>Pharmacokinetics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F (bioavailability)</td>
<td>25% (fasted) (39% by a fatty meal)</td>
<td>86%</td>
</tr>
<tr>
<td>t_{max}</td>
<td>0.25-2.3 h</td>
<td>1-3 h</td>
</tr>
<tr>
<td>C_{max}</td>
<td>208 µg/L</td>
<td>1.8±0.7 µg/mL</td>
</tr>
<tr>
<td>Protein binding</td>
<td>&lt;1%</td>
<td>&lt;4%</td>
</tr>
<tr>
<td>Elimination</td>
<td>Unchanged Glomerular filtration (mainly) Tubular secretion</td>
<td>Unchanged Glomerular filtration and tubular secretion (86%) Feces (14%)</td>
</tr>
<tr>
<td>t_{1/2}</td>
<td>Plasma: 0.5-0.6 days Intracellular: 6.25 days (range 2.5-7.5)</td>
<td>Plasma: 0.3-0.4 days Intracellular: 1.6 days (range 1.2-2.3)</td>
</tr>
<tr>
<td>CL/F</td>
<td>0.51 L/h/kg</td>
<td>4.99-6.33 mL/min/kg</td>
</tr>
<tr>
<td>Clearance affected by</td>
<td>Renal function loss</td>
<td>Renal function loss</td>
</tr>
<tr>
<td>CYP₄₅₀ Substrate</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Transporter Substrate</td>
<td>OAT1/3</td>
<td>MATE 1</td>
</tr>
<tr>
<td>(Controversial)</td>
<td>MRP1/4/5</td>
<td>CNT 1</td>
</tr>
<tr>
<td></td>
<td>BCRP</td>
<td>OCT1/2</td>
</tr>
</tbody>
</table>
Table 2.3 Summary of the clinical pharmacology of tenofovir and emtricitabine (cont’d).

<table>
<thead>
<tr>
<th></th>
<th>Tenofovir</th>
<th>Emtricitabine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DDIs</strong></td>
<td>Didanosine (TFV-MP, PNP inhibitor)</td>
<td>Deoxycytidine kinase</td>
</tr>
<tr>
<td></td>
<td>Atazanavir (not known)</td>
<td>Uridylate-cytidylate kinase</td>
</tr>
<tr>
<td><strong>Phosphorylating Enzyme</strong></td>
<td>Adenylate kinase 2</td>
<td>Pyruvate Kinase</td>
</tr>
<tr>
<td></td>
<td>Nucleoside diphosphate kinase</td>
<td>3’-phosphoglycerate kinase</td>
</tr>
<tr>
<td><strong>Plasma vs PBMC</strong></td>
<td>Participate unequally</td>
<td>Participate unequally</td>
</tr>
<tr>
<td><strong>Pharmacodynamics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Resistance</strong></td>
<td>K65R</td>
<td>M184V/I</td>
</tr>
<tr>
<td><strong>Immunomodulatory effect</strong></td>
<td>(+)IL-1β, IL-10, TNF-α that interfere with HIV replication</td>
<td>(+) T cell, macrophage</td>
</tr>
<tr>
<td><strong>Side Effects</strong></td>
<td>• Startup syndrome (nausea, abdominal cramping, vomiting, dizziness, headache, and fatigue)</td>
<td>• Transitory CNS complaints (sleep disturbance with insomnia, abnormal dreaming, mood changes and depression) at first few days</td>
</tr>
<tr>
<td></td>
<td>• Renal toxicity (accumulation within proximal renal tubules leads to mitochondrial damage)</td>
<td>• Nausea, Diarrhea</td>
</tr>
<tr>
<td></td>
<td>• Loss of bone mineral density (BMD)</td>
<td>• Rash</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hyperpigmentation of palms &amp; soles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Headache</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Flu-like syndrome</td>
</tr>
<tr>
<td><strong>Extend of adverse effects</strong></td>
<td>Mild to Moderate</td>
<td>Mild to Moderate</td>
</tr>
<tr>
<td><strong>Dosage adjustment</strong></td>
<td>CL_{CR}&lt;60 mL/min/1.73m^2</td>
<td>CL_{CR}&lt;50 mL/min/1.73m^2</td>
</tr>
</tbody>
</table>
2.3.5 Atripla

2.3.5.1 Coformulation of Atripla

Atripla™ (Gilead Sciences, Foster City, CA, and Bristol-Myers Squibb, Newark, NJ, USA) is a coformulation of 600 mg efavirenz (EFV, Sustiva®, Bristol-Myers Squibb), 200 mg emtricitabine (FTC), and 300 mg tenofovir disoproxil fumarate (TDF) [182]. The current guidelines recommend that the treatment of HIV includes two NRTIs as “back bone” agents and one antiviral from another class as an “anchor” agent. It was the first FDA-approved (July 2006) single daily tablet (“one-pill-daily”) form of HIV-treatment, with the goal of reducing pill burden and benefitting adherence. It represented the most simplified antiviral dosing schedule available at that time [126]. This research dissertation included Atripla™, but focused on TDF/FTC. Therefore, a brief description of efavirenz pharmacology follows.

2.3.5.2 Efavirenz

Of all three compounds included in Atripla, EFV is the one that had been available for the longest time period (approved by FDA in Sep 1998). EFV is a nonnucleoside reverse transcriptase inhibitor (NNRTI), which acts as a noncompetitive HIV-1 RT inhibitor [182]. EFV does not significantly inhibit mitochondrial DNA polymerase-γ, nor does it inhibit polymerase α, β, or δ [183]. The EC<sub>90-95</sub> of EFV in vitro is 1.7-25 nmol/L. It is a highly efficacious antiviral drug against HIV-1 [184, 185]. The signature resistance mutation is K103N, which leads to class resistance to most NNRTIs [186].

2.3.5.3 Clinical pharmacokinetics considerations

The bioequivalence of TDF/FTC and TDF/FTC/EFV coformulated single tablet and their individual components have also been demonstrated, supporting the coadministration and lack of interactions among them [187-189]. Similar to TDF, EFV is
affected by food intake, manifesting as a 28% greater AUC in high-fat meal vs fasting conditions [183]. It is suggested that Atripla be taken once daily on an empty stomach (before sleep) to minimize side effects. Steady state plasma EFV is achieved after 6-7 doses. Its long half-life (40-55 hours) enables its once-daily dose administration, facilitating the once-daily single tablet coformulation with TDF/FTC. EFV is highly bound (>99%) to plasma protein, mainly albumin. EFV is metabolized predominantly via CYP3A4 and CYP2B6, producing hydroxylated metabolites which undergo glucuronidation. The metabolites do not have antiviral activities. EFV is an inducer of CYP3A and an inhibitor of CYP2C9, CYP 2C19, and CYP 3A4. Possible DDI should be closely monitored in patients on medications cleared by these enzymes. 14-34% of EFV dose is eliminated via kidney as metabolites and 16-61% of EFV is excreted in the feces unchanged [183, 190]. EFV is not affected by renal impairment, however, Atripla is restricted by the renal disposition of TDF/FTC and should not be used in renal dysfunction. Patients who have eGFR<50 mL/min/1.73m\(^2\) should consider alternative therapy. Since EFV is metabolized in the liver, Atripla™ should be used with caution in patients who have hepatic impairment.

2.3.5.4 Tolerability and toxicity

Generally, Atripla™ is moderately well tolerated by HIV-infected patients. Due to teratogenic effects of EFV, Atripla™ is not recommended in women of child-bearing potential, pregnant, or breast-feeding mothers [191, 192]. Two other clinically relevant side effects of EFV include skin rash (mild to moderate) and neurological adverse effects [193]. Skin rash typically occurs within the first two weeks of treatment (26%), and central nervous system (CNS) side effects include dizziness and somnolence (~50%). The CNS effects usually appears during the first and second day of administration, and may resolve after 2-4 weeks of treatment [194, 195]. Other neurological effects such as
sleep disturbance are generally well tolerated in patients [196]. Lactic acidosis and severe hepatomegaly have been reported in patients taking Atripla™, and should be closely monitored especially in those patients with pre-existing hepatic impairment [192].

The following research dissertation evaluated the cellular pharmacology of TFV and FTC in healthy volunteers and HIV-infected individuals (who also received EFV). This work included dNTP measurements and a PK-PD model that linked plasma with cellular TFV-DP/FTC-TP, and finally linked these active anabolite concentrations with changes in dNTP. The following chapters describe the steps used in the research, and the results.
CHAPTER III

DEVELOPMENT AND VALIDATION OF A LC-MS/MS QUANTITATIVE METHOD FOR ENDOGENOUS DEOXYNUCLEOSIDE TRIPHOSPHATES IN CELLULAR LYSATE

3.1 Introduction

The endogenous deoxynucleoside triphosphate (dNTP) pool includes deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and thymidine triphosphate (TTP). The endogenous dNTP pool components are the building blocks of DNA, and they play important roles in cell division, proliferation, and differentiation. The balanced concentrations of the four components in the dNTP pool, as well as the overall pool sizes, maintain these essential functions of nucleated cells.

The endogenous dNTP pool is regulated by complex enzymatic pathways that can be targeted by drugs. For example, antimetabolites as chemotherapy agents, affect the enzymes involved in the anabolism of dNTPs, systematically suppressing the dNTP pool and thereby cancer cell proliferation. Fluorouracil is an example of a suicide inhibitor for thymidylate synthase that depletes thymidine nucleotides [197, 198]. Also, methotrexate competitively inhibits dihydrofolate reductase, suppressing folate and eventually the de novo synthesis of purine and pyrimidine bases [199], whereas mercaptopurine interferes with hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and phosphoribosylpyrophosphate (PRPP) amidotransferase, eventually suppressing purine nucleotide biosynthesis [200]. Given that these drugs target the

dNTP pool, the quantitation of these moieties can help inform antimetabolite biological effects.

Additionally, numerous nucleos(t)ide analogs (NA) are marketed as antivirals and these agents compete with dNTPs (owing to their similar structures) and inhibit virus genetic material replication. For example, aciclovir, an anti-herpes antiviral, has an active metabolite that competes with deoxyguanosine triphosphate at the binding site of HSV DNA polymerase [201] and, tenofovir – an HIV drug used for HIV infection pre-exposure prophylaxis (PrEP) and treatment – competes with deoxyadenosine triphosphate at the HIV reverse transcriptase (RT) active site. If the NA is incorporated this results in HIV-DNA chain termination as these moieties lack the 3’ hydroxyl needed for chain elongation [202]. For these and other nucleos(t)ide analogs, the ratios between nucleos(t)ide analog and the corresponding dNTP define their pharmacologic efficacy [157]. Thus, it is important to have methodologies to investigate the dNTP disposition in individuals receiving nucleos(t)ide analogues.

Historically, the quantitation of the dNTP pool presented challenges because of sensitivity needs, chromatography problems, and matrix issues for endogenous compounds. Traditional detectors such as UV [203, 204] and diode array [205, 206] are generally not sensitive enough to quantify dNTPs in clinical samples, which contain limited cell numbers. The high polarity and chemical similarities of dNTP (and NTP) require ion-pairing or similar approaches [207], which in our hands, increase run times, limit sensitivity, and introduce chromatography variability [208]. Here, we report a novel LC-MS/MS quantitative method of each individual dNTP (dATP, dCTP, dGTP, and TTP) from cellular lysate, which circumvents many of these challenges.
3.2 Methods

3.2.1 Chemicals and Materials

Chemicals were acquired from Sigma-Aldrich Chemical, St. Louis, MO: A deoxynucleotide set, 10 mM (deoxyadenosine triphosphate: Product#: D6920, MW: 535.15; deoxycytidine triphosphate: Product#: D7045, MW: 511.12; deoxyguanosine triphosphate: Product#: D7170, MW: 573.13; and thymidine triphosphate: Product#: T7791, MW: 504.15). Internal standards were purchased from Cambridge Isotope Laboratories, Inc. Andover, MA including 2′-deoxyadenosine-15N5 (dA-IS, Cat#: PR-15211, MW: 256.21), 2′-deoxycytidine-15N5 (dC-IS, Cat#: NLM-3897-0, MW: 230.19), 2′-deoxyguanosine:H2O-15N5 (dG-IS, Cat#: NLM-3899-CA-0, MW: 290.22), and Thymidine-13C10, 15N2 (T-IS, Cat#: CNLM-3902-0, MW: 254.14). Analytical grade reagents were obtained from: Fisher Scientific, Fairlawn, NJ: methanol, 2-propanol, acetic acid, and potassium chloride; Sigma-Aldrich Chemical: alkaline phosphatase; and JT Baker, Phillipsburg, NJ: acetonitrile. Ultrapure (UP) water was prepared in house from deionized water with a Barnstead Nanopure System (Thermo Fisher Scientific, Waltham, MA). Consumables included Waters Sep-Pak Accell Plus QMA Cartridge, 3cc/500 mg (Water Corporation, Milford, MA); Phenomenex Strata-X-CW 33 µm Polymeric Weak Cation Mixed Mode Cartridge 3cc/200mg (Phenomenex, Inc., Torrance, CA); and blood products for the lysed cellular matrix (Bonfils, Denver, CO).

3.2.2 Analytical Approach

Standard and QC stocks were created in a concentration of 50 pmol/µL (stored at -80°C) in ultrapure water from Sigma® Deoxynucleotide Set 10mM (dATP, dCTP, dGTP, and TTP). Purity and potency of the compounds were assessed, as previously described [208] and were further diluted in 70:30 methanol: water (70% methanol), which was also called the cell lysis solution, to working standards ranging between 50 and 2500
fmol/sample, which were aliquoted and stored at -80°C. QC preparation stocks were also created in ultrapure water at a concentration of 50 pmol/µL, then diluted to concentrations of 150, 600, and 2000 fmol/sample in 70% methanol. Sample was defined as 200 µL in cell lysis solution. These solutions did not include cells because cellular lysate matrix contains endogenous dNTPs, which would interfere with quantitation.

To help qualify analytical runs, this method also included a quality assurance (QA) sample in each run. This was a blank lysed PBMC matrix sample (from Bonfils, Denver, CO, USA) with an aliquot assayed in each run to demonstrate consistent results, similar to an incurred sample reanalysis (ISR). A combined lot of PBMC lysate in 70% methanol was processed resulting in “stock” solution consisting of 10×10⁶ cells/mL. This solution was then aliquoted into 1 million cell samples and all were stored at -80°C. One aliquot was quantified in each run to assess the assay reproducibility. The average value and coefficient of variation (CV) were assessed, with acceptance criteria of ≤15%.

For each analytical run, two 70% methanol solutions (as blank samples), standards, QCs, and the QA were extracted. Separation of intracellular monophosphate (MP), diphosphate (DP), and triphosphate (TP) fractions was accomplished using a potassium chloride concentration gradient (MP: 5mL × 75mM KCl; DP: 7mL × 90mM KCl; TP: 2mL × 1M KCl) with Waters QMA Solid Phase Extraction (SPE) cartridges. The triphosphate fraction was then dephosphorylated using excess phosphatase. Both alkaline and acid phosphatase from Sigma® were assessed. Peak area responses from 10, 15, 30, or 60 minute incubations were tested for completion of dephosphorylation. Following dephosphorylation, internal standard (IS) working stock solution (20 µL) was added to all except the blank without IS sample. Samples were desalted and concentrated using Phenomenex Strata-X-CW SPE cartridges, using an extraction
process optimized for deoxynucleosides. The Strata-X-CW SPE was prepared with 1 × 2.0 mL methanol and 1 × 2.0 mL ultrapure water with 1 min × 200 g centrifugation. Samples were then applied to the cartridge, and centrifuged for 3 min × 100 g. The cartridge was then washed with 2 × 2.0 mL ultrapure water at 1 min × 200 g centrifugation plus a 1 × 0.25 mL methanol addition (this latter step significantly facilitated the drying process). Analytes were then eluted using 3 × 0.5 mL methanol for 1 min × 200 g centrifugation. Samples were dried for 30 minutes under nitrogen at 50°C in a Zymark TurboVap (Zymark Corp., Hopkinton, MA, USA). The sample was reconstituted using 100 µL ultrapure water, vortex mixed, and transferred to a 150 µL low volume insert. A 20 µL aliquot of reconstituted solution was injected onto the ultrahigh pressure liquid chromatography (UHPLC-MS/MS) system. Figure 3.1 briefly summarizes the extraction process, and shows the extra quality assurance (QA) sample application.

A Thermo Quantum Ultra® triple quadrupole mass spectrometer coupled with a HESI II® probe was used for detection. The Waters Acquity® UHPLC system (Waters corporation, Milford, MA) included Binary Solvent Manager (BSM) and Sample Manager (SM). SM used a 250 µL sample syringe, 50 µL sample loop, and data were captured with Xcalibur™ 2.2 SP1.48. A 100 × 2.1 mm Phenomenex Kinetex 2.6µ PFP 100Å analytical column (Phenomenex, Torrance, CA) was used for chromatographic separations. The mobile phase consisted of 2% isopropanol and 0.1% acetic acid in ultrapure water at an isocratic flow of 400 µL/min. The column temperature was 50°C, and the sample temperature was 15°C. Each injection was followed by a strong (70:20:10 Methanol–Water–isopropanol) and weak needle wash (50:50 acetonitrile–water), to eliminate carry over. The source was operated in positive ionization mode.
Figure 3.1 Sample processing procedure overview.
The spray voltage was 3500 V, vaporizer temperature 400°C, sheath gas (nitrogen) 60 arbitrary units, aux gas (nitrogen) 20 arbitrary units, capillary temperature 175°C, chromfilter peak width 6.0 s, collision gas (argon) pressure 1.0 mTorr, T lens (V) were 150, 159, 144, and 173 for dA, dC, dG, and T. Collision energy (V) were 10, 13, 11, and 11 for dA, dC, dG, and T. Resolution was set at 0.7 FWHM for both Q1/Q3, scan width 0.002 m/z, scan time 0.035 s, and centroid data type collected. The run time was three minutes. The SRM precursor/product transitions (m/z) were as follows: dA (252.092/136.100), dA-IS (257.210/141.000), dC (228.112/112.081), dC-IS (231.200/115.100); dG (268.081/152.100), dG-IS (273.102/157.100); and T (243.081/127.085), T-IS (255.081/134.085).

3.2.3 Validation

This method was validated using Food and Drug Administration (FDA) guidance, with acceptance criteria of ±15% (LLOQ: ±20%) for both accuracy (compared with nominal as percentage deviation (%dev)) and precision (as CV) determinations at all concentrations. Accuracy and precision were determined by replicate analysis (n=6) at each QC level described above, plus a QC prepared at the LLOQ level (50 fmol/sample), in three separate analytical runs. Standard curve performance in these runs was also assessed. Matrix effect (ME), recovery (RE), and processing efficiency (PE) were determined following the experiments described by Matuszewski et al [209]. Since the biological matrix (cellular lysate) already contained the analytes (endogenous dNTPs), to investigate the ME, RE, and PE of the desalting and concentration process, we used the internal standards peak area (stable labeled isotopes of each analyte) as previously described by Machon et al [210]. Six different lots of PBMC lysate matrix were extracted, and three levels of stable labeled internal standard working solutions were prepared at concentrations of 150, 600, and 2000 fmol/sample. The slopes CVs (calculated from six
lots of matrix) of internal standard (dNTP-IS) peak areas from post extraction spike
sample (set 2) and extracted sample (set 3) were used for assessment (an analyte:IS
ratio was not available).

3.2.4 Minimization of Contamination, Specificity and Selectivity

The endogenous dNTP pool measurement at an ultra-sensitive level (lower limit
of quantitation: 50 fmol/sample) requires minimizing the risk of environmental
contamination, which is particularly important for method specificity and selectivity. A
separate set of glassware and reagents were exclusively used for this assay. All
solutions were either freshly made with an expiration day less than 72 hours (e.g.
potassium chloride solution), or aliquoted and frozen at -20°C (e.g. enzyme buffer
solution). Consumables such as tubes, pipette tips, transfer tips, and vials were
disposed of immediately after use. Gloves were frequently changed during extractio
process. Centrifuges that had been shared with other personnel was wiped with
deionized water before use.

Specificity was determined by injecting extracted 70% methanol lysate solution
and monitoring for dNTPs. The high standard (2500 fmol/sample) with no internal
standard and blank with internal standard were used to evaluate cross-talk between
dNTP and dNTP internal standard. Carry over was evaluated by assessing signal in a
blank water injection following the cross-talk samples. Additionally, blank and blank
internal standards were included with each analytical run to monitor for specificity and
selectivity.

3.2.5 Conditional Stability

Conditional dNTP stability in 70% methanol was determined by assessing
freeze/thaw stability and room temperature stability using triplicate quality control (QC)
samples at 150 and 2000 fmol/sample (QL and QH). Freeze thaw stability in three cycles, from -80˚C to ambient, was assessed, and room temperature stability was investigated by maintaining triplicate QC samples at room temperature for up to 24 hours prior to extraction. Result mean values were compared to triplicate control sample mean values. Extracted sample stability was determined by assessing six replicates of extracted sample using QL and QH. Samples were tested by maintaining in the autosampler (15˚C) for 5 days prior to reinjection. Result mean values were compared to the first day injection data averages. Long term stability was assessed using triplicate QC samples in 300 fmol/sample, which were maintained at -80˚C for up to 2 years. Result mean values were compared to triplicate fresh extracted control sample averages.

Conditional dNTP stability in water was determined for prep stock solutions. Freeze/thaw (3 cycles) stability, room temperature stability up to 24 hours were assessed. Dilution were made prior to analysis, and result averages from triplicated extracted samples were compared to the nominal value (2000 fmol/sample).

Conditional dNTP stability in biological matrix was assessed using the quality assurance sample (QA, one combined lot of PBMC lysate stored at -80˚C). Freeze/thaw (3 cycles) stability, room temperature stability at a longer time period up to 9 days, and long term stability (-80˚C) up to 2 years were evaluated. Triplicate treated sample averages were compared to mean values from 22 separate analytical runs (see section 3.3.5). Results from conditional stability were assessed with the acceptance criteria of ≤15%.

3.2.6 Method Application

This method was used to determine repeated measurements of the deoxy-nucleoside triphosphate pool in peripheral blood mononuclear cell lysates from 40 subjects (n=279 total samples). The clinical protocol was approved by the institutional
review broad (IRB) of the University of Colorado, and participants provided informed consent. Among these subjects, 19 were infected with HIV, 21 were HIV-negative. Participants were receiving daily co-formulated 300 mg Tenofovir disoproxil fumarate (TDF) and 200 mg Emtricitabine (FTC), plus daily 600 mg Efavirenz in HIV-infected group, as part of a pharmacokinetic study. Typically, 1.5 million cells were assayed.

3.3 Results

3.3.1 Accuracy and Precision

Standard curves were best fitted by quadratic regression \( y = ax^2 + bx + c \) with 1/concentration weighting, and ranged from 50 to 2500 fmol/sample. Standard performance is shown in table 3.1. Accuracy was within ±13.0% and precision ≤ 14.3% for back-calculated standards. The calibration curve \( r^2 \) were ≥ 0.9965.

Intra- and inter-assay accuracy and precision based on the QCs are shown in table 3.2. Intra-assay accuracy was within ±12.3% and precision within 15.2% for LLOQ, and accuracy within ±8.6% and precision within 11.1% for other QCs. Inter-assay accuracy was within ±9.3% and precision within 13.2% for all QC levels, including the LLOQ.

3.3.2 Matrix Effect, Recovery, and Process Efficiency

The recovery of the endogenous deoxynucleoside triphosphate moiety off the Waters QMA SPE were previously determined to be ≥95.2% [9]. Acid phosphatase from Sigma® was assessed and found to be containing deoxynucleosides, therefore, alkaline phosphatase was adopted to dephosphorylate the nucleotide fractions. Peak area responses were equivalent during incubation ranging from 10 to 60 minutes, demonstrating complete dephosphorylation. A 15-minute incubation was adopted.
Table 3.1 Accuracy and precision of calibration standards from n=3 analytical runs.

<table>
<thead>
<tr>
<th>Nominal Value (fmol/sample)</th>
<th>50</th>
<th>100</th>
<th>250</th>
<th>500</th>
<th>750</th>
<th>1000</th>
<th>1500</th>
<th>2500</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP %CV</td>
<td>5.0</td>
<td>6.0</td>
<td>10.2</td>
<td>2.9</td>
<td>5.8</td>
<td>3.1</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>%dev</td>
<td>5.9</td>
<td>-5.2</td>
<td>-2.9</td>
<td>-0.4</td>
<td>-2.2</td>
<td>3.5</td>
<td>-0.3</td>
<td>-0.2</td>
</tr>
<tr>
<td>dCTP %CV</td>
<td>4.7</td>
<td>10.5</td>
<td>7.9</td>
<td>3.0</td>
<td>8.2</td>
<td>5.3</td>
<td>3.0</td>
<td>1.0</td>
</tr>
<tr>
<td>%dev</td>
<td>5.3</td>
<td>-0.6</td>
<td>-5.3</td>
<td>-1.7</td>
<td>-1.0</td>
<td>3.0</td>
<td>0.6</td>
<td>-0.5</td>
</tr>
<tr>
<td>dGTP %CV</td>
<td>3.8</td>
<td>14.3</td>
<td>9.2</td>
<td>4.6</td>
<td>7.0</td>
<td>2.0</td>
<td>3.2</td>
<td>0.8</td>
</tr>
<tr>
<td>%dev</td>
<td>4.7</td>
<td>0.1</td>
<td>-3.9</td>
<td>-1.7</td>
<td>-4.1</td>
<td>4.3</td>
<td>1.1</td>
<td>-0.6</td>
</tr>
<tr>
<td>TTP %CV</td>
<td>7.0</td>
<td>7.5</td>
<td>3.2</td>
<td>1.6</td>
<td>5.6</td>
<td>6.3</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>%dev</td>
<td>12.0</td>
<td>-13.0</td>
<td>-2.2</td>
<td>-4.6</td>
<td>-0.5</td>
<td>3.6</td>
<td>1.0</td>
<td>-0.6</td>
</tr>
</tbody>
</table>

Table 3.2 Inter- and intra-assay accuracy and precision of quality control samples prepared at known concentrations.

<table>
<thead>
<tr>
<th>Nominal Value (fmol/sample)</th>
<th>Inter-assay Statistics, n=18</th>
<th>Intra-assay Statistics (min, max), n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>dATP %CV</td>
<td>8.1</td>
<td>7.2</td>
</tr>
<tr>
<td>%dev</td>
<td>5.5</td>
<td>-2.1</td>
</tr>
<tr>
<td>dCTP %CV</td>
<td>13.2</td>
<td>9.0</td>
</tr>
<tr>
<td>%dev</td>
<td>1.0</td>
<td>-2.6</td>
</tr>
<tr>
<td>dGTP %CV</td>
<td>7.8</td>
<td>7.8</td>
</tr>
<tr>
<td>%dev</td>
<td>9.3</td>
<td>-0.3</td>
</tr>
<tr>
<td>TTP %CV</td>
<td>13.0</td>
<td>11.2</td>
</tr>
<tr>
<td>%dev</td>
<td>4.2</td>
<td>-1.3</td>
</tr>
</tbody>
</table>
Matrix effect, recovery, and process efficiency are detailed in table 3.3. The slopes %CV (n=6) from post extraction spike samples (set 2) were 2.3% for dATP, 3.9% for dGTP, 3.9% for TTP, and 4.1% for dCTP; while from extracted samples (set 3) were 2.9% for dATP, 3.8% for dGTP, 3.8% for TTP, and 49.3% for dCTP. The %CV was high in dCTP set 3 samples but not in set 2 samples, which was because the IS peak areas were used instead of analyte/IS ratios (see section 3.2.3). The dCTP recovery is lowered by the addition of the 1 × 0.25 mL methanol wash prior to elution to facilitate drying process in Strata-X-CW process (see section 3.2.2). The variation in the dCTP recovery caused high %CV in slopes for set 3 samples, however, in practice, the analyte/IS ratio will correct for the variability of recovery.

3.3.3 Specificity and Selectivity

The overlay chromatographs of blank, blank and internal standard, and LLOQ sample is shown in figure 3.2. No more than 20% (IS: 5%) of the LLOQ level (50 fmol/sample) was found in the blank lysate solution matrix samples (≤12%), nor was any significant cross-talk or carry over observed for any dNTP or the internal standard (≤0.94%), showing the method to be specific and selective for dNTPs and dNTPs internal standard.

3.3.4 Analyte Stability

The stability of dNTP pool components in 70% methanol, water, and cellular lysate is shown in table 3.4. Treated samples were within ±13.2% of control for all tested conditions, except for dGTP QL samples after 24-hour room temperature treatment (-18.5% of control), suggesting QC samples require freezer storage. The dNTP room temperature stabilities were up to 6 hours for QCs in 70% methanol, 24 hours for prep stocks in water, and 9 days for QA samples (cellular lysate). Both QC and QA samples were stable for up to 2 years at -80°C.
Table 3.3 Matrix effect (ME), recovery (RE), and process efficiency (PE).

<table>
<thead>
<tr>
<th>%</th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>TTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ME</td>
<td>RE</td>
<td>PE</td>
<td>ME</td>
</tr>
<tr>
<td>Nominal Value (fmol/sample)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>104.1</td>
<td>98.8</td>
<td>102.8</td>
<td>64.3</td>
</tr>
<tr>
<td>600</td>
<td>102.9</td>
<td>97.1</td>
<td>99.9</td>
<td>62.4</td>
</tr>
<tr>
<td>2000</td>
<td>101.7</td>
<td>96.2</td>
<td>97.8</td>
<td>65.3</td>
</tr>
<tr>
<td>Mean</td>
<td>102.9</td>
<td>97.4</td>
<td>100.2</td>
<td>64.0</td>
</tr>
</tbody>
</table>

Data shown are averages from six matrix lots.
Figure 3.2 Typical overlaying chromatogram of the blank sample (bottom), blank with internal standard (middle), and lower limit of quantitation sample (top, 50 fmol/sample). RT: Retention Time; LLOQ: Lower Limit of Quantitation Sample; BLK-IS: Blank Sample with Internal Standard; BLK-BLK: Blank Sample. The SRM precursor/product transitions (m/z) are as follows: deoxycytidine (228.112/112.081); deoxyguanosine (268.081/152.100); thymidine (243.081/127.085); and deoxyadenosine (252.092/136.100).
Table 3.4 Accuracy of samples evaluated for conditional stability.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>%difference</th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>TTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC in 70% Methanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze/Thaw</td>
<td>3 cycles</td>
<td>-5.8</td>
<td>-4.4</td>
<td>1.3</td>
<td>-2.9</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>3 hours</td>
<td>-3.4</td>
<td>-5.8</td>
<td>-9.6</td>
<td>-5.4</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>6 hours</td>
<td>-5.3</td>
<td>-4.9</td>
<td>-5.9</td>
<td>-5.1</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>24 hours</td>
<td>-12.2</td>
<td>-2.7</td>
<td>-9.7</td>
<td>-5.5</td>
</tr>
<tr>
<td>Autosampler</td>
<td>5 days</td>
<td>3.6</td>
<td>2.7</td>
<td>-6.9</td>
<td>-4.9</td>
</tr>
<tr>
<td>Long Term*</td>
<td>2 years</td>
<td>1.4</td>
<td>-0.7</td>
<td>0.9</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Prep. Stocks in Water

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>%difference</th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>TTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze/Thaw</td>
<td>3 cycles</td>
<td>8.8</td>
<td>-2.0</td>
<td>0.4</td>
<td>5.3</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>24 hours</td>
<td>4.7</td>
<td>-3.5</td>
<td>0.2</td>
<td>4.7</td>
</tr>
</tbody>
</table>

QA Sample: PBMC Lysate

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>%difference</th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>TTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze/Thaw</td>
<td>3 cycles</td>
<td>-7.7</td>
<td>-8.8</td>
<td>-10.0</td>
<td>-8.5</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>24 hours</td>
<td>-1.3</td>
<td>1.2</td>
<td>-4.2</td>
<td>6.8</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>3 days</td>
<td>2.0</td>
<td>2.7</td>
<td>-4.1</td>
<td>-3.1</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>6 days</td>
<td>3.9</td>
<td>6.2</td>
<td>-2.2</td>
<td>-0.1</td>
</tr>
<tr>
<td>Room Temp.</td>
<td>9 days</td>
<td>2.6</td>
<td>4.9</td>
<td>0.0</td>
<td>-1.1</td>
</tr>
<tr>
<td>Long Term</td>
<td>2 years</td>
<td>-4.0</td>
<td>2.2</td>
<td>-4.3</td>
<td>11.6</td>
</tr>
</tbody>
</table>

*compared to nominal value (300 fmol/sample)
3.3.5 Clinical Application

Median (interquartile range) concentrations in peripheral blood mononuclear cells from study participants were 143 (116, 169) for dATP, 737 (605, 887) for dCTP, 237 (200, 290) for dGTP, and 315 (220, 456) for TTP, respectively, in femtomole per million cells (see figure 3.3). Typical chromatographs for a clinical research sample is shown in figure 3.4.

For the QA sample, for which 1 million cells were extracted from a single PBMC pool, a total of 22 separate analytical runs were analyzed. The average level of dATP, dCTP, dGTP, and TTP in femtomole per million cells was 270, 313, 152, and 198, and the CVs were 7.67%, 5.15%, 9.17%, and 8.84%.

3.4 Discussion

Currently, most quantitative methodologies of the dNTP pool adopted a traditional strategy: measurement of the target analytes directly using ion-pairing [204, 207, 211], anion exchange [203, 205, 206] chromatographic techniques, or periodate oxidation procedure (to remove NTP) [203, 212] for separation. Our experiences with ion-pairing technologies led to carryover issues likely from residual ion-pairing binding on the system [213]. Thus, we took a different strategy using an indirect approach: first, we separated mono-, di-, and triphosphate moieties using solid phase extraction; next, by collecting and dephosphorylating the triphosphate fractions, we acquired molar equivalent deoxynucleosides; then, another solid phase extraction was optimized exclusively for deoxynucleosides; last, a sensitive liquid chromatography-tandem mass spectrometry quantitative method was developed for these deoxynucleosides. This method requires a longer extraction process, however, compared to other
Figure 3.3 Bar plot of medians and interquartile ranges of dNTP concentrations in PBMC. dCTP: deoxycytidine triphosphate; TTP: thymidine triphosphate; dGTP: deoxyguanosine triphosphate; dATP: deoxyadenosine triphosphate.
Figure 3.4 Typical subject PBMC sample chromatogram. Resulting in a concentration of dCTP: 307, dGTP: 143, TTP: 207, and dATP: 264 in femtomole per million cells. The SRM precursor/product transitions (m/z) are as follows: deoxycytidine (228.112/112.081), deoxycytidine-^{15}N_5 (231.200/115.100); deoxyguanosine (268.081/152.100), deoxyguanosine-^{15}N_5 (273.102/157.100); thymidine (243.081/127.085), thymidine-^{13}C_{10} (255.081/134.085); and deoxyadenosine (252.092/136.100), deoxyadenosine-^{15}N_5 (257.210/141.000).
methodologies that measure triphosphates directly, an indirect strategy has several advantages. We were able to simplify the liquid chromatography by separating deoxynucleosides, facilitating a faster, sharper, and cleaner chromatography with isocratic mobile phase. We were able to achieve excellent sensitivity represented by lower LLOQ (50 fmol/sample), compared with other LC-MS/MS methodologies: LLOQ =0.25 pmol/sample reported by Cohen et al. [207], and LLOQ =0.3-0.4 pmol/sample reported by Hennere et al [212]. Potential interferences were minimized from similar compounds such as dGTP and ATP (and nucleoside analog drugs such as zidovudine-triphosphate), which have the same molecular weight and major transition product [213]. This approach is also able to quantify monophosphates and diphosphates with similar advantages. This indirect quantitative method can be used for cellular lysate samples of different cell types, as previously described [9].

For an endogenous compound analytical method, the development and validation require special considerations, such as how to handle the biological matrix and minimize the environmental contamination. In this case, biological matrix (cellular lysates) contains the endogenous dNTP pool, so it cannot be used for matrix effect assessment or quality control sample preparation. For this reason, we used lysis solution as the assay matrix and evaluated peak areas of stable labeled internal standards to assess matrix effects [210]. To further assess the overall extraction performance, we introduced a quality assurance sample (an aliquot from one combined lot of PBMC lysate). These samples assessed performance of dNTP standards and QCs in every run. Finally, environmental contamination was a problem that may affect the assay accuracy and precision, introducing systematic bias, particularly at the lower concentrations. Multiple procedural steps were used in this method to prevent contamination.
The assay was used to quantify the dNTP pool in peripheral blood mononuclear cell lysate from study participants. Median (interquartile range) concentrations were 143 (116, 169) for dATP, 739 (606, 889) for dCTP, 238 (201, 291) for dGTP, and 315 (220, 458) for TTP, respectively, in femtomole per million cells. These results generally agree with previous results from Hawkins et al who reported a median (interquartile range) concentration in femtomole per million cells of 154 (10, 474) for dATP, and 111 (53, 359) for dGTP, in HIV-infected patients [72]. Other studies reported higher concentrations such as Hennere et al. who reported a TTP level in femtomole per million cells range from 1800 to 6700, and dCTP from 550 to 3970 in patients receiving d4T, AZT, and 3TC [212], as well as Goicoechea et al who reported medians range 3238–4638 for dATP and 2464–4026 for dGTP in femtomole per million cells [60]. The reasons for these differences might due to the analytical methodologies, underscoring the need for additional research in this area.

On the benchtop, the dGTP quality control did not pass at 24 hours. This sample type was prepared in 70% methanol suggesting that pure triphosphate solutions may degrade at room temperature. Importantly, the QA sample, which represents a biological cell lysate in 70% methanol, was stable at room temperature for 9 days, suggesting more robust stability in the biological matrix. Both sample types were stable long term at -80°C.

In conclusion, the quantitation of dNTPs is challenging. The described quantitative method addressed many of these challenges and was used to quantify deoxycytidine triphosphate, deoxyguanosine triphosphate, thymidine triphosphate, and deoxyadenosine triphosphate concentrations in cellular lysates from clinical research individuals. This method will be highly useful for in vivo studies of the endogenous dNTP
pool disposition, such as in subjects who receive antimetabolites or nucleos(t)ide analogues.
CHAPTER IV

ANALYSIS OF THE ENDOGENOUS DEOXYNUCLEOSIDE TRIPHOSPHATE POOL IN HIV POSITIVE AND NEGATIVE INDIVIDUALS RECEIVING TENOFOVIR-EMTRICITABINE

4.1 Introduction

The coformulated medication consisting of 300 mg tenofovir (TFV) disoproxil fumarate (TDF) and 200 mg emtricitabine (FTC) is marketed as an antiviral combination pill for treatment and pre-exposure prophylaxis (PrEP) of HIV-infection [158]. TFV is a nucleotide analog, and its diphosphate anabolite (TFV-DP) has a structure similar to 2’-deoxyadenosine-5’-triphosphate (dATP); FTC is a nucleoside analog, and its trisphosphate (FTC-TP) has a structure similar to 2’-deoxycytidine-5’-triphosphate (dCTP). TFV-DP and FTC-TP compete with dATP and dCTP (natural substrates) at the active site of HIV reverse transcriptase (RT), effectively inhibiting the biosynthesis of HIV genetic material. Once incorporated, they terminate the elongation of the HIV DNA chain, due to the lack of a 3’hydroxyl group to incorporate the next component [214]. The ratio between drug concentration and the corresponding dNTP affects the pharmacologic efficacy of TDF/FTC, as a high ratio has been associated with a greater antiviral activity [215, 216].

It is well known that nucleos(t)ide analogs (NA) can affect the endogenous dNTP pool, including dATP and dCTP, as well as 2’-deoxyguanosine-5’-triphosphate (dGTP), and thymidine-5’-triphosphate (TTP). NAs may compete with the host enzyme system

for phosphorylation, as well as influence the complex dNTP pool metabolism pathways. For example, *in vitro*, TFV or its anabolites competitively inhibit creatine kinase (CK), phosphoglycerate kinase (PGK), and purine nucleotide phosphorylase (PNP) [67, 69], and upregulate 5’ecto-nucleotidases and 5’nucleotidase (NT) [68]. Although FTC has limited information available, a similar deoxy-pyrimidine nucleoside analog: lamivudine (3TC), inhibits deoxycytidine kinase (dCK) [142, 217]. In addition, zidovudine (AZT), a thymidine analog, has been shown to inhibit thymidylate kinase 2 (TK2) [70, 71]. In HIV-infected patients, dATP increased during TDF/ABC (abacavir) dual therapy compared to TDF monotherapy [60], and decreased during didanosine (ddI)-containing therapy relative to TDF-containing therapy [72]. Taken together, it is important to investigate the effects of TDF/FTC therapy on the endogenous dNTP pool in clinical settings.

The use of TDF/FTC for PrEP, as well as long-term treatment, requires a low toxicity profile for an acceptable risk-benefit balance [10]. TDF/FTC are generally safe and well tolerated [218], however, uncommon side effects have been reported, particularly in HIV-infected patients. For example, TDF is rarely associated with mitochondrial dysfunction in renal proximal tubule cells [76, 104]. Mitochondrial dysfunction can arise from and imbalance among purine and pyrimidine nucleotides [71, 219, 220]. Additionally, the TDF/ddI combination is correlated with paradoxical CD4 T cell decline [221] that may be explained by PNP inhibition and subsequent apoptosis, which is associated with excessive intracellular dATP and dGTP [220, 222]. FTC moderately reduces cell proliferation *in vitro* [223], which may be associated with imbalanced dNTP pools.

The characterization of the dNTP pool changes in patients receiving TDF/FTC would enable quantitation of the analog:dNTP ratio for pharmacologic efficacy and provides possible mechanisms of adverse effects. The goal of this pharmacodynamic study was to investigate the time profile of the dNTP pool, from baseline to TDF/FTC
pharmacological intracellular steady-state, in both HIV-positive and HIV-negative individuals.

4.2 Methods

4.2.1 Participants and Study Design

The clinical protocol was approved by the Colorado Multiple Institutional Review Board (COMIRB), and participants provided informed consent (Cell-PrEP, ClinicalTrials.gov Identifier: NCT01040091). HIV-negative adults were enrolled in an intensive clinical pharmacology study of daily co-formulated TDF/FTC treatment for 30 days, followed by 30 days of washout. HIV-positive adults initiated TDF/FTC/EFV (efavirenz) treatment for 60 days (and beyond). All participants were tested for hepatitis B virus and were excluded if they were positive. Individuals were excluded if they were pregnant (or planning pregnancy), breastfeeding, had a body weight less than 110 pounds, MDRD estimated glomerular filtration rate (eGFR) less than 60mL/min/1.73m², history of pathologic bone fractures, albuminuria creatinine ratio more than 30, or history of kidney disease. Participants’ age, weight, sex, race, and body mass index (BMI) were recorded upon enrollment in the study. Peripheral blood mononuclear cells (PBMC) samples were taken at baseline, and at 8 hours post-dose on days 1, 3, 7, 20, 30, and 60 in all participants. The HIV-negative group had two additional visits on days 35 and 45 during the washout period. The study design is illustrated in figure 4.1.

4.2.2 PBMC Processing

A previously described method was used for PBMC processing [9]. Blood was drawn into a CPT tube. After mixing, the blood tube was spun at 1800 g for 30 min at room temperature to separate plasma, PBMC and red blood cells (RBC). The buffy layer
Figure 4.1 Clinical study design. TDF: tenofovir disoproxil fumarate; FTC: emtricitabine; EFV: efavirenz; 1-60: study visits in days.
(PBMC) between the plasma and separation medium was collected into a 15 mL centrifuge tube. After RBC lysis to eliminate potential RBC contamination, the sample was washed with an equal volume of phosphate buffer saline (PBS). The cell sample was then spun and the cell pellet was resuspended in 5 mL of PBS for automated cell counting (Countess Invitrogen™, Thermo Fisher Scientific Corporation, Carlsbad, CA). Finally, the cells were spun again to pellet, and lysed in 500 µL 70:30 methanol: water. The lysate was stored at -80°C until analysis.

4.3.3 The dNTP Pool Quantitation

The analytical method utilized a strong anion exchange isolation of mono-(MP), di-(DP), and tri-phosphates (TP) from the intracellular matrix [9]. The TP fraction was then dephosphorylated to the parent moiety (deoxynucleoside, dN) yielding a molar equivalent to the original deoxynucleotide intracellular concentration. A reverse phase and weak cation exchange mixed mode purification was then used for desaltation and concentration. Endogenous dNTP levels were then quantified using a validated LC/MS/MS method with an analytical range of 50-2500 fmol/sample [224].

4.3.4 Statistical Analysis

Data were natural log (ln) transformed for statistical analysis. The intracellular dNTPs (dATP, dCTP, dGTP, and TTP) were summarized in geometric means and 95% confidence intervals (95% CI). All statistical analyses were performed using SAS® (version 9.4, SAS Institute Inc., Cary, NC, USA).

To investigate the effects of HIV-infection on the dNTP pool, the baseline data were analyzed. A Welch’s t-test was used to compare the dNTP between HIV-negative vs HIV-positive group and an F-test was used to compare the difference of variances. Linear regression was performed to probe for the effects of subject variables at baseline using continuous variables such as age, weight, and BMI, while a Welch’s t-test and
analysis of variance (ANOVA) were performed on categorical variables such as sex and race.

To evaluate the effects of TDF/FTC on the dNTP pool, longitudinal measurements from baseline to TDF/FTC pharmacological steady-state in PBMC (day 0 to 30) were used in both HIV-positive and HIV-negative groups. Time profiles of the dNTP pool were described and analyzed using mixed model segmented linear regression (SAS: MIXED procedure) [225].

**Mixed model:**

\[ y = X\beta + Z\gamma + \varepsilon \] \hspace{1cm} (4.1)

- \( y \): vector of longitudinal observations of \( \ln(\text{dNTP}) \);
- \( X \): matrix of time;
- \( \beta \): vector of known fixed-effect parameters;
- \( Z \): matrix of random effects;
- \( \gamma \): normally distributed vector of unknown random-effects parameters;
- \( \varepsilon \): normally distributed vector of unknown random errors.

**Segmented linear regression model:**

\[ \hat{y} = \beta_0 + X_{\text{time}} \cdot \beta_1 + X_{\text{time, after change point}} \cdot \beta_2 \] \hspace{1cm} (4.2)

- \( \hat{y} \): vector of model estimated population mean of \( \ln(\text{dNTP}) \);
- \( X_{\text{time}} \): vector of time;
- \( X_{\text{time, after change point}} = \min \left( (X_{\text{time}} - X_{\text{time, change point}}), 0 \right) \); \( X_{\text{time, change point}} \) estimated by model goodness of fit;
- \( \beta_0 \): intercept (the baseline value);
- \( \beta_1 \): first slope;
- \( \beta_1 + \beta_2 \): second slope;
- \( \beta_2 \): change in slope after the change point.

The model estimated baseline, first slope, the change point (of two slopes), and the second slope. The change point was estimated by iteration (0-30 days, graduated by 0.05 in days), based on model goodness of fit (Akaike Information Criteria, AIC) [226]. The variance structures (e.g. compound symmetry, heterogeneous compound symmetry, Autoregressive model, unknown structure) were assessed and selected also
by the model goodness of fit. The differences between the change point vs baseline, day 30 vs baseline, and the change point vs day 30 were tested using ESTIMATE statement, and results were summarized in percentage change (%). Subject covariates were tested using a likelihood ratio test (LRT).

To evaluate the change of the dNTP pool during the washout period in the HIV-negative group, day 35, 45, and 60 data were compared to day 30, and day 60 data were also compared to baseline. A paired t-test was used to compare the differences in two selected time points. To investigate the long-term treatment effects of TDF/FTC in the HIV-positive group, baseline and day 60 data were compared.

4.3 Results

4.3.1 Study Population

A total of 40 participants were enrolled in the study. Among them, 21 were HIV-negative, and 19 were HIV-positive patients. 34 subjects completed all study visits, but six participants, two HIV-negative and four HIV-positive subjects (one female and five males) stopped early. One participant (HIV-positive) had a baseline sample but withdrew prior to any dosing visits, and the other five prior to the day 30 visit. One HIV-positive participant did not have a baseline sample but completed all visits. The study drug was generally well-tolerated. The demographic characteristics of the study population are summarized in Table 4.1.

4.3.2 Baseline Characteristics of the dNTP Pool

Data are presented in geometric means and 95% CIs in table 4.2. At baseline in PBMC (n=39), it was observed that the HIV-negative group generally had higher dNTP pool levels compared to the HIV-positive group, besides dATP. However, these differences were not statistically significant (p≥0.21). Statistically significant differences
Table 4.1 Demographic characteristics of the study population.

<table>
<thead>
<tr>
<th>characteristic</th>
<th>number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV status (%)</td>
<td>21 (53)</td>
</tr>
<tr>
<td>negative</td>
<td>21 (53)</td>
</tr>
<tr>
<td>positive</td>
<td>19 (47)</td>
</tr>
<tr>
<td>gender (%)</td>
<td>13 (33)</td>
</tr>
<tr>
<td>female</td>
<td>13 (33)</td>
</tr>
<tr>
<td>male</td>
<td>27 (67)</td>
</tr>
<tr>
<td>race (%)</td>
<td>19 (47)</td>
</tr>
<tr>
<td>white</td>
<td>19 (47)</td>
</tr>
<tr>
<td>black</td>
<td>16 (40)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>5 (13)</td>
</tr>
<tr>
<td>median eGFR (range) mL/min/1.73m²</td>
<td>93.3 (66.0-130.8)</td>
</tr>
<tr>
<td>median age (range) years</td>
<td>31 (20-52)</td>
</tr>
<tr>
<td>median weight (range) kg</td>
<td>81.1 (56.5-127)</td>
</tr>
<tr>
<td>median BMI (range) kg/m²</td>
<td>26.6 (19.9-37.7)</td>
</tr>
<tr>
<td>participants (withdrawals)</td>
<td>40 (6)</td>
</tr>
</tbody>
</table>

eGFR: estimated glomerular filtration rate. BMI: body mass index.
Table 4.2 Baseline characteristics of the dNTP pool, in femtomole per million cells.

<table>
<thead>
<tr>
<th></th>
<th>geometric mean (95% CI) in fmol/10^6 cells</th>
<th>variance of log transformed data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV-negative</td>
<td>HIV-positive</td>
</tr>
<tr>
<td>dCTP</td>
<td>858 (710, 1037)</td>
<td>804 (724, 893)</td>
</tr>
<tr>
<td>TTP</td>
<td>436 (322, 589)</td>
<td>380 (312, 462)</td>
</tr>
<tr>
<td>dATP</td>
<td>150 (120, 187)</td>
<td>154 (141, 167)</td>
</tr>
<tr>
<td>dGTP</td>
<td>287 (234, 352)</td>
<td>251 (230, 274)</td>
</tr>
</tbody>
</table>

*F-test to compare variances.

95% CI: 95% confidence interval. dCTP: 2’-deoxycytidine-5’-triphosphate. TTP: Thymidine-5’-triphosphate. dATP: 2’-deoxyadenosine-5’-triphosphate. dGTP: 2’-deoxyguanosine-5’-triphosphate.
in variances were observed in all four dNTPs (p≤0.04) with higher variability in the HIV-negative group. No subject covariates (including age, weight, BMI, gender, and race) had statistically significant effect associated with baseline dNTPs (p≥0.17).

4.3.3 Changes in the dNTP Pool on TDF/FTC Therapy

Figure 4.2 shows data from each individual, the model predicted population means and the 95% confidence intervals. Model parameter statistics are summarized in table 4.3. For all four models, a heterogeneous compound symmetry (CSH) covariate structure best described the data. Throughout the study, no effects of subject covariates on intercepts or slopes (as interaction terms) were observed (p≥0.24).

Change in dCTP Level: The change point of slopes was day 1.55 with a decrease of 20% (95% CI -28%, -11%; p<0.0001) from baseline. Compared with baseline, dCTP was decreased by 15% (95% CI -23%, -6.4%; p=0.001) at day 30. After day 1.55, concentrations increased slightly between the change point and day 30, but was not statistically significant (5.8%, 95%CI -2.4%, 15%; p=0.17).

Change in TTP Level: The change point was reached at day 1.4 with a decrease of 37% (95% CI -46%, -26%; p<0.0001) from baseline. This 37% decrease remained at day 30 (95% CI -47%, -27%; p=0.001). The change between day 1.4 and day 30 was not statistically significant (-1.2%, 95%CI -15%, 14%; p=0.86).

Change in dATP Level: The change point of slopes was reached at 2.9 days with a decrease of 14% (95% CI -21%, -4.8%; p=0.003) from baseline. After that, although concentrations increased slightly, no statistically significant change between day 2.9 and day 30 was detected (6.5%, 95%CI -2.3%, 16%; p=0.15). A decreasing trend was observed when day 30 was compared to baseline, with a reduction of 8.3% (95%CI: -17%, 0.80%; p=0.07).
Figure 4.2 The dNTP changes during TDF/FTC therapy. 95% CI: model 95% confidence interval. dCTP: β'-deoxycytidine-5'-triphosphate. TTP: Thymidine-5'-triphosphate. dATP: 2'-deoxyadenosine-5'-triphosphate. dGTP: 2'-deoxyguanosine-5'-triphosphate.
### Table 4.3 Summarization of model parameter statistics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate Baseline</th>
<th>First Slope</th>
<th>Second Slope</th>
<th>Change in Slope</th>
<th>Change Point*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln(dCTP)</td>
<td>6.73</td>
<td>-0.14</td>
<td>0.0020</td>
<td>0.14</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>-0.21, -0.077</td>
<td>-0.00086, 0.0048</td>
<td>0.077, 0.21</td>
<td>na</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.17</td>
<td>&lt;0.0001</td>
<td>na</td>
</tr>
<tr>
<td>ln(TTP)</td>
<td>6.03</td>
<td>-0.33</td>
<td>-0.00044</td>
<td>0.33</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>-0.44, -0.22</td>
<td>-0.0055, 0.0046</td>
<td>0.21, 0.44</td>
<td>na</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.86</td>
<td>&lt;0.0001</td>
<td>na</td>
</tr>
<tr>
<td>ln(dATP)</td>
<td>5.08</td>
<td>-0.052</td>
<td>0.0023</td>
<td>0.054</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>-0.085, -0.018</td>
<td>-0.00085, 0.0055</td>
<td>0.019, 0.089</td>
<td>na</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.0001</td>
<td>0.0026</td>
<td>0.15</td>
<td>0.0027</td>
<td>na</td>
</tr>
<tr>
<td>ln(dGTP)</td>
<td>5.61</td>
<td>-0.15</td>
<td>0.0031</td>
<td>0.16</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>-0.24, -0.070</td>
<td>0.00018, 0.0061</td>
<td>0.072, 0.24</td>
<td>na</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
<td>0.038</td>
<td>0.0003</td>
<td>na</td>
</tr>
</tbody>
</table>

*estimated by iteration.

95% CI: 95% confidence interval of model estimates. Baseline in ln(fmol/10^6 cells). First slope, second slope, and change in slope in ln(fmol/10^6 cells)/day. Change point in days. na: not applicable.
Change in dGTP Level: The change point of slopes was reached at day 1.35 of therapy with a decrease of 19% (95% CI -27%, -8.9%; p=0.0004) from baseline. Compared with baseline, there was a decrease of 11% (95% CI -20%, -0.67%; p=0.03) at day 30. A statistically significant increase was detected from the change point (day 1.35) to day 30, amounting to 9.6% (95% CI 0.61%, 19%; p=0.04).

4.3.4 Change in the dNTP Pool in the Washout Period

The HIV-negative group was assigned to a 30-day washout period after 30 days of treatment. Compared to day 30 data, which represented the pharmacological steady-state of TDF/FTC in PBMC, deoxy-pyrimidine triphosphates increased throughout the washout period. A statistically significant increase of 20% (95%CI 3.8%, 40%; p=0.02) in dCTP was observed 15 days after the initiation of the washout period. TTP increased by 83% (95%CI 42%, 136%; p=0.0001) at day 5, 72% (95%CI 34%, 121%; p=0.0004) at day 15, and 82% (95%CI 33%, 149%; p=0.002) at day 30 of the washout period.

However, for deoxy-purine triphosphates, statistically significant changes in dATP and dGTP were not observed (p≥0.38). Day 60 data compared to baseline was not statistically significant in dCTP (geometric means: 934 vs 1038 fmol/10^6 cells; n=12; p=0.48) and TTP (geometric means: 496 vs 641 fmol/10^6 cells; n=11; p=0.14). However, statistically significant differences were observed for dATP and dGTP, with geometric means of 130 vs 181 fmol/10^6 cells (n=10; p=0.04), and 243 vs 343 fmol/10^6 cells (n=10; p=0.04). These results suggest that dCTP and TTP returned to baseline levels around 5-15 days after treatment discontinuation, while dATP and dGTP remained below baseline until at least day 30 of the washout period.

4.3.5 Change in the dNTP Pool after two-month treatment

In the HIV-positive group, the day 60 results were compared to baseline. No statistically significant difference was observed in all four components (n=15; p≥0.28).
The geometric means (95% CI) in fmol per million cells were 747 (631, 886) for dCTP, 354 (266, 470) for TTP, 159 (131, 193) for dATP, and 270 (226, 323) for dGTP, which were comparable to baseline levels (see table 4.2). This suggested that the dNTP pool recovered after treatment in HIV-infected patients.

4.4 Discussion

In this study, we used PBMC to investigate the effects of TDF/FTC on the dNTP pool of HIV-positive and negative adults. PBMC include cells that are the major target of TDF/FTC (e.g. CD4 T-cells). Also, these nucleated cells represent dNTP pool kinetics driven by enzymes such as PNP, which have high activity in PBMC [67].

At baseline, we observed generally lower dNTP (besides dATP), but not statistically significantly difference, in treatment naïve HIV-infected compared with HIV-negative participants. However, the variance of the dNTP pool was higher in the HIV-negative group compared with the HIV-positive group. Although a higher T-cell activation and cell proliferation might initially lead to dNTP pool upregulation [227, 228], the pathological chronic cell activation during HIV-infection [229] may induce anergy in T-cells [230]. It is also reported that compensatory dNTP consuming mechanisms associated with NTPDase-1 and SAMHD-1 are upregulated especially in uncontrolled HIV-1 infection [231, 232]. These biological effects may have reduced the variability in dNTP in HIV infected participants. However, the small sample size (n=39) of our study limited the ability to probe possible effects of HIV-infection and subject covariates on baseline dNTP pools. Future studies require a larger sample size to further address these questions.

In HIV-infected patients, Hawkins et al reported a lower dATP level with didanosine (ddl) including treatment compared with TDF including treatment (medians: 67.2 vs 186 fmol/10⁶ cells; p=0.001), but could not exclude the possibility that TDF is
also related to dATP reduction [72]. Goicoechea et al reported an increasing trend in dATP and dGTP with ABC/TDF dual therapy, however, in their 7-day monotherapy period, they observed a decreasing trend in dGTP (medians: 4026 vs 2464 fmol/10^6 cells; p=0.68) [60]. The Goicoechea study reported higher dATP and dGTP levels compared with the Hawkins et al and the present study. In a macaque model, Durand-Gasselin et al. reported no change in dATP and dGTP level from 2-24 hours post oral TDF and subcutaneous TFV treatment, but this study was limited by single dose administration, shorter observation period (≤24 hours), and the lack of a baseline observation [233]. Taken together, the present study represents a novel assessment of dNTP changes in PBMC during TDF/FTC therapy.

We observed that TDF/FTC therapy was associated with slight to moderate decreases in dNTP levels in both HIV-negative and HIV-positive individuals. A mixed model segmented linear regression analysis was a useful approach to analyzing the dynamic changes in the dNTP pool during TDF/FTC treatment [234, 235]. In comparison to nonlinear regression models, the statistical analysis of the second slope from segmented linear regression model enabled us to investigate the possible change point and return of dNTP during therapy (a statistically significant return was detected in dGTP). This work indicates that the dNTP decrease within a few days (<3 days) of TDF/FTC initiation. However, the change point was estimated based on the model goodness of fit, and these temporal approximations were limited by sampling times. The detected effects of TDF/FTC on the dNTP pool were (largest to smallest) TTP > dCTP > dGTP > dATP, indicating that TDF/FTC had larger effects on deoxy-pyrimidine triphosphate compounds (dCTP and TTP) than deoxy-purines (dGTP and dATP). If these were driven by FTC-TP, it could due to the higher intracellular FTC triphosphate concentrations compared to TFV diphosphate [64]. We anticipate that lamivudine may induce similar effects given the pharmacological similarity with emtricitabine [142, 217].
Studies are needed to assess on the effects of other NA such as abacavir and zidovudine.

In terms of potential mechanisms, the reduction of dNTP pool could be explained by TFV-associated upregulation of dephosphorylation enzyme 5’-ectonucleotidase and 5’-nucleotidase [68]. TFV also suppresses the ATP generating enzymes CK and PGK [69], indirectly inhibits kinases that use ATP as the phosphate donor, such as nucleotide diphosphate kinase (NDPK) [236]. Only in dGTP did we observe a statistically significant but minor return toward baseline values by day 30 after reaching the change point. Partial PNP inhibition by TFV phosphates might play a role in dGTP upregulation [66], perhaps counteracting some of the reduction effects. However, the decreased dATP and dGTP levels observed in this study does not support PNP inhibition as a dominant effect of TDF, which would theoretically induce an increase of dATP and dGTP. Although there are limited data for FTC, our findings may be explained by the inhibition of dCK which had been shown for another deoxycytidine analog: 3TC [10].

During the washout period in the HIV-negative group, dCTP and TTP returned to baseline levels, while dGTP and dATP stayed slightly below the baseline. TFV-DP exhibits a much longer intracellular half-life compared to FTC-TP, and previous research in this cohort observed quantifiable intracellular TFV-DP concentrations at day 30 of the washout phase, whereas FTC-TP was below the limit of quantitation [237]. This is consistent with the suppression of dCTP and TTP being related to FTC-TP, while dATP and dGTP to TFV-DP. Future studies should investigate the individual effects TDF and FTC on the dNTP pool in the HIV-negative participants.

For long-term treatment in HIV-positive group, day 60 data showed less reduction in the dNTP pool level compared to the plateau or the change point, which indicates that in our participants, the endogenous dNTP pool slowly returned to baseline with long-term treatment. We still need more information to understand other factors that
may affect the dNTP pool in PBMC after long-term treatment in HIV-infected patients, such as disease progression reversal, immune system restoration, and immune cell activation changes [10, 238].

In conclusion, during TDF/FTC therapy, the dNTP pool slightly to moderately decreased within 3 days after the initiation of TDF/FTC therapy, and generally remained below baseline at day 30. Potential clinical implications need to be evaluated in the future including assessment of the analog:dNTP ratio over time, which influences the pharmacological efficacy of TDF/FTC [60, 215, 216].
CHAPTER V

MODEL LINKING PLASMA AND INTRACELLULAR TENOFOVIR/EMTRICITABINE WITH DEOXYNUCLEOSIDE TRIPHOSPHATES

5.1 Introduction

Tenofovir (TFV) disoproxil fumarate (TDF) and emtricitabine (FTC) are co-formulated as Truvada®, which is approved for human immunodeficiency virus 1 (HIV-1) infection treatment as part of combination antiretroviral therapy, as well as pre-exposure prophylaxis [239]. TDF is a prodrug, which undergoes ester hydrolysis on first pass by the gut and the liver and circulates in plasma as TFV predominantly [48, 240]. TFV and FTC are nucleos(t)ide analogs (NA) of deoxyadenosine monophosphate (dAMP) and deoxycytidine (dC). Each undergoes cellular uptake and anabolism to their active intracellular forms: TFV-diphosphate (TFV-DP) and FTC-triphosphate (FTC-TP) [69]. TFV-DP and FTC-TP compete with corresponding endogenous deoxynucleoside triphosphates (dNTP) at the active site of HIV reverse transcriptase (RT), thus inhibiting genetic material biosynthesis. If incorporated into the proviral DNA, TFV-DP and FTC-TP terminate chain elongation [240].

The accumulation of intracellular (IC) TFV-DP/FTC-TP is presumably driven by plasma TFV/FTC concentrations. However, the formation of intracellular TFV-DP/FTC-TP is complicated, as it requires a hybrid of endocytosis, active transport, diffusion, and enzymatic reactions. [48, 241]. These complexities contribute to nonlinearities in the pharmacokinetics (PK) relationship between plasma and intracellular TFV-DP

*This work has been published in the journal of PLoS One.
Currently, many studies have characterized the PK of plasma TFV/FTC [49, 78, 85, 243-247] and intracellular TFV-DP/FTC-TP [57, 60, 64, 72, 237, 248, 249], individually. However, only a few PK link models have investigated the relationship between the plasma TFV and the intracellular TFV-DP, all of which used steady-state observations only. As a result, these models were restricted by the lack of accumulation phase data [79, 250, 251].

In target cells such as CD4 T-cells, TFV-DP and FTC-TP compete with corresponding natural substrates of HIV RT, which are deoxyadenosine triphosphate (dATP) and deoxycytidine triphosphate (dCTP), respectively. The analog:dNTP molar ratios are associated with antiviral efficacies [60, 250]. However, as NAs, TFV and FTC also have the potential to disturb the dNTP pool, which consists of dATP, dCTP, deoxyguanosine triphosphate (dGTP), and thymidine triphosphate (TTP), given the interactions with the same enzymes in deoxypurine/deoxypyrimidine anabolic and metabolic pathways [66-68, 222].

We previously characterized the dNTP pool reductions during TDF/FTC therapy, in the same participants included in this communication [252, 253], and used non-compartmental analysis (NCA) to describe TFV-DP/FTC-TP concentration-time profiles [237, 253]. However, there is a need to understand the pharmacokinetic-pharmacodynamic (PKPD) relationships and to model non-linearities between plasma and intracellular TFV/FTC along with dNTP changes [50, 60, 72].

Creating such models will enable PK-PD simulations of alternative dosing strategies and interpretations for various clinical scenarios. For example, nonlinear PK introduces difficulties in calculating clinical relevant half-lives via traditional methods. Sahin et al proposed the operational multiple dosing half-life, which is based on simulations of different dosing intervals (tau) [254]. In addition, for PK-PD simulations
can be applied to alternative TDF/FTC dosing strategies such as on-demand preexposure prophylaxis (PrEP) studied by Molina et al, who reported an 86% protective rate against HIV-infection using IPERGAY dosing strategy (oral administration of two pre-coitus doses followed by two post-coitus doses) [162]. With dNTP data, PK-PD simulation can use target in vitro EC$_{50}$ and EC$_{90}$ for analog:dNTP ratio such as those proposed by Cottrell [50].

The main objective of this study was to develop a PK-PD model that characterized the nonlinearities of TFV-DP and FTC-TP using integrated compartmental PK models linking plasma TFV/FTC with intracellular TFV-DP/FTC-TP concentrations, including changes in dNTPs. The model was then used to estimate operational multiple dosing half-lives ($t_{1/2,op}$) [254] and to simulate the analog:dNTP time profiles for the preexposure prophylaxis (PrEP) IPERGAY dosing [162].

### 5.2 Materials and Methods

#### 5.2.1 Study Design

This study was a prospective, observational, intensive, PKPD study in HIV-positive and negative adults. The study was approved by the Colorado Multiple Institutional Review Board (COMIRB), and participants provided written informed consent before participation (Cell-PrEP; ClinicalTrials.gov Identifier: NCT01040091; study duration: 03/2010–08/2013). HIV-negative subjects received daily oral co-formulated TDF 300 mg (TFV 136 mg) and FTC 200 mg for 30 days and HIV-positive subjects received daily co-formulated TDF 300 mg/FTC 200 mg plus efavirenz (EFV) 600 mg for 60 days. HIV-negative subjects had washout visits on days 35, 45, and 60 and HIV-positive subjects had one follow-up visit on day 60 (therapy was continued in
this group). All participants had their baseline sample taken before the initiation of treatment for dNTPs. Subjects were asked to fast overnight, beginning at 10 pm before their dosing visits. On days 1 (first-dose) and 30, blood was collected at 1, 2, 4, 8, and 24 hours post-dose, and on days 3, 7, and 20, blood was collected pre-dose, 2 and 8 hours post-dose. Single blood samples were collected on days 35, 45, and 60 [237, 252, 253]. The sample size was chosen to detect a ~60% increase (63%) in TFV-DP in HIV-negative versus HIV-infected persons using a significance level of 0.05, a power of 80%, a coefficient of variation for TFV-DP of 50%, and a two-sample t-test. This outcome was assessed in another communication [253]. The study design is illustrated in figure 4.1.

5.2.2 Bioanalysis

Samples included plasma and peripheral blood mononuclear cells (PBMC). Blood was collected in EDTA tubes for plasma separation. CPT vacutainers were used to isolate PBMC, which were counted with an automated countess® cytometer (Invitrogen™, Thermo Fisher Scientific Corporation, Carlsbad, CA) and lysed [9, 237, 253]. TFV and FTC in blood plasma were assayed using a validated LC/MS/MS methodology with a lower limit of quantification (LLOQ) of 10 ng/mL [255]. TFV-DP and FTC-TP concentrations in cellular lysates were assayed with a validated LC/MS/MS method with an LLOQ of 2.5 fmol/sample for TFV-DP and 0.1 pmol/sample for FTC-TP [9]. The components of the dNTP pool (dATP, dCTP, dGTP, and TTP) were analyzed using a validated LC/MS/MS methodology, with an LLOQ of 50 fmol/sample (this method is described in chapter 3) [256, 257]. For PBMC samples, values were converted to femtomole per million cells (fmol/10⁶ cells, based on the number of cells per sample). The dNTP pool components were quantified in PBMC samples from baseline, 1, 2, 4, 8, 24 hours post-dose on day 1, and 8 hours post-dose in all other visits. TFV/FTC in plasma and TFV-DP/FTC-TP in PBMC were assayed at all time points [237, 252, 253].
5.2.3 Model Development

Concentration-time data were analyzed using the first-order conditional estimation with interaction (FOCEI) method of the nonlinear mixed effects modeling, NONMEM® (version 7.3, ICON plc., Ellicott City, MD, USA). Below limit of quantitation (BLQ) samples were treated as missing [258, 259]. Most BLQ samples for TFV-DP/FTC-TP were in the washout phase; for TFV-DP, 11 at day 60 and one at day 45; for FTC-TP, 19 at day 60, four at day 45; and one at day 35. On occasion, TFV-DP was BLQ within the first dose (five occasions), but other samples in the first dose were quantifiable for the subject (five samples were collected per subject). Plasma was always BLQ in the washout phase (all time points except five FTC at day 35). To develop link models, simultaneous (parameters from two models estimated at the same time) and sequential link (parameters from one model were estimated in advance and fixed) methods were both investigated [260, 261]. The plasma TFV model was linked to the intracellular TFV-DP model, then TFV-DP model was linked to endogenous dATP and dGTP (deoxypurines) models, given TFV is a deoxypurine analog and could affect deoxypurine turnover. The FTC models were linked in a similar fashion except with endogenous dCTP and TTP (deoxypyrimidines), as FTC is a deoxypyrimidine analog and could affect deoxypyrimidine turnover.

Classical one-, two-, and three-compartmental models were investigated to develop plasma TFV/FTC models. The plasma models were then linked to the intracellular TFV-DP/FTC-TP model. Because units from the two models carried different meanings (ng/mL vs. fmol/10^6 cells), mass movement was avoided. Instead, plasma TFV/FTC concentrations (regarded as “exposure”) were used to stimulate TFV-DP/FTC-TP formation (regarded as “response”). Models tested included the first-order formation model described by Burns et al [251], the saturable formation model described by Duwal
et al [242], the indirect response model described by Baheti et al [65], and the saturation (tolerance) model described by Porchet et al [262]. One-, two-, and three-compartmental models were investigated to describe the elimination phase of TFV-DP/FTC-TP.

TFV-DP/FTC-TP concentrations were then linked to the endogenous dNTP pool change (response). The concentrations of TFV-DP/FTC-TP were used to inhibit the zero-order rate constant of the corresponding dNTP production ($k_{in}^0$). Direct link and indirect response link, as well as $E_{\text{max}}$ models and sigmoidal $E_{\text{max}}$ models (hill coefficient), were investigated [263, 264]. The baseline response (dNTP at time zero, $R_0$) is the determinant of indirect responses and a source of variation in pharmacodynamics analysis [265]. Thus, we assessed the three different estimation methods as described by Dansirikul et al [266] for the endogenous dNTP pool $R_0$ that arose from a single measurement. These were method 1: estimation using deviation ($\omega^2$) from the population mean baseline values, method 2: estimation using deviation ($\sigma^2$) from the individual observed baseline values, and method 3: estimation using both weighted population mean and individual observed baseline values.

Several random effect models were also evaluated to describe interindividual variability (IIV) and residual variability (RV), such as additive, proportional, and exponential models. To assess the model goodness of fit, the objective function value (OFV) was used, with a lower value indicating a better fit.

Acceptable models were selected based on the following criteria: successful minimization; successful covariate step; absolute value of gradients less than 1 in the last iteration; the %RSE (relative standard error) less than 30% for fixed effects in base model, while %RSE less than 50% for random effects; 95% confidence intervals for fixed and random effects not including 0; model conditional number (generated in covariate
step) not exceeding $10^p$ ($p$: number of parameters); no significant correlation among parameters ($r<0.8$); and eta bars not statistically significant from zero ($p>0.05$).

5.2.4 Covariate Selection

The effects of covariates on the model parameters were evaluated. A linear model was used for categorical variables such as HIV-infection status, sex, and race. For continuous variables such as estimated glomerular filtration rate (eGFR), age, weight, and body mass index (BMI), subject variables were centered at medians. Then, linear, natural log, and power models were evaluated [267]. The covariates were assessed by likelihood ratio test (LRT), with a forward selection at $\alpha=0.01$ (OFV decreased by 6.64), then a backward elimination at $\alpha=0.001$ (OFV increased by 10.8).

5.2.5 Model Evaluation

The goodness of fit diagnostics was generated and visually assessed using Xpose [268] (version 4.5.3, http://xpose.sourceforge.net/) and R (http://www.r-project.org/), which includes plots of observations vs. population predictions, observations vs. individual predictions, absolute values of individual weighted residuals vs. individual predictions, and weighted residuals vs. time (basic diagnostic plots). No substantial pattern was allowed in these diagnostic plots. In addition, an overlay plot of observed values and predicted values vs. time was also used to assess the model performance.

Visual predictive checks (VPC) were performed using NONMEM–PDxPOP (version 5.1, ICON plc., Ellicott City, MD, USA) and R. VPCs were conducted using 100× study population ($n=4000$) as the sample size. VPCs of the first-dose (day 1), as well as day 1-60 in the HIV-negative group (to capture washout) for intracellular models (TFV-
DP, FTC-TP, dATP, dCTP, dGTP, and TTP), were generated. An overlay plot of observed values as well as 90% percentiles, 80% percentiles, and medians were generated and evaluated. Observations outside 90% confidence intervals were also calculated, and a proportion of less than 15% was considered acceptable.

Model evaluation by bootstrapping (n=1000) was carried out with PDxPOP and R. The 95% percentiles (2.5% and 97.5%) and the bootstrapping success rates were generated and assessed. A success rate that exceeded 90% was required for an acceptable model.

5.2.6 Simulations and Model Applications

To demonstrate the application of the model, simulations were carried out for the calculation of operational multiple dosing half-lives \( t_{1/2,op} \) and the characterization of the change of analog:dNTP molar ratios over time for on-demand PrEP purpose using the IPERGAY doing strategy [162, 254].

Given the nonlinearities of TFV-DP/FTC-TP disposition (see results), simulations were performed to calculate the intracellular PK (TFV-DP/FTC-TP) operational multiple dosing half-lives \( t_{1/2,op} \), proposed by Sahin et al [254]. Simulations of population mean concentrations were performed. For TFV-DP, simulations of different dosing intervals \( \tau \) from 1 day to 9 days (graduated by 1 day) were performed. Dosing intervals of 0.2, 0.5, 1, 1.5, 2, and 2.5 days were performed for FTC-TP. A total of 50 doses were simulated to ensure the acquisition of steady-state. Maximum concentrations from the first-dose \( C_{max,fd} \), maximum concentrations at steady-state \( C_{max,ss} \), and minimum concentrations at steady-state \( C_{min,ss} \) were then determined for the different \( \tau \) values. The operational half-lives were defined as the \( \tau \) where the \( C_{max,ss}:C_{max,fd}=2 \) (suggesting the dosing interval achieved doubled concentrations at steady-state.
compared with first-dose), as well as the $C_{\text{max,ss}}:C_{\text{min,ss}}=2$ (suggesting the dosing interval led to 50% elimination at trough compared with peak concentrations at steady-state).

For the IPERGAY trial design for on demand PrEP dosing strategy, Monte-Carlo simulations ($n=1000$) were performed. The IPERGAY dosing consisted of two doses (600/400 mg TDF/FTC), 24 hours to 2 hours before coitus, followed by one dose (300/200 mg TDF/FTC) at 24 hours after coitus and another dose at 48 hours post-coitus. Statistical analysis of simulation results was carried out using SAS® (version 9.4, SAS Institute Inc., Cary, NC, USA). Simulations of dosing relative to a coitus event in a virtual HIV-negative population were set for a time course of one-week (7 days), and data generated in every 0.1 days post-dose, as well as at $10^{-4}$, $10^{-3}$ and $10^{-2}$ days after the first-dose (to capture the initial accumulation). Medians and percentiles (5th percentile (P_5) to 95th percentile (P_95), graduated by 5) of molar ratios of TFV-DP:dATP and FTC-TP:dCTP were calculated, then compared with the CD4 T-cell viral suppression $EC_{50}$ and $EC_{90}$ (molar ratio) in vitro, reported by Cottrell et al [50].

### 5.3 Results

#### 5.3.1 Study Demographics

21 HIV-negative adults and nineteen HIV-positive patients ($n=40$) were enrolled in the study. Thirteen participants were female, and 27 were male. Nineteen were Caucasians, 16 were African Americans, and five were Hispanic. The median (range) age was 31 (20-52) years. The median eGFR (range) was 93.3 (66.0-131) mL/min/1.73m². The medians (ranges) weight and BMI were 81.1 (56.5-127) kg and 26.6 (19.9-37.7) kg/m², respectively. The clinical trial flow chart is shown in Figure 5.1. TDF
and FTC were well tolerated throughout the study (see Figure 5.1). 34 (among 40) subjects completed all study visits, two HIV negative and four HIV-positive subjects stopped the study early [237, 252], but all available longitudinal data prior to dropout were included in the model development. Study demographics is summarized in table 4.1.

### 5.3.2 Population Pharmacokinetics Modeling of Plasma TFV/FTC

The best fit was obtained using a classical first-order absorption two-compartmental model (subroutines ADVAN4 TRANS4) for both plasma TFV and FTC. Model structures are illustrated in Figure 5.2 left portion (compartment 1-3). Differential equations of the plasma model are listed in equations 5.1-5.3. In these models, bioavailability ($F$) adjusted central compartment volume of distribution ($V_c/F$), clearance ($CL/F$), intercompartmental clearance ($Q/F$), and peripheral compartment volume of distribution ($V_p/F$) were estimated. The absorption rate constant ($K_a$) was estimated, but a lag time ($t_{lag}$) was not.

---

gut: \[
\frac{dA(1)}{dt} = -K_a \times A(1) \tag{5.1}
\]

central: \[
\frac{dA(2)}{dt} = K_a \times A(1) - \frac{CL}{V_c} \times A(2) - \frac{Q}{V_c} \times A(2) + \frac{Q}{V_p} \times A(3) \tag{5.2}
\]

peripheral: \[
\frac{dA(3)}{dt} = \frac{Q}{V_c} \times A(2) - \frac{Q}{V_p} \times A(3) \tag{5.3}
\]
Figure 5.1 Clinical trial flow chart. LFTs: liver function tests.
Exponential random effect model on interindividual variability (IIV) and residual variability (RV) resulted in lowest OFV. The IIV for the $V_c/F$, $CL/F$, and $Q/F$ were estimated in the TFV plasma model. In the FTC plasma model, the IIV in $V_c/F$, $CL/F$, and the $V_p/F$ were estimated. The model estimated terminal half-lives (beta half-life, $t_{1/2,z}$) of TFV and FTC were 17.3 hours (95%CI: 15.7, 19.1) and 26.8 hours (95%CI: 25.6, 28.1).

In the FTC plasma model, sex was statistically significant ($p=0.009$; ΔOFV=-6.76; ΔIIV$V_c/F$=-2.8%), as males had 24.4% (95% CI: 7.25%, 41.6%) higher average (typical value, $tv$) central compartment volume of distribution ($tvV_c/F=99.4+24.3\times sex$; male=1, female=0). No subject variable was statistically significant in the TFV plasma model.

The goodness of fit plots demonstrated that the model provided a good description of the data (Figures 5.3-5.5). The VPCs are shown in Figures 5.6 and 5.7 upper left panels. Model parameters, as well as bootstrapping outcomes, are listed in the top portions of tables 5.1 and 5.2.

Other model evaluation results are shown in table 5.3.

5.3.3 Population Pharmacokinetics Modeling of TFV-DP/FTC-TP

Individual PK parameters from the plasma models were fixed for the intracellular TFV-DP/FTC-TP model development (sequential link). Model structures are illustrated in Figure 5.2 upper right portion (compartment 4-6). Differential equations for intracellular TFV-DP/FTC-TP models are listed in equations 5.4-5.6.
Figure 5.2 Model schematic. Red box represents plasma. Blue box represents cells. Solid outlines suggest observations available in these compartments. Dashed outlines indicate virtual compartments. Dashed arrows indicate where mass movements were avoided during model development. PBMC: peripheral blood mononuclear cells. IC: intracellular TFV: tenofovir. FTC: emtricitabine. TFV-DP: tenofovir diphosphate. FTC-TP: emtricitabine triphosphate. dATP: deoxyadenosine triphosphate. dGTP: deoxyguanosine triphosphate. dCTP: deoxycytidine triphosphate. dTTP: thymidine triphosphate. $F$: bioavailability. $K_a$: absorption rate constant. $Q$: intercompartmental clearance. $V_c$: central compartment volume of distribution. $V_p$: peripheral compartment volume of distribution. $K_f$: first-order formation rate constant of intracellular TFV-DP/FTC-TP stimulated by plasma TFV/FTC. $SC_{50}$: concentration in a virtual saturation compartment at which formation rate was inhibited by 50%. $R$: recycling portion constant in %. $K_{el}$: elimination rate constant of intracellular TFV-DP/FTC-TP. $K_{in}$: zero-order production rate constant of intracellular dNTPs. $K_{out}$: elimination rate constant for intracellular dNTPs. $EC_{50}$: concentration of intracellular TFV-DP/FTC-TP at which $K_{in}$ is inhibited by 50%.
Figure 5.3 Dependent variables (observed values) and predicted values vs time plots. IC: intracellular. PRED: predicted values. DV: dependent variables.
Figure 5.4 Basic goodness of fit plots of TFV plasma model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. $|\text{WRES}|$: absolute values of individual weighted residuals.
Figure 5.5 Basic goodness of fit plots of FTC plasma model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. $|\text{WRES}|$: absolute values of individual weighted residuals.
Figure 5.6 TFV-deoxypurine models visual predictive check. Circles indicate observations. Solid lines indicate model 90% prediction intervals. Dashed lines indicate model estimated medians and 80% prediction intervals. The results of the first-dose inserted with the results of the 60 days in the HIV-negative group (had a washout period from day 30-60) for intracellular TFV-DP, dATP and dGTP are shown. TFV: tenofovir. TFV-DP: tenofovir diphosphate. dATP: deoxyadenosine triphosphate. dGTP: deoxyguanosine triphosphate.
Figure 5.7 FTC-deoxypyrimidine models visual predictive check. Circles indicate observations. Solid lines indicate model 90% prediction intervals. Dashed lines indicate model estimated medians and 80% prediction intervals. The results of the first-dose inserted with the results of the 60 days in the HIV-negative group (had a washout period from day 30-60) for intracellular FTC-TP, dCTP and TTP are shown. FTC: emtricitabine. FTC-TP: emtricitabine triphosphate. dCTP: deoxycytidine triphosphate. TTP: thymidine triphosphate.
Table 5.1 TFV-deoxypurine model parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Population Mean</th>
<th>95% Confidence Interval</th>
<th>Interindividual Variability</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
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<td></td>
<td>Estimate</td>
<td>%RSE</td>
<td>NONMEM</td>
<td>Bootstrap</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TFV plasma</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_a$ (day$^{-1}$)</td>
<td>80.1</td>
<td>13.5</td>
<td>58.9, 101</td>
<td>56.9, 124</td>
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<td>$V_c/F$ (L)</td>
<td>390</td>
<td>10.1</td>
<td>313, 467</td>
<td>295, 465</td>
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<td>CL/F (L/day)</td>
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<td>4.94</td>
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<td>1290, 1540</td>
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<td>$V_d/F$ (L)</td>
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<td>6.29</td>
<td>769, 985</td>
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</tr>
<tr>
<td>Q/F (L/day)</td>
<td>5390</td>
<td>5.96</td>
<td>4760, 6020</td>
<td>4580, 6310</td>
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<td>$\sigma$ (exponential)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>TFV-DP IC</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_f$ (day$^{-1}$)</td>
<td>1.4</td>
<td>19.1</td>
<td>0.877, 1.92</td>
<td>0.872, 2.39</td>
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<td>SC$_{50}$,TFV</td>
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<td>11.6</td>
<td>5.06, 8.04</td>
<td>5.08, 9.94</td>
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<tr>
<td>$K_w$ (day$^{-1}$)</td>
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<td>11.8</td>
<td>0.175, 0.281</td>
<td>0.181, 0.311</td>
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<tr>
<td>R (%)</td>
<td>5.82</td>
<td>11.9</td>
<td>4.47, 7.17</td>
<td>4.63, 8.86</td>
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<td>$\sigma$ (proportional)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>dATP</strong></td>
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<td></td>
</tr>
<tr>
<td>$R_0$ (fmol/10$^6$cells)</td>
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<td>4.08</td>
<td>143, 167</td>
<td>142, 167</td>
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<tr>
<td>EC$_{50}$,TFV-DP</td>
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<td>7.66</td>
<td>361, 2890</td>
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<td>(fmol/10$^6$cells)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\sigma$ (exponential)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>dGTP</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R_0$ (fmol/10$^6$cells)</td>
<td>245</td>
<td>4.86</td>
<td>222, 268</td>
<td>241, 273</td>
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<td>EC$_{50}$,TFV-DP</td>
<td>54.6</td>
<td>18.8</td>
<td>12.4, 240</td>
<td>14.3, 268</td>
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<tr>
<td>(fmol/10$^6$cells)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>gamma</td>
<td>0.928</td>
<td>26.4</td>
<td>0.448, 1.41</td>
<td>0.46, 1.8</td>
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<tr>
<td>$\sigma$ (exponential)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.76</td>
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IC: intracellular
Table 5.2 FTC-deoxypyrmidine model parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Population Mean</th>
<th>95% Confidence Interval</th>
<th>Interindividual Variability</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
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<td>Estimate</td>
<td>NONMEM</td>
<td>Bootstrap</td>
<td>Estimate</td>
</tr>
<tr>
<td></td>
<td>%RSE</td>
<td>95% CI</td>
<td>%RSE</td>
<td>%CV</td>
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<tr>
<td><strong>FTC plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( K_a ) (day(^{-1}))</td>
<td>55.7</td>
<td>13.8</td>
<td>40.6, 70.8</td>
<td>41.9, 2.7E+9</td>
</tr>
<tr>
<td>( V_c/F ) (L)</td>
<td>99.4</td>
<td>5.91</td>
<td>87.9, 111</td>
<td>87.2, 112</td>
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<tr>
<td>CL/F (L/day)</td>
<td>482</td>
<td>3.98</td>
<td>444, 520</td>
<td>440, 514</td>
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<tr>
<td>( V_p/F ) (L)</td>
<td>166</td>
<td>13.5</td>
<td>122, 210</td>
<td>107, 239</td>
</tr>
<tr>
<td>Q/F (L/day)</td>
<td>141</td>
<td>8.94</td>
<td>116, 166</td>
<td>110, 191</td>
</tr>
<tr>
<td>Sex on ( V_c/F ) (linear)</td>
<td>24.3</td>
<td>35.9</td>
<td>7.21, 41.4</td>
<td>6.21, 41.6</td>
</tr>
<tr>
<td>( \sigma ) (exponential)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>FTC-TP IC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( K_f ) (day(^{-1}))</td>
<td>41.6</td>
<td>9.52</td>
<td>33.8, 49.4</td>
<td>35.6, 52.2</td>
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<tr>
<td>( S_{50, FTC} )</td>
<td>3320</td>
<td>12.2</td>
<td>2530, 4110</td>
<td>2590, 4120</td>
</tr>
<tr>
<td>( V_{el} ) (day(^{-1}))</td>
<td>1.6</td>
<td>4.89</td>
<td>1.45, 4.75</td>
<td>1.43, 1.74</td>
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<tr>
<td>R (%)</td>
<td>16.0</td>
<td>11.4</td>
<td>12.4, 19.6</td>
<td>13.0, 21.1</td>
</tr>
<tr>
<td>HIV on ( K_f ) (Linear)</td>
<td>31.3</td>
<td>26.9</td>
<td>14.8, 47.8</td>
<td>15.4, 50.3</td>
</tr>
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<td>( \sigma ) (proportional)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>dCTP</strong></td>
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<td></td>
</tr>
<tr>
<td>( R_0 ) (fmol/10(^6)cells)</td>
<td>771</td>
<td>3.97</td>
<td>711, 831</td>
<td>716, 825</td>
</tr>
<tr>
<td>( E_{50, FTC-TP} ) (fmol/10(^6)cells)</td>
<td>44400</td>
<td>2.86</td>
<td>24300, 80800</td>
<td>26900, 98700</td>
</tr>
<tr>
<td>( \sigma ) (exponential)</td>
<td>0.229</td>
<td>5.2</td>
<td>47.9</td>
<td>0.206, 0.252</td>
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<tr>
<td><strong>TTP</strong></td>
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<td></td>
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</tr>
<tr>
<td>( R_0 ) (fmol/10(^6)cells)</td>
<td>335</td>
<td>9.67</td>
<td>271, 399</td>
<td>271, 396</td>
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<tr>
<td>( E_{50, FTC-TP} ) (fmol/10(^6)cells)</td>
<td>18800</td>
<td>2.69</td>
<td>11200, 32900</td>
<td>11800, 32900</td>
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<td>( \sigma ) (exponential)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
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IC: intracellular
Table 5.3 Other model evaluation results.

<table>
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<tr>
<th>Model Output</th>
<th>Bootstrapping</th>
<th>VPC</th>
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<tr>
<td></td>
<td>Condition Number</td>
<td>Success Rate</td>
</tr>
<tr>
<td>TFV plasma</td>
<td>25.3</td>
<td>99.2%</td>
</tr>
<tr>
<td>FTC plasma</td>
<td>79.6</td>
<td>94.3%</td>
</tr>
<tr>
<td>TFV-DP IC</td>
<td>24.9</td>
<td>99.3%</td>
</tr>
<tr>
<td>FTC-TP IC</td>
<td>27.8</td>
<td>99.5%</td>
</tr>
<tr>
<td>dATP</td>
<td>6.0</td>
<td>95.7%</td>
</tr>
<tr>
<td>dCTP</td>
<td>5.5</td>
<td>99.7%</td>
</tr>
<tr>
<td>dGTP</td>
<td>4.4</td>
<td>99.2%</td>
</tr>
<tr>
<td>TTP</td>
<td>7.5</td>
<td>99.9%</td>
</tr>
</tbody>
</table>

VPC: visual predictive check.
A hybrid of first-order formation and saturation link model best described the data. In this model, a saturation compartment (compartment 5) was used, in which high plasma concentrations \( \frac{A(2)}{V_c} \) slowed the first order rate constants of TFV-DP/FTC-TP formation \( K_f \). The elimination rate constant in the saturation compartment was fixed to the plasma central compartment elimination rate constant \( \frac{CL}{V_c} \) to avoid over-parameterization, assuming that the saturation effect disappeared based on plasma TFV/FTC disappearance. An \( SC_{50} \) was introduced in the saturation model, which indicated the concentration in the virtual saturation compartment that leads to 50% inhibition of TFV-DP/FTC-TP formation. The stimulation constant for both the saturation compartment and the TFV-DP/FTC-TP compartment (IC central, compartment 4) was estimated with the same rate constant of formation \( K_f \) to avoid over-parameterization. A two-compartmental model best described TFV-DP and FTC-TP elimination. This was modeled with a "recycling" compartment (compartment 6). Due to the collinearity \( r>0.9 \) between \( K_{46}, K_{64} \) (rate of movement from compartment 4 to 6, 6 to 4), and \( K_{40} \) (elimination rate of compartment 4), a "recycling" ratio constant \( \%R \), ranged from 0 to 1) was used. Given the high correlation, the intercompartmental distribution rate constants
\( (K_{46} \text{ and } K_{64}) \) between the IC central (compartment 4) and the “recycle” compartment (compartment 6) were defined as \( R \times K_{el} \), and the IC central elimination rate constant \( (K_{40}) \) defined as \( (1-R) \times K_{el} \). In effect, this modeling created a second elimination phase for TFV-DP and FTC-TP, consistent with a “recycling” (re-phosphorylation) or a “deep” (slow turnover) compartment.

The model used user-defined differential equations (subroutines ADVAN13 TRANS1). TFV-DP and FTC-TP shared the same model structure. The exponential random effect model on IIV and proportional random effect model on RV resulted in the lowest OFV. The IIV was estimated in \( K_f \) and \( K_{el} \) for IC central compartment in both TFV-DP and FTC-TP models. In the intracellular FTC-TP model, HIV infection status was found to be statistically significant in regard to the formation rate constant \( (tvK_f = 41.6 + 31.3 \times HIV; \text{ positive}=1, \text{ negative}=0), \) as HIV-infected patients had 75.2% (95% CI: 35.6%, 115%) higher average formation rate (\( p<0.00001; \Delta \text{OFV}=-20.5; \Delta \text{IIV}_{K_f}=-5.6\% \)). No statistically significant subject variable was observed in the TFV-DP model.

The alpha half-lives (\( \ln(2)/\lambda_1 \), where \( \lambda_1 \) is the slope of the concentration decline in the initial phase) of TFV-DP and FTC-TP were 73.4 hours (95% CI: 61.8, 87.1) and 8.8 hours (95% CI: 8.2, 9.4). The beta half-lives (associated with compartment 6, \( t_{1/2,\beta} = \ln(2)/\lambda_2 \), where \( \lambda_2 \) is the slope of the concentration decline in the terminal phase) were 55.6 days (95% CI: 46.9, 66.0) and 76.8 hours (95% CI: 71.7, 82.2). However, these beta half-lives only represented a small fraction of concentration-time profiles, as the “recycling” ratios for the description of the second elimination phase were only 5.82% and 16.0% (see tables 5.1 and 5.2). The goodness of fit plots showed good descriptions of the data (Figures 5.3, 5.8, and 5.9). The VPCs are illustrated in Figures 5.6 and 5.7 upper right panels. Model parameters and bootstrapping outcomes are listed in Tables 5.1 and 5.2 middle portions. Other model evaluation results are shown in table 5.3.
5.3.4 Population Pharmacodynamics Modeling of dATP/dGTP and dCTP/TTP

To develop dNTP models, individual PK parameters of TFV-DP/FTC-TP models were first fixed (sequential link). In the previous study of this data, dNTPs were shown to decrease during TDF/FTC treatment [252]. Thus, intracellular TFV-DP/FTC-TP models were linked to the reduction of the zero-order production rate constant ($K_{\text{in}}^0$) of the corresponding intracellular endogenous dNTPs. The indirect response $E_{\text{max}}$ model best described the data. An $EC_{50}$ was introduced in the model, which indicated concentrations of TFV-DP/FTC-TP that led to 50% inhibition of the corresponding dNTP production, and the typical values were estimated as $tvEC_{50}=\text{EXP}(\text{THETA})$ to improve precision and to ensure positive estimations. Model structures are shown in Figure 5.2 lower right portion, as response compartment (compartment 7). The equation was (equation 5.7):

$$\frac{dA(7)}{dt} = K_{\text{in}}^0 \times (1 - \frac{E_{\text{max}} \times A(4)}{A(4) + EC_{50}}) - A(7) \times K_{\text{out}}$$ \hspace{1cm} (5.7)

To avoid overparameterization, the elimination rate constant ($K_{\text{out}}$), as well as maximum drug effect ($E_{\text{max}}$) were fixed to 1. $K_{\text{in}}^0$, and the baseline dNTP ($R_0$) were interconvertible ($K_{\text{in}}^0=R_0 \times K_{\text{out}}$). To estimate the initial individual status of response compartment (compartment 7), in other words, baseline level of dNTP, the model below was chosen (equation 5.8).

$$R0i = (\overline{R}0 \cdot \frac{\sigma^2}{\omega^2 + \sigma^2} + R0i, o \cdot \frac{\omega^2}{\omega^2 + \sigma^2}) \cdot \text{e}^{\text{ηiRV} \cdot \frac{\omega^2}{\omega^2 + \sigma^2}}$$ \hspace{1cm} (5.8)
Figure 5.8 Basic goodness of fit plots of intracellular TFV-DP model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. $|\text{WRES}|$: absolute values of individual weighted residuals.
Figure 5.9 Basic goodness of fit plots of intracellular FTC-TP model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. |\text{IWRES}|: absolute values of individual weighted residuals.
Where $R0_i$ is the model estimated individual baseline level of dNTP. $(R0)^\circ$ is the model estimated population mean level of dNTP. $R0_i,o$ is the observed individual dNTP level at baseline. $\omega$ is the standard deviation (sd) of model estimated IIV of dNTP level at baseline. $\sigma$ is the sd of model estimated residual error of dNTP level throughout the observation period (0-60 days). $\eta_i,RV$ represents the random effect of residual error (a normal distribution with mean=0 and sd=$\sigma$). The effect on dGTP waned over time, and this was described using a sigmoidal $E'_{max} \sim time$ model. However, due to the small sample size and high data variability, this term was simplified to $1/(1+time^\gamma)$ (eq. 5.9), where $\gamma$ is a power exponent that modifies the effect of time mathematically).

$$
\text{dGTP: } \frac{dA(7)}{dt} = K_{in}^o \times \left(1 - \frac{1}{1 + Time^\gamma} \times \frac{A(4)}{A(4) + EC_{50}}\right) - A(7) \times K_{out}
$$

(5.9)

The best fit was obtained using the user defined model (subroutines ADVAN13 TRANS1). For dATP, dCTP, and TTP models, the IIV was estimable on $R_0$ and $EC_{50}$. For the dGTP model, the IIV was only estimable for $R_0$. The exponential random effect model on IIV and RV achieved the lowest OFV. No subject covariate was found to be statistically significant. The goodness of fit plots showed a good description of the data (Figures 5.3, 5.10-5.13). The VPCs are illustrated in Figures 5.6 and 5.7 lower panels. Model parameters and the bootstrapping 95% CIs are listed in Tables 5.1 and 5.2 lower portions. The model estimated (median (5% and 95% percentile)) decreases were 11% (0.45%, 53%) in dATP; 14% (2.6%, 35%) in dCTP and 24% (4.5%, 62%) in TTP. The dGTP reached a low point around day 2.5 with a reduction of 13% (6.9%, 21%), then returned to the baseline value; other components in the dNTP pool returned to baseline during the washout period. Other model evaluation results are shown in Table 5.3.
Figure 5.10 Basic goodness of fit plots of intracellular dATP model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. |iWRES|: absolute values of individual weighted residuals.
Figure 5.11 Basic goodness of fit plots of intracellular dCTP model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. \(|\text{iWRES}|\): absolute values of individual weighted residuals.
Figure 5.12 Basic goodness of fit plots of intracellular dGTP model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. |iWRES|: absolute values of individual weighted residuals.
Figure 5.13 Basic goodness of fit plots of intracellular TTP model. Red line represents average values. Black line represents theoretical values. Data from the same individual are showed in blue circles and are connected by lines. $|\text{iWRES}|$: absolute values of individual weighted residuals.
5.3.5 Simulations and Model Applications

5.3.5.1 Calculation of operational multi-dosing half-lives

Given nonlinear PK relationships between plasma TFV/FTC and intracellular TFV-DP/FTC-TP, an operational half-life ($t_{1/2,op}$) was determined [254]. Simulations were performed to generate ratios ($C_{\text{max,ss}}:C_{\text{max,fd}}$ and $C_{\text{max,ss}}:C_{\text{min,ss}}$) from different dosing intervals ($tau$). The curves generated from these ratios are illustrated in Figure 5.14. The nonlinearity of TFV-DP and FTC-TP accumulation resulted in the interception of the two curves at ratio≠2. The intercepts at x-axis where $C_{\text{max,ss}}:C_{\text{max,fd}}=2$ and $C_{\text{max,ss}}:C_{\text{min,ss}}=2$ were defined as the multiple dosing half-lives ($t_{1/2,op}$). Based on $C_{\text{max,ss}}:C_{\text{max,fd}}=2$, the $t_{1/2,op}$ of TFV-DP and FTC-TP were 4.2 days and 4.8 hours. Based on $C_{\text{max,ss}}:C_{\text{min,ss}}=2$, the $t_{1/2,op}$ of TFV-DP and FTC-TP were 6.7 days and 33 hours.

5.3.5.2 Simulations of IPERGAY trial design

Given the importance of analog:dNTP on NA efficacy, and findings of reduced dNTPs over time, simulations of medians and selected percentiles of analog:dNTP ratios for the IPERGAY trial design are illustrated in Figure 5.15.

When the dose was taken 24 hours pre-coitus, the model predicted that 85% of the virtual population had a TFV-DP:dATP molar ratio above $EC_{50}$ (0.086) at the time of coitus and this persisted for 7 days. The percentage dropped to 50% for 2 hours pre-coital dosing. When evaluating using $EC_{90}$, 20% of simulated individuals were above $EC_{90}$ when dosing was 24 hours pre-coitus, but this dropped to 5% when the dose was taken 2 hours before coitus, illustrating the slow accumulation of TFV-DP.
Figure 5.14 Intracellular operational multiple dosing half-lives. $C_{\text{max,fd}}$: maximum concentration at first dose. $C_{\text{max,ss}}$: maximum concentration at steady-state. $C_{\text{min,ss}}$: minimum concentration at steady-state. TFV-DP: tenofovir diphosphate. FTC-TP: emtricitabine triphosphate. Y-axis: ratios. X-axis: dosing interval. Dashed lines in red represent the curve of $C_{\text{max,ss}}:C_{\text{max,fd}}$. Solid lines in blue represent the curve of $C_{\text{max,ss}}:C_{\text{min,ss}}$. X values at which dashed lines or solid lines intercept at y=2 represent operational multiple dosing half-lives.
Figure 5.15 Protection with pre- and post-coital PrEP dosing. Left panels illustrate TFV-DP:dATP molar ratios. Right panels illustrate FTC-TP:dCTP molar ratios. All simulations included another two doses at 24 hours and 48 hours after coitus. Upper panels illustrate 24 hours dosing pre-coitus. Lower panels illustrate 2 hours dosing pre-coitus. P_n: n\textsuperscript{th} percentile. TFV-DP: tenofovir diphosphate. FTC-TP: emtricitabine triphosphate. dATP: deoxyadenosine triphosphate. dCTP: deoxycytidine triphosphate.
At the time of coitus, virtually all individuals (>95%) had FTC-TP:dCTP above $EC_{90}$ (0.07) and $EC_{50}$ (0.022), and this persisted for at least seven days, regardless of the timing of the pre-coital dosing.

5.4 Discussion

The PK links between plasma TFV/FTC and intracellular TFV-DP/FTC-TP are complicated due to nonlinearities. The accumulation phases are driven by endocytosis, diffusion, and active transport of plasma TFV/FTC, as well as multiple kinases and phosphorylases that orchestrate the intracellular phosphorylation and dephosphorylation [48, 241]. Duwal et al [242], Baheti et al [65], and Burns et al [251] successfully described steady-state kinetics of TFV-DP/FTC-TP using a saturable formation model, an indirect response model, and a first-order formation model. However, all these models failed to minimize in our study, which included observations from first-dose to steady-state. The reason may be that plasma TFV/FTC fluctuates in roughly similar (little accumulation) and wide ranges, whereas intracellular TFV-DP/FTC-TP slowly accumulates. When plotting “exposure” (plasma TFV/FTC) vs “response” (intracellular TFV-DP/FTC-TP) from first-dose to steady-state, we observed “handshape” relationships (examples are shown in Figure 5.16) instead of “S-shape” relationships (suggesting direct effects) or hysteresis loops (suggesting indirect effects), similar to observations of Porchet et al for nicotine tolerance [262]. These “handshape” relationships suggested saturation of phosphate formation for high plasma concentrations, as previously described by Madrasi et al [241]. This effect hints at a
Figure 5.16 An example of the plasma TFV/FTC vs the intracellular TFV-DP/FTC-TP “handshape” plot. Arrows indicate the progression of time after treatment initiation.
constantly changing saturable formation rate of intracellular TFV-DP/FTC-TP by plasma TFV/FTC. We addressed this with a novel TDF/FTC PK model link method—a hybrid of first-order formation and saturation—to describe the formation of intracellular TFV-DP/FTC-TP. However, these models might be limited by the coadministration of TDF/FTC in our study, which limited the ability to assess potential interactions in vivo, as some in vitro studies suggest a metabolic interaction of intracellular TFV-DP and FTC-TP [148]. Studies with the individual drugs are needed to address potential intracellular interactions.

In addition, a possible “recycling” or “deep” compartment for TFV-DP/FTC-TP distribution was identified, manifesting as biphasic elimination. The complex elimination processes are driven by cell trafficking from peripheral tissue with peripheral blood, as well as multiple enzymes that phosphorylate/dephosphorylate nucleoside analogs [10, 11, 269, 270]. Triphosphates that are eliminated (dephosphorylated) may be re-phosphorylated. The biphasic elimination was detected during model development, as a one-compartmental model underpredicted the day 15-30 data in the washout period (day 45-60 of the overall study). As a result, a “recycle” compartment (IC peripheral, compartment 6) was introduced in our model. A three-compartmental model did not converge, due to limited sampling and overparameterization. A two-compartmental model (utilizing compartment 6) best described the data, assuming that part (%R, range from 0 to 1) of the eliminated intracellular TFV-DP/FTC-TP “recycled” (“re-phosphorylated”), or reappeared from a “deep” compartment back into the IC central compartment (compartment 4). However, this compartment was described via a mathematical approach, and the biological interpretation is limited by the study duration (30 doses in HIV-negative group), sparse washout period sampling (5, 15, and 30 days in washout in HIV-negative group only), and the possible influence of the treatment of
samples that were below the limit of quantitation (BLQ) as missing, which is a loss of information reducing power and potentially biasing estimates for this portion of the curve [258, 259]. We chose not to impute BLQ values (e.g. between 0 and the LLOQ) as this may also lead to bias [259]. Further study of this “deep” compartment is needed.

During model link development, simultaneous and sequential link methods were both assessed. Simultaneous link models did not minimize after >48 hours of computation (intel® Xeon® Processor E5-2637 v3 at 3.50 Ghz). Therefore, we chose the sequential link method to develop the model link, as these approaches show similar robustness [261].

These models detected two covariates that were statistically significant. In the plasma FTC model, the male participants had a higher volume of distribution of the central compartment (compartment 2) compared with female participants, which might be explained by the higher lean weight in males into which FTC distributes. In the intracellular FTC-TP model, HIV-positive individuals had a higher FTC-TP $K_f$ but not $K_{el}$ compared with HIV-negative individuals. Seifert et al. reported that FTC-TP area under the concentration-time curve (AUC) was higher at the first-dose in HIV-infected patients (first-dose concentration is affected by $K_0$), but this effect disappeared at steady-state (presumably $K_{el}$ is rate limiting at steady-state concentrations) [237]. Our study was limited by small sample size (n=40) and sampling time, which did not allow us to estimate the IIV for some parameters (e.g. $K_a$ and $SC_{50}$) in the model and to detect statistically significant effects of subject covariates, particularly the HIV-infection status. Other studies suggest lower intracellular NA triphosphates in HIV-negative individuals, so further work is needed to better define such effects [271].

For plasma TFV/FTC model development, a classical two-compartmental model was used, similar to previous studies [65, 79, 242, 243, 246, 247, 251]. The TFV PK
parameters were within the range of literature [65, 79, 251]. Compared with Valade et al [246, 247], FTC PK parameters were all higher (CL/F (L/day): 482 vs 362; Vc/F (L): 99.4 vs 42.3; Vp/F (L): 166 vs 55.4) except Q/F was similar (141 vs 138 (L/day)). $K_a$ was reported to be difficult to estimate [79, 242, 246, 247, 251]. However, in our study, even with sampling starting at 1 and 2 hours (TFV $t_{\text{max}}$: 0.5-3.0 hours, FTC $t_{\text{max}}$: 1.5-3.0 hours [48, 49, 141, 183, 188]), $K_a$ estimation was possible, although $t_{\text{lag}}$ was not [65, 243]. In bootstrapping analysis, extreme values in 95% CI were observed for a few parameters (tables 5.1 and 5.2), including the upper bound of $K_a_{\text{FTC}}$, and the lower bounds of IIV for $V_c/F_{\text{TFV}}$, $V_p/F_{\text{FTC}}$ and $EC_{50_{\text{FTC-TP,dCTP}}}$. These results suggest possible limitations in the sampling times and sample size of our study. To assess the effect of parameter instabilities in bootstrapping analysis, sensitivity analyses were performed by fixing $K_a_{\text{FTC}}$=55.7/day and not estimating the IIV for $V_c/F_{\text{TFV}}$, $V_p/F_{\text{FTC}}$ and $EC_{50_{\text{FTC-TP,dCTP}}}$. Similar model parameter estimations were obtained (%difference<27.6) and the general conclusions of these models remained. A much higher interindividual variability in TFV plasma $V_C$ was observed (CV=53.7%) to FTC plasma $V_c$ (CV=17.9%), which might be introduced by the low bioavailability of TFV (F=25%).

We previously found reduced dNTPs in this study cohort but had not linked drug concentrations to these effects [252]. In this analysis, PKPD models linking the TFV-DP–dATP/dGTP and the FTC-TP–dCTP/TTP were developed using an indirect response model. Given the limited sample size and high variability in dNTPs, several PD parameters ($E_{\text{max}}$ and $K_{\text{out}}$) were fixed to 1 to avoid overparameterization. Because the PD model heavily depended on the baseline response (initial status of compartment 7), to effectively estimate the baseline response, different baseline models were assessed as described by Dansirikul et al [266]. This approach incorporated both the population means and observed values into the estimation, which best described the data. Future
studies should consider repeated measurements prior to treatment initiation to improve the estimation of baseline. In the TFV-DP–dGTP link model, a transient effect that reduced over time was observed [252]. This may represent purine nucleoside phosphorylase (PNP) inhibition by intracellular TFV phosphates which would theoretically raise dGTP [67]. However, a larger sample size is required to probe these responses of dGTP in the future.

The half-lives of TFV and FTC were within the range of previous studies [85, 133, 244, 249, 272, 273] (TFV: 11.6-31 h; FTC: 3.0-40.5 h). The alpha half-life of TFV-DP (73 hours) was within the range of literature (53.3-125 hours[65, 242, 251]). The beta half-life (55.6 days) may be an overestimation as it was limited by the sparse sampling in washout phase and the approach of handling BLQ as missing values. However, it only accounted for a small portion ($R_{TFV-DP} = 5.8\%$) of the concentration change in the washout. Non-compartmental analysis (NCA) reported a half-life of 106 hours (TFV-DP) and 54 hours (FTC-TP) in these same HIV-negative subjects in the 30-day washout (n=17) [274]. Others reported similar half-lives in prolonged observations in washout phase. For example, Hawkins et al, in their trial with a 28-day TFV-DP washout, reported a half-life of 150 h. Dickinson et al reported 116 h (TFV-DP) and 37 h (FTC-TP) half-lives in their 7-day washout observation[249]. Jackson et al reported 164 h (TFV-DP) and 39 h (FTC-TP) in their 10-day washout observation [244]. In a two-compartmental model, the apparent half-lives are the mixture of alpha and beta half-lives. Given this and the non-linearities, the simulation based operational multiple dosing half-lives ($t_{1/2,op}$) might be informative.

Models enabled us to better investigate these complexities by applying the $t_{1/2,op}$ calculation proposed by Sahin et al [254]. They recommended generating both curves of $C_{max,ss}:C_{min,ss}$ and $C_{max,ss}:C_{max,fd}$ from different dosing intervals ($tau$), and
showed that for linear systems, the two curves will intercept at ratio=2 (such as plasma diazepam [254]). In our study, the interception did not occur at ratio=2 due to the nonlinearities, resulting in two $t_{1/2, op}$. Nonlinearities in accumulation favors a higher first-dose concentration ($C_{\text{max,} fd}$), and a lower steady-state concentration ($C_{\text{max,} ss}$), resulting in a lower position of the $C_{\text{max,} ss}:C_{\text{min,} ss}=2$ curve. Results of $t_{1/2, op}$ calculated from $C_{\text{max,} ss}:C_{\text{min,} ss}=2$ were comparable to current literature. The $t_{1/2, op}$ (161 hours) of intracellular TFV-DP supports the prolonged viral suppression for up to a week after the treatment discontinuation, reported by Deeks et al [53], and is also similar to the 150 hours reported by Hawkins et al [57] and 180 hours reported by Pruvost et al [58]. The $t_{1/2, op}$ for FTC-TP (33 hours) is also comparable to other literature values (37-39 hours) [133, 244, 249].

These models also enabled simulations of the analog:dNTP time courses for IPERGAY dosing [162]. Models estimated that two tablets pre-coitus plus two tablets post-coitus had 5-20% of >90% protective effect and 50-85% of >50% protective effect from TDF. The relatively slow accumulation of TFV-DP on day 1 may be a disadvantage of TDF for on demand PrEP, but the FTC-TP:dCTP ratios were well above $EC_{90}$ after the first-dose. This suggests that FTC contributes significantly to the protective on demand PrEP effect of TDF/FTC and is consistent with 86% efficiency from IPERGAY. In these simulations, drug concentrations dominated the molar ratio (analog:dNTP) change, while the relatively small changes in dNTPs did not contribute much to the ratio change. This simulation is limited by the assumption of a simple/direct relationship between analog:dNTP and efficacy, and that the intracellular data were generated from PBMC only. TFV-DP/FTC-TP levels may differ among cell types, such as in tissue mononuclear cells in rectal and female genital tissues that are the major target for PrEP [50, 275]. The dNTP pool also varies among different cell types [50]. Thus, our simulation in PBMC
might not represent the cells in target tissues. Nevertheless, PBMC includes CD4 T-cells, which is one of the major target cells for HIV and antiretroviral agents. Also, model development was based on oral dosing, thus simulations may not extrapolate to topical dosing scenarios, in which tissues, concentration gradients, and model structure are different, and other biological factors may be important to consider, such as local pH and the microbiome.

In conclusion, we developed PKPD models linking plasma and intracellular TFV/FTC with the dNTP pool. Observations from first-dose to steady-state were necessary for model development to describe complex accumulation processes. A hybrid of first-order formation and saturation was used to describe nonlinearities of intracellular TFV-DP/FTC-TP formation. An indirect response model was used to describe the interaction between TFV-DP/FTC-TP and dNTPs. These models can be helpful to understand the time profiles of analog:dNTP ratio changes in different clinical trial designs and to calculate the operational multiple dosing intracellular half-lives. Future study is required to investigate the possible mechanisms of these findings and to test other covariates.
CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

The antiviral efficacy of TFV/FTC is defined by the intracellular TFV-DP/FTC-TP and the analog:dNTP ratios, creating a clinically relevant need to study these triphosphates moieties, given they may interact because they share the same metabolism/anabolism pathways. Of particular importance is to study these effects in vivo, as in vitro studies do not reliably translate in vivo. In this research dissertation, we approached this problem by first successfully developing a reliable and sensitive quantitative method for the measurement of dNTPs (including dATP, dCTP, dGTP, and TTP) in cell lysates from humans (Chapter 3). This method was then used to quantify the dNTP pool levels from the “Cell-PrEP” study of daily TDF-FTC in humans in which we observed reductions in the dNTP pool components from baseline, using a segmented mixed model linear regression analysis (Chapter 4). Finally, in order to understand the kinetics of this interaction, we linked dNTP changes to intracellular TFV-DP/FTC-TP concentrations using compartmental PK-PD modeling.

The PK-PD model was created using multiple steps. First, the formation of intracellular TFV-DP/FTC-TP was modeled by stimulation from plasma TFV/FTC concentrations, using a hybrid of first-order formation and saturation equations, resulting in six compartments in total. Then intracellular TFV-DP/FTC-TP concentrations were used to inhibit the production of dNTPs using indirect response link models, in which models estimated the EC\textsubscript{50} (effective concentrations of TFV-DP/FTC-TP that led to 50% decrease in dNTPs production), which ultimately summarized the effects of TFV/FTC on dNTPs (Chapter 5). The creation of this model is important to guide research of
TDF/FTC in the future. For example, we used the model to simulate the time course of
dNTP:drug-triphosphate for the IPERGAY dosing strategy. Similar simulations could be
used for other novel TDF/FTC dosing strategies, and for the same with the new pro-drug
tenofovir-alafenamide. This model is a new contribution to the TDF/FTC literature and it
represent a new modeling strategy that could be applied to other nucleoside analogs.

In addition, we also learned about limitations, which taught us lessons that can
be applied in future studies. First, a sampling time starting at 1 hour was not early
enough to capture the absorption phase of plasma TFV/FTC. While we did not consider
Ka a focus in our PK models, it still prevented a robust estimation of this parameter.
Second, the single measurements at 5, 15, and 30 days of washout limited our ability to
probe the biphasic elimination of intracellular TFV-DP/FTC-TP. More frequent sampling
in the washout would have been helpful. More importantly, we made a decision to treat
samples that were below the limit of quantitation (BLQ) as missing. We understood that
this led to loss of information and potential biasing for this part of the concentration time
curve. Different approaches have been advocated for handling BLQ values (each with
strengths and weaknesses), and these should be explored in future work.

Another issue was high interindividual and intraindividual variability of dNTPs,
suggesting a larger sample size would be needed for covariate testing for future studies.
In addition, repeated measurements (n>1) at baseline would increase the robustness of
PKPD modeling using NONMEM, as in this study we had to use a model incorporating
both weighted population mean and weighted observed values at baseline to estimate
individual baseline values because the classic estimation methodologies of NONMEM
failed to produce reasonable estimations. Furthermore, the high variability of dNTPs
might be due to different sampling conditions, such as morning vs afternoon and fed vs
fasted. A blinded control arm who received placebo would help sort this out.
Variability in bioavailability can lead to PK variability for drugs with low bioavailability such as TFV ($F=25\%$). For this reason, we asked participants to arrive to their PK visits fasted. While we cannot confirm subjects were fasted, we only observed relatively high interindividual variability in TFV plasma $V_c$ ($CV=53.7\%$), and residual variability was acceptable ($CV=27.3\%$).

This study quantified the time course of dNTPs changes as the inhibition of dNTP production. However, the affected intracellular pathway(s), which includes enzymes and transporters were not investigated in this clinical PK-PD modeling study. Future research is required to understand these interactions. For example, *in vitro* studies may be performed to help understand the the change of endogenous dNTPs, including the affinity and effect of intracellular TFV/FTC (and their anabolites) on enzymes that participate in the anabolism and metabolism of dNTPs.

A benefit of this work is that the quantitative method for dNTPs in cell lysates (chapter 3) can be used for future studies for the measurement dNTP in humans, such as in the oncology and immunology fields. We can also use what we learned from this method to develop other quantitative methodologies that can simultaneously detect both the nucleos(t)ide analog and endogenous dNTPs, using similar extraction and purification SPE methods, optimized LC/MS/MS conditions, and standard operating procedures to minimize environmental contamination.

We found the segmented mixed model linear regression analysis to be useful for probing dNTP changes (chapter 4), and advocate this approach for other future pharmacodynamics studies that assess two distinct slopes. Compared with traditional t-tests and nonlinear regression models, this model was particularly useful and biostatistically sound for detecting small changes of dNTPs.
Finally, this modeling strategy (chapter 5) opens new opportunities for using simulations without performing actual experiments. Examples include simulations for tenofovir alafenamide regimens and the HPTN067/ADAPT study design, which include three different dosing strategies: daily dosing, twice-weekly dosing + an extra dose after sex, or event-driven dosing (one dose up to 48 hours before sex, another 2 hours after sex). In addition, the parameters and model structures developed here can contribute to the construct of comprehensive physiologically based pharmacokinetics-pharmacodynamics (PBPK/PD) models that incorporate all available in vitro and in vivo research results to optimally characterize the physiological basis of drug therapy in vivo.

In conclusion, this study quantified the effect and time course of TFV/FTC on dNTPs in vivo. This new model and approach used state-of-the-art analytical chemistry, biostatistical analysis, and population PKPD modeling and simulation. This work represents a significant contribution to our knowledge of TDF/FTC in vivo and sets the groundwork for future investigations of these drugs or similar agents in other therapeutic areas.
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APPENDIX A

Validation of a Sensitive LC/MS/MS Method for the Determination of Telaprevir and its R-isomer in Human Plasma
Validation of a sensitive LC/MS/MS method for the determination of telaprevir and its R-isomer in human plasma

Xinhui Chen, Lane R. Bushman, Kevin J. McAllister, Peter L. Anderson* and Jennifer J. Kiser

ABSTRACT: The purpose of this study was to validate a reversed-phase high-performance liquid chromatographic (HPLC) tandem mass spectrometry (MS/MS) assay for the determination of telaprevir and its R-isomer in acidified and nonacidified human plasma. The chromatographic baseline separation of telaprevir and telaprevir-R was performed on a Waters XBridge™ BEH Shield C18, 2.1 x 75 mm column with a 2.5 µm particle size, under isocratic conditions consisting of a mobile phase of 50:45:5 water-acetonitrile-isopropanol with 1% ammonia at 0.2 ml/min. This method utilized a stable isotope internal standard with 11 deuterium atoms on the structure of the telaprevir molecule (telaprevir-d11). An internal standard for the telaprevir-R (telaprevir-R-d11) was also prepared by incubating telaprevir-d11 in basic solution, which facilitated isomer inter-conversion. The detection and quantitation of telaprevir, telaprevir-R, telaprevir-IS and telaprevir-R-IS was achieved by positive ion electrospray (ESI+) MS/MS detection. The assay quantifiable limit was 5.0 ng/ml when 0.100 ml of acidified human plasma was extracted. Accuracy and precision were validated over the calibration range of 5.0-9000 ng/ml. It was demonstrated using patient samples that, contrary to previous recommendations, quantitation of telaprevir does not require acidified plasma. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: telaprevir; analytical methodology; hepatitis C virus; LC/MS/MS; clinical pharmacology

Introduction

Since its discovery in 1989, the hepatitis C virus (HCV) has infected approximately 130-200 million individuals worldwide. Mortality owing to HCV has surpassed that of HIV in the USA, accounting for 10,000-15,000 deaths annually (Zanetti, 1999). The virus is transmitted mainly by exposure to blood, including blood transfusion (prior to 1992), shared needles and reused medical supplies, and more uncommonly, through sexual exposures. An estimated 1.6% of the US population is infected; those quarters of whom do not know they have HCV infection (Alter, 1999). Baby boomers (individuals born between 1946 and 1964) make up about 30% of the US population, but they account for two-thirds of the people in the USA infected with HCV (Gravitz, 2011). Chronic infection with HCV is a major risk factor for the development of cirrhosis and hepatocellular carcinoma worldwide (Leverson, 2006). Until recently, the standard of care for HCV infection consisted of ribavirin combined with pegylated interferon alpha. However, approximately 60% of patients did not achieve sustained virologic response after 48 weeks of therapy (McHutchinson et al., 2009). The recent addition of direct-acting antiviral agents such as the NS3/4A protease inhibitor telaprevir to the standard ribavirin and pegylated interferon alpha treatment significantly increased the rates of sustained virologic response for patients with genotype 1 disease, including those who previously failed to achieve viral eradication with pegylated interferon and ribavirin alone (Zeuzem et al., 2011).

Telaprevir is administered twice or three times daily with a non-low-fat meal in combination with ribavirin and pegylated interferon alpha. It is extensively metabolized by the liver, and it is involved in several important drug-drug interactions, especially those involving the CYP3A pathway, which is a major route of drug metabolism in man (Kiser et al., 2012). Given these pharmacologic characteristics, there is a potential role for therapeutic drug monitoring in the clinical use of telaprevir (Dolton et al., 2013). To facilitate clinical pharmacology studies of telaprevir, simple, fast and sensitive analytical methods are needed, but few methods are currently available (Famik et al., 2009; D’Avolio et al., 2013; Penczala et al., 2013).

The goal of this study was to develop and validate an analytical method for telaprevir that achieved baseline separation of telaprevir/telaprevir-R under isocratic conditions, generated a telaprevir-R internal standard from telaprevir internal standard, and evaluated telaprevir quantitation in acidified vs nonacidified plasma.

* Correspondence to: P. L. Anderson, University of Colorado Denver, School of Pharmacy, Department of Pharmaceutical Sciences, Aurora, Colorado, USA. Email: peter.anderson@ucdenver.edu

University of Colorado Denver, School of Pharmacy, Department of Pharmaceutical Sciences, Aurora, Colorado, USA

Abbreviations used: BLOQ, below limit of quantitation; ESI+, electrospray ionization positive polarity; HCV, hepatitis C virus; MTRA, method test article; QL, high level quality control; QL, low level quality control; QM, medium level quality control; SIM, selected ion monitoring; telaprevir-S, isotopic telaprevir; ULOQ, upper limit of quantitation; LLOQ, lower limit of quantitation.
Experimental

Chemicals and reagents
Telaprevir (99.3% pure), telaprevir-R (96.5% pure) and isotopic telaprevir (11-S) telaprevir-S were generously provided by Vertex Pharmaceuticals Inc. (Cambridge, MA, USA). The chemical structures of telaprevir (MW 679.85; the diastereomer position and the isotopic deuterium-labeled position of internal standard are shown in Fig. 1. HPLC-grade methanol, isopropanol, acetonitrile, formic acid and ammonia were acquired from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure (UP) water was prepared in house from deionized water with a Barnstead Nanopure System (Thermo Fisher Scientific, Waltham, MA, USA). Human K$_2$-EDTA anti-coagulant plasma was obtained from the Biological Specialty Corporation (Colmar, PA, USA).

LC/MS/MS instrumentations and conditions
The HPLC system utilized a Surveyor LC autosampler and LC Pump (Thermo Scientific, San Jose, CA, A.Waters XBridge$^{	ext{TM}}$ BEH (Waters Corp., Milford, MA, USA) analytical column was used for chromatographic separations. The mobile phase consisted of freshly prepared 1% amonia in a 45:55 acetonitrile-isopropanol-water (v/v/v) solution, delivered at a flow rate of 3.200 mL/min. At the end of each analytical run, the column was washed with 50 mL acetonitrile-water (v/v). The analytical column was maintained at 50°C, and extracted samples were kept at 15°C while inside the autosampler. The autosampler needle was washed with 0.1% formic acid in a 75:25 acetonitrile-water (v/v) solution between injections. A TSQ Quantum triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) was used in positive ion electrospray (ES$^+$) mode. All analytes and internal standards were detected in MS/MS selected reaction monitoring (SRM) mode using optimized parameters: spray voltage of 1500 V, capillary temperature of 275°C, collision energy of 27 V, and tube lens setting of 140. Precursor product transitions of 680.30/322.00 and 691.300/322.00 were monitored for telaprevir/telaprevir-R and telaprevir-S/telaprevir-R-S, respectively. The MS/MS spectra were the same as that reported in previous studies (Frenki et al., 2009; Panchala et al., 2013) and telaprevir-R/S had the same spectra as telaprevir-S. Data acquisition, processing, and storage were performed using Xcalibur software, version 1.3 (Thermo Scientific, San Jose, CA, USA). Calculations were based on peak area ratios of analyte to internal standard. Concentrations were interpolated from a quadratic least squares regression calibration curve based on 1/concentration$^2$ weighting for both analytes.

Preparation of calibration standards, internal standard, acidified plasma and quality controls
Telaprevir, telaprevir-R and telaprevir-S were all prepared in separate 1 ng/mL stock solutions, and then combined to prepare appropriate working standard stocks consisting of both analytes. Internal standard working solutions were prepared by adding telaprevir-S stock solution in 1% ammonia solution for ≥3 h to facilitate interconversion from telaprevir-S to telaprevir-R/S. After drying down and reconstitution in methanol, a total concentration of 1000 ng/mL solution combining telaprevir-S and telaprevir-R/S in an approximate 1:1 ratio was achieved. This solution was used as the working internal standard stock solution. All stock and working solutions were stored in screw cap 13 × 100 mm tubes with polytetrafluoroethylene-faced rubber liner cap at –20°C and prepared as necessary.

Acidified plasma was prepared by adding 250 µL of 20% formic acid solution to 5 mL K$_2$-EDTA plasma, which yielded a concentration of 1% formic acid in plasma. Telaprevir/telaprevir-R combined quality controls (QC) were prepared separately in acidified plasma from the QC prep stocks to make final concentrations of 15 ng/mL (low-level quality control, LQ), 300 ng/mL (medium-level quality control, MQ) and 4000 ng/mL (high-level quality control, HQ). The QC samples were stored at –20°C. A 5 ng/mL QC was prepared similarly and used to validate the lower limit of quantitation, as described below. All experiments utilized acidified plasma unless otherwise noted.

Sample preparation
Plasma telaprevir/telaprevir-R standards were prepared in 13 × 100 mm test tubes on a daily basis by spiking the appropriate working standard stock solution into 100 µL of blank acidified plasma, resulting in plasma telaprevir/telaprevir-R standard (calibration) concentrations of 5, 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 ng/mL. Unknowns and QC plasma samples (100 µL) were added to test tubes, followed by addition of 10 µL of working internal standards solution. One milliliter methanol tert-butyl ether (MTBE) was added to all samples followed by vortexing for at least 10 s, then centrifugation at 5000 rpm for 5 min followed by a dry ice bath for at least 5 min. The organic layer was then decanted to 12 × 75 mm test tubes. Samples were dried under nitrogen, reconstituted with 100 µL of 50:50 methanol-water solution, and transferred to labeled vials containing 150 µL, low-volume inserts (Waters Corporation, Milford, MA, USA). The injection volume was 5 µL.

Method validation
Validation included an evaluation of the following characteristics: assay accuracy and precision, calibration curve performance, recovery and matrix effects, dilution accuracy and precision, analyte stability and assay specificity/selectivity. These validation experiments followed standard acceptance criteria for bioanalytical method validation (Shah et al., 2000).

Accuracy and precision.
Intra- and inter-day accuracy and precision were determined by the performance of four concentrations of QC, LLOQ, 5 ng/mL; MQ, 15 ng/mL; OM, 300 ng/mL; and OH, 4000 ng/mL. The 5 ng/mL QC was run in five replicates on three days; and all other QC levels were run in six replicates on three separate days. Accuracy was evaluated and reported by calculating the percentage deviation from the nominal concentration. Precision was determined by calculating the coefficient of variation (CV) of replicates within one sample run (intra-day) and between sample runs (inter-day). The accuracy and precision at each concentration level should not exceed 15%, except for the LLOQ, which should not exceed 20%.

Calibration curve.
Calibration curve performance was assessed by evaluating deviation of standards from the back-calculated curve, and evaluating the slope, intercept, and coefficient of determination (r²) of the weighted 1/concentration⁰ regression fit. At least seven standards were required for a valid calibration curve where ≥20% from the nominal value was acceptable at the LLOQ and ≥±15% from the nominal value accepted at all other concentrations. If a calibrator did not meet these criteria, it was dropped from the calibration curve and the curve was recalculated.

Matrix effects and recovery study.
Validation included an assessment of matrix effects on the quantitation of both analytes. To determine
Figure 2. Representative LC/MS/MS chromatograms. The retention time for telaprevir is approximately 3.9 min, followed by telaprevir-R at 4.3 min. (A) Overlay figure of blank acidified plasma sample, blank acidified plasma sample spiked with telaprevir-IS and telaprevir-R-IS, and blank acidified plasma sample spiked with telaprevir/telaprevir-R at the lower limit of quantitation (LLOQ: 5.0 ng/mL); (B) blank acidified plasma sample spiked at the telaprevir/telaprevir-R upper limit of quantitation (ULOQ, 5000 ng/mL); and (C) a subject unknown acidified sample. Telaprevir/telaprevir-R concentration was 494.95 ng/mL. For (B) and (C) the top chromatograms in the analyte while the bottom chromatogram is the internal standard; telaprevir elutes first and is in the left chromatograms, followed by telaprevir-R in the right chromatograms. The IS only sample (A) shows a small and insignificant (approximately 1% of the LLOQ) telaprevir/telaprevir-R peak.
If endogenous compounds in plasma suppressed or enhanced analyte ionization during detection, potential matrix effects were tested following the method of Mackenzie et al. (2003). Three sets of samples (set 1, set 2, and set 3) were prepared containing telaprevir/telaprevir-R standards of 15, 300, and 4000 ng/mL in five replicates, with each replicate in sets 2 and 3 using a different plasma lot. The 1 set 1 samples ( neat samples) consisted of analyte and internal standard added to 5% methanol solution for a total volume of 100 mL. In set 2 (post-extract spiked), the analyte and internal standard were spiked into a matrix extracted from blank acidified plasma. For set 3 samples, the analyte and internal standard were spiked into acidified plasma and then extracted as described above. A comparison of set 1 and set 2 samples yielded a measure of observed matrix effects. A comparison of set 2 and 3 demonstrated analytic recovery from the extraction process. The difference between set 1 and set 3 samples described the overall efficiency of the analytical process. The effects of different plasma lots on the assay were determined by comparing the regression line slopes and peak area ratios for each different lot of plasma, as well as by examining the precision of the analytic and internal standard areas and ratios for each sample set and plasma lot.

**Dilution accuracy and precision.** In order to determine the accuracy and precision (n=3) of measuring telaprevir/telaprevir-R in diluted plasma, a QC was prepared at a concentration of 750 ng/mL, then diluted to 375 ng/mL (5x) and to 187.5 ng/mL (10x) with blank acidified plasma. These samples were allowed a difference of ±15% from the expected value to be acceptable.

**Stability.** The stability of both analytes in EDTA plasma was tested by subjecting QCs to different test conditions. Freeze-thaw stability of the QCs was tested in triplicate with QL (15 ng/mL) and QH (400 ng/mL) after three freeze-thaw cycles. The samples were allowed to thaw completely and remained at room temperature for at least one hour. The samples were returned to freezer storage conditions (−20°C) for at least 24 hours prior to removal for the next freeze-thaw cycle. The stability of telaprevir and telaprevir-R in plasma at room temperature was tested for thawed QC samples (QL and QH in triplicate) maintained at room temperature for 4, 8, 12, and 24 hours. The samples were stored at 4°C in a sealed vial and were removed at the end of each interval. The stability of the analytes was determined by comparing the concentration of the unreacted or reference QC samples that were run within the same analytical run.

**Chromatography.** Isocratic chromatography was optimized so that baseline separation of telaprevir and telaprevir-R was achieved. Representative LC-MS/MS chromatograms are shown in Fig. 2. In Fig. 2(A) is an overlay figure of a blank plasma sample, a blank plasma sample spiked with internal standard, and an extracted ULOQ (5000 ng/mL) sample; Fig. 2(B) shows an extracted sample at the assay’s ULOQ (5000 ng/mL); and Fig. 2(C) shows a typical extracted patient sample—the concentration of telaprevir/telaprevir-R was 494/959 ng/mL. In Fig. 2(B) and (C), analytes are shown in the top part, with the internal standard for each in the bottom window. Telaprevir eluted first, at approximately 3.9 min, followed by telaprevir-R at 4.3 min.

**Results and discussion.**

**Table 1. Summary statistics for telaprevir and telaprevir-R QCs during the six separate analytical runs used for accuracy and precision determination.**

<table>
<thead>
<tr>
<th></th>
<th>Telaprevir Interassay statistics</th>
<th>Telaprevir-R Interassay statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal concentration</td>
<td>5.00</td>
<td>1.50</td>
</tr>
<tr>
<td>Mean</td>
<td>5.32</td>
<td>1.64</td>
</tr>
<tr>
<td>SD</td>
<td>0.80</td>
<td>1.25</td>
</tr>
<tr>
<td>CV (%)</td>
<td>15.0</td>
<td>8.6</td>
</tr>
<tr>
<td>Deviation (%)</td>
<td>0.4</td>
<td>2.9</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>18</td>
</tr>
</tbody>
</table>
Validation results

Accuracy and precision. Inter- and intra-assay precision and accuracy based on QCs are shown in Table 1. Deviations from nominal (accuracy) were ≤11.3/10.9% for telaprevir/telaprevir-R QCs. Precision values were ≤5.6/6.1% respectively. Accuracy and precision for LLOQ were ≤15.4/17.2% and ≤16.2/15.2% for telaprevir/telaprevir-R, respectively. The precision and accuracy for all of the five tested QC levels and LLOQ were within the acceptable range, demonstrating an accurate and precise methodology.

Calibration curve. The calibration range was linear in the range of 5–2500 ng/mL before demonstrating some nonlinearity between 2500 and 5000 ng/mL, thus promoting the fit with the weighted (1/x²) quadratic formula. The standard curve performance was validated over the range 5–5000 ng/mL with typical r² values within 0.9983 and 0.9986. Average calibrator accuracy and precision were 2.07 and −0.01% for telaprevir, and 2.21 and −0.01% for telaprevir-R.

Matrix effect and recovery study. The liquid–liquid extraction protocol yielded a recovery of 58.1–67.4% (mean 63.8%) for telaprevir and 51.4–60.9% (mean 57.1%) for telaprevir-R (Table 2). The recoveries were below those reported in Penchala et al. (2013) using a similar extraction technique, leading to recoveries of >90%. The reasons for this difference were not investigated further because the extraction produced accurate and precise results. The matrix effect was consistent over the concentration range, with a mean 10.2% suppression for telaprevir and telaprevir-IS, and 10.6% suppression for telaprevir-R and telaprevir-R-IS. The overall process efficiency was 57.2% for telaprevir and 51.8% for telaprevir-R. Finally, the CV between the different plasma lots used was ≤13.8% for telaprevir and 10.4% for telaprevir-R, indicating that different plasma sources did not adversely affect quantitation.

Dilution accuracy and precision. Two- and four-fold dilution experiments yielded accuracy of ≤5.3% and precision of ≤3.7% vs expected concentrations, well within acceptance criteria. Thus, samples above the LLOQ can be diluted up to 4-fold.

Stability. Stability of telaprevir/telaprevir-R was evaluated under a wide variety of conditions. QC samples were stable in plasma through at least three freeze-thaw cycles at −20°C.

Table 2. Summary of matrix effect, recovery and process efficiency of telaprevir/telaprevir-R

<table>
<thead>
<tr>
<th></th>
<th>ME</th>
<th>RE</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>IS</td>
<td>Analyte</td>
<td>IS</td>
</tr>
<tr>
<td>Telaprevir Mean</td>
<td>89.8</td>
<td>89.9</td>
<td>63.8</td>
</tr>
<tr>
<td>SD</td>
<td>6.0</td>
<td>6.2</td>
<td>4.9</td>
</tr>
<tr>
<td>CV (%)</td>
<td>6.7</td>
<td>6.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Telaprevir-R Mean</td>
<td>89.4</td>
<td>90.4</td>
<td>57.1</td>
</tr>
<tr>
<td>SD</td>
<td>4.1</td>
<td>3.5</td>
<td>5.0</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.6</td>
<td>3.9</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Figure 3. Telaprevir and telaprevir-R bench-top stability. Each sample is compared with the mean of control (T=0). Dashed lines show ±15% acceptance criteria. Telaprevir was stable to 24 h and telaprevir-R was stable to 8 h.

Table 3. Conditional stability of telaprevir/telaprevir-R in acidified plasma

<table>
<thead>
<tr>
<th>Condition</th>
<th>Analyte</th>
<th>Nominal concentration (ng/mL)</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
<th>Treated</th>
<th>Precision</th>
<th>Accuracy</th>
<th>Control</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench-top</td>
<td>Telaprevir (24 h)</td>
<td>15.0</td>
<td>12.2</td>
<td>0.4</td>
<td>2.4</td>
<td>−18.4</td>
<td>14.0</td>
<td>0.5</td>
<td>−12.5</td>
<td>−3.4</td>
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<tr>
<td></td>
<td></td>
<td>4000</td>
<td>3399</td>
<td>16.2</td>
<td>0.5</td>
<td>−15.0</td>
<td>3854</td>
<td>29.1</td>
<td>−11.8</td>
<td>−3.7</td>
</tr>
<tr>
<td></td>
<td>Telaprevir-R (8 h)</td>
<td>15.0</td>
<td>15.4</td>
<td>0.5</td>
<td>3.1</td>
<td>2.4</td>
<td>16.7</td>
<td>0.5</td>
<td>−7.8</td>
<td>−2.2</td>
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<tr>
<td></td>
<td></td>
<td>4000</td>
<td>3752</td>
<td>31.1</td>
<td>0.8</td>
<td>−6.2</td>
<td>3893</td>
<td>37.8</td>
<td>−3.6</td>
<td>−1.0</td>
</tr>
<tr>
<td>Autosampler</td>
<td>Telaprevir</td>
<td>15.0</td>
<td>14.9</td>
<td>0.4</td>
<td>2.6</td>
<td>−0.4</td>
<td>14.3</td>
<td>0.3</td>
<td>−4.6</td>
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<td></td>
<td></td>
<td>4000</td>
<td>3555</td>
<td>88.8</td>
<td>2.5</td>
<td>−11.1</td>
<td>3711</td>
<td>51.8</td>
<td>−4.2</td>
<td>−1.6</td>
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<tr>
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<td>Telaprevir-R</td>
<td>15.0</td>
<td>14.1</td>
<td>0.6</td>
<td>4.0</td>
<td>−6.2</td>
<td>14.2</td>
<td>0.2</td>
<td>−1.2</td>
<td>−0.4</td>
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<td></td>
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<td>4000</td>
<td>3445</td>
<td>87.6</td>
<td>2.5</td>
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<td>3569</td>
<td>81.2</td>
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<td>Freeze-thaw</td>
<td>Telaprevir</td>
<td>15.0</td>
<td>15.3</td>
<td>1.4</td>
<td>9.3</td>
<td>2.2</td>
<td>15.8</td>
<td>0.6</td>
<td>−3.1</td>
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<td>4000</td>
<td>3405</td>
<td>32.2</td>
<td>0.9</td>
<td>−14.9</td>
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<td>53.2</td>
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<td>−0.4</td>
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<tr>
<td></td>
<td>Telaprevir-R</td>
<td>15.0</td>
<td>15.7</td>
<td>1.5</td>
<td>9.4</td>
<td>−4.6</td>
<td>16.1</td>
<td>0.9</td>
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<td>−0.8</td>
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<td></td>
<td>4000</td>
<td>3482</td>
<td>29.2</td>
<td>0.8</td>
<td>−12.9</td>
<td>3509</td>
<td>8.3</td>
<td>−0.8</td>
<td>−0.2</td>
</tr>
</tbody>
</table>

Note: although the accuracy was out of range (≥15%), the percentage difference with control was within range.
When kept at room temperature on the bench top, telaprevir was stable for 24 h when compared with control (T = 0) and 12 h when compared with both control and nominal, and telaprevir-R was stable for 8 h when compared with both control and nominal (Fig. 3 and Table 3). Extracted samples were also stable when maintained at 15°C in the autosampler for at least 8 days.

To examine the stability of telaprevir and telaprevir-R in acidified vs nonacidified plasma, 15 paired acidified and nonacidified samples were tested. No sample differed by more than ±17.7% for telaprevir, which passed the incurred analysis acceptance threshold of 30% (Table 4). However, for telaprevir-R the mean (range) difference was 44.9% (−2.9 to 162%), indicating that acidification is necessary for telaprevir-R. Of note, the 15 paired samples arose from pre-steady state and steady-state time points. This was important because the ratio of telaprevir/telaprevir-R changed from first dose to steady-state (described below). This changing ratio of telaprevir-R/total did not impact the lack of acidification effect on telaprevir quantitation.

### Specificity and selectivity

In terms of assay selectivity, the blank with internal standards showed no telaprevir-S or telaprevir-R-S signal in the analytic channels, while the blank without internal standard showed that no signal came from the sample matrix (Fig. 2A). Furthermore, the extraction of a sample spiked with the highest concentration telaprevir/telaprevir-R standard (5000 ng/mL), but without internal standard, showed no signal for telaprevir or telaprevir-R in the telaprevir-S or telaprevir-R-S SRM channel. Analysis of six different sources of blank and EDTA plasma showed no analyte or internal standard peaks, indicating that the source of plasma did not contribute to analyte or internal standard signals. No interferences were observed with ribavirin and pegylated-interferon using clinical samples.

### Clinical application

One-hundred and fifty plasma samples from HCV positive subjects were analyzed for telaprevir and telaprevir-R concentrations using this methodology. Because of the variable times post-dose and post-initiation of telaprevir therapy, a summary of concentrations was evaluated for this communication. The medians (interquartile ranges) of telaprevir and telaprevir-R were 1841 (1031, 2848) and 1121 (642, 1842) ng/mL, respectively (Fig. 4). The overall average of telaprevir-R/total ratio was 33.9%, but this ratio increased from 24% at the first dose to 40% at steady state. Observed telaprevir and telaprevir-R concentrations in these 150 samples were within the range of published values from 249 to 4812 ng/mL (Sarrazin et al., 2007; Marcellin et al., 2011; Garg et al., 2013). Method reproducibility was demonstrated by reanalyzing incurred samples (n = 15). Samples were all within the 30% threshold. The mean (range) difference was 4.7% (−4.1% to 11.6%) for telaprevir and 8.5% (−2.9 to 22.1%) for telaprevir-R (Table 5).

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**Table 4.** Acidification effect analysis data

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Telaprevir (ng/mL)</th>
<th>Telaprevir-R (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acidified</td>
<td>Nonacidified</td>
</tr>
<tr>
<td>1</td>
<td>435</td>
<td>435</td>
</tr>
<tr>
<td>2</td>
<td>972.2</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>2835</td>
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BLQ, Below limit of quantitation.
Table 5. Incurred sample analysis data

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Conclusions

A sensitive, simple, efficient and reliable method for determining telaprevir/telaprevir-R concentrations in acidified human plasma was validated. This method accomplished specific goals that were set at the onset of method development, including baseline separation of telaprevir and telaprevir-R with isocratic conditions, generation of a separate IS for telaprevir-R, and evaluation of the effects of acidified vs nonacidified plasma. To accomplish baseline separation of telaprevir we used isopropanol as a modifier and ammonia as an ion pairing agent in the mobile phase. We found that it was important to use the smallest diameter tubing available while shortening the tubing to the shortest length to minimize horizontal dispersion effects. We also increased the column temperature to facilitate the baseline separation in the isocratic condition. Although the strong basic mobile phase (pH between 11 and 12) and elevated column temperatures were close to the limits of the column (pH 12), the isocratic liquid chromatography conditions were reproducible and allowed for relatively short analytical run times, without the need for re-equilibration, ultimately increasing sample throughput.

Because telaprevir-IS co-eluted with telaprevir but not telaprevir-R, we endeavored to generate a telaprevir-R internal standard that would co-elute with telaprevir-R to more accurately and precisely quantify telaprevir-R. We were able to achieve this goal by incubating telaprevir-IS in 1% ammonia solution to facilitate the conversion of telaprevir-IS to telaprevir-R-IS. Note that we tested the effects of the mobile phase, which included 1% ammonia on this interconversion, and did not observe an effect. The final telaprevir-IS/telaprevir-R-IS ratio was close to 1:1. With the use of telaprevir-R-IS, we were able to achieve higher accuracy and precision for the quantitation of telaprevir-R.

Telaprevir and telaprevir-R QC samples in acidified plasma have been shown to be stable at room temperature until 6 h (Famk et al., 2009) and 16 h (Penchala et al., 2013) in previous studies. Our research, with observation up to 120 h, demonstrated telaprevir QC stability at room temperature for up to 24 h when compared with control (T=0). We also found telaprevir-R QC samples were only stable at room temperature for up to 8 h (Figure 3). An explanation for this difference in QC sample stability may arise from the equilibrium ratio of telaprevir and telaprevir-R, which is at an average of approximately 3:2 in patient plasma. This suggests unequal driving forces favoring the conversion from telaprevir-R to telaprevir.

An incurred sample reanalysis showed reproducibility using clinical samples. An analysis that compared quantitation in acidified vs nonacidified plasma that was subjected to room temperature for up to 8 h demonstrated that quantitation of telaprevir does not require acidification, while telaprevir-R does require acidification. Our results for telaprevir stability in patient samples were similar to the previous study of Penchala et al. (2013), however telaprevir-R showed a more substantial change of concentrations in our study, possibly owing to longer exposure to room temperature. Our study also assessed more patient samples for the analysis. Penchala et al. (2013) concluded that, in patient samples, the ratio of telaprevir/telaprevir-R changed significantly in nonacidified plasma, leading to the recommendation to use acidified plasma. Taken together, these findings show that telaprevir is stable in nonacidified patient samples when processed within 8 h, which enables new research focused on telaprevir, such as protein binding determinations, that was not possible previously when samples had to be acidified. It is important to note that such studies would not be able to include telaprevir-R or the telaprevir/telaprevir-R ratio, because telaprevir-R was not stable in those conditions. Excluding telaprevir-R may be justifiable in some cases because the clinical relevance of telaprevir-R is relatively small compared with telaprevir (one-thirtieth the antiviral activity).

The assay was successfully applied to 150 clinical samples, showing excellent comparability to published literature values.
Telaprevir LC/MS/MS method

Also it was found that the ratio of telaprevir/telaprevir-R increased from the first dose to steady state where the ratio reached a level of approximately 52, respectively. Thus, the current methodology is suitable for studies of telaprevir, telaprevir-R and the corresponding ratio.

In summary, a new analytical method was developed and validated that enables rapid and sensitive analysis of telaprevir and telaprevir-R. This method was used to show that nonaspirin plasma was suitable for telaprevir determinations, enabling new research investigations for telaprevir in the future.

Acknowledgments

We wish to thank Vortech Pharmaceuticals Inc. for providing the reference standards and internal standard, the subjects who participated in the clinical studies and the University of Colorado Clinical Translational Research Center core laboratory and nursing staff who assisted with sample processing and the clinical protocols, respectively. Funding source was DKO82621 (Kisar). The authors declare no conflict of interest.

References


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APPENDIX B

Complete SAS Code
DATA endo;
    INFILE 'D:\Desktop\2dEndo SAS data sheet.csv' DSD FIRSTOBS=2;
    INPUT Id Subject Time HIV_Status TFV FTC dCTP dGTP TTP dATP HLADR_CD4 HLADR_CD8 Race Age Sex eGFR Weight BMI;
    RETAIN LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine Pool LnPurine LnPyrimidine LnPool;
    Purine=SUM(dGTP,dATP);
    Pyrimidine=SUM(TTP,dCTP);
    Pool=SUM(dGTP,dATP,TTP,dCTP);
    LnPool=LOG(Pool);
    IF TFV=0 THEN LnTFV=0;
    ELSE LnTFV=LOG(TFV);
    IF FTC=0 THEN LnFTC=0;
    ELSE LnFTC=LOG(FTC);
    LndCTP=LOG(dCTP);
    LndGTP=LOG(dGTP);
    LntTP=LOG(TTP);
    LndATP=LOG(dATP);
    LnPurine=LOG(Purine);
    LnPyrimidine=LOG(Pyrimidine);
    RUN;

*Print Data;
PROC PRINT DATA=endo;
RUN;

*Examine means of dNTPs by different time points;
PROC MEANS DATA=endo;
CLASS Time;
VAR dCTP dGTP TTP dATP LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine LnPurine LnPyrimidine HLADR_CD4 HLADR_CD8;
OUTPUT OUT=endomean MEAN (dCTP dGTP TTP dATP LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine LnPurine LnPyrimidine HLADR_CD4 HLADR_CD8) = MEANdCTP MEANdGTP MEANtTP MEANdATP MEANLnTFV MEANLnFTC MEANLndCTP MEANLndGTP MEANLnTTP MEANLndATP MEANPurine MEANPyrimidine MEANLnPurine MEANLnPyrimidine MEANHLADR_CD4 MEANHLADR_CD8;
RUN;

*Summary table of means;
PROC PRINT DATA=endomean;
RUN;

*Plot the change of means;
PROC SGPLOT DATA=endomean;
    SERIES X=TIME Y=MEANdCTP / MARKERS;
    TITLE 'dCTP change';
RUN;
PROC SGPLOT DATA=endomean;
    SERIES X=TIME Y=MEANdGTP / MARKERS;
    TITLE 'dGTP change';
RUN;
PROC SGPLOT DATA=endomean;
    SERIES X=TIME Y=MEANtTP / MARKERS;
    TITLE 'TTP change';
RUN;
PROC SGPLOT DATA=endomean;
    SERIES X=TIME Y=MEANdATP / MARKERS;

TITLE 'dATP change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANPurine / MARKERS;
   TITLE 'Purine change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANPyrimidine / MARKERS;
   TITLE 'Pyrimidine change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANLndCTP / MARKERS;
   TITLE 'LndCTP change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANLnTTP / MARKERS;
   TITLE 'LnTTP change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANLndATP / MARKERS;
   TITLE 'LndATP change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANLndGTP / MARKERS;
   TITLE 'LndGTP change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANLnPurine / MARKERS;
   TITLE 'LnPurine change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANLnPyrimidine / MARKERS;
   TITLE 'LnPyrimidine change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANHLADR_CD4 / MARKERS;
   TITLE 'HLADR_CD4 change';
RUN;
PROC SGPLOT DATA=endomean;
   SERIES X=TIME Y=MEANHLADR_CD8 / MARKERS;
   TITLE 'HLADR_CD8 change';
RUN;

*Examine the Distribution of Data;
PROC UNIVARIATE DATA=endo;
   VAR dCTP dGTP TTP dATP LnTFV LnFTC LndCTP LndGTP LnTTP LnATP Purine Pyrimidine LnPurine LnPyrimidine;
   CLASS Time;
RUN;
PROC MEANS DATA=endo Kurtosis Skewness;
   VAR Pool dCTP dGTP TTP dATP Purine Pyrimidine LnPool LnTFV LnFTC LndCTP LndGTP LnTTP LnATP LnPurine LnPyrimidine;
   CLASS Time;
RUN;

*Regression Analysis of Primary HIV Status;
PROC REG DATA=endo;
   WHERE Time=0;
   MODEL Age=HIV_Status;
   MODEL Sex=HIV_Status;
   MODEL Weight=HIV_Status;
   MODEL BMI=HIV_Status;
   MODEL Race=HIV_Status;
   TITLE Variables Tests;
RUN;
PROC REG DATA=endo;
   WHERE Time=0;
   MODEL Age=Sex;
   MODEL Age=Weight;
   MODEL Age=BMI;
   MODEL Sex=BMI; *Significant!!;
   MODEL Sex=Weight;
   MODEL BMI=Weight; *Significant!!;
   MODEL Race=Age;
   MODEL Race=Sex;
   MODEL Race=Weight;
   MODEL Race=BMI;
   TITLE Variables Tests;
RUN;

*Look at continuous variables using mixed model;
PROC MIXED DATA=endo;
   CLASS HIV_Status;
   MODEL HLADR_CD4=HIV_Status / S;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject;
RUN;
PROC MIXED DATA=endo;
   CLASS HIV_Status;
   MODEL HLADR_CD8=HIV_Status / S;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject;
RUN;
PROC MIXED DATA=endo;
   MODEL HLADR_CD8=HLADR_CD4 / S;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject;
RUN;

*Baseline Regression Analysis on endo;
*Compare baseline with HLADR+ Status;*no significant found!;
PROC REG DATA=endo;
   WHERE Time=0;
   *Pool;
   MODEL Pool=HLADR_CD4;
   MODEL Pool=HLADR_CD8;
   *Purine;
   MODEL Purine=HLADR_CD4;
   MODEL Purine=HLADR_CD8;
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   MODEL Pyrimidine=HLADR_CD8;
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MODEL dGTP=HLADR_CD8;
*TTP;
MODEL TTP=HLADR_CD4;
MODEL TTP=HLADR_CD8;
*dATP;
MODEL dATP=HLADR_CD4;
MODEL dATP=HLADR_CD8;
TITLE Baseline Regression Analysis on endo;
RUN;

*Compare baseline values with age, weight;*No significant effects!;
PROC REG DATA=endo;
  WHERE Time=0;
  *Pool;
  MODEL Pool=age;
  MODEL Pool=weight;
  *Purine;
  MODEL Purine=age;
  MODEL Purine=weight;
  *Pyrimidine;
  MODEL Pyrimidine=age;
  MODEL Pyrimidine=weight;
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  MODEL dGTP=age;
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  *dATP;
  MODEL dATP=age;
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  TITLE Baseline Regression Analysis on endo;
RUN;

*Compare baseline values with BMI, eGFR;*No significant effects!;
PROC REG DATA=endo;
  WHERE Time=0;
  *Pool;
  MODEL Pool=BMI;
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  MODEL Purine=eGFR;
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MODEL TTP=eGFR;  
*dATP;  
MODEL dATP=BMI;  
MODEL dATP=eGFR;  
TITLE Baseline Regression Analysis on endo;  
RUN;  

*Compare endo with HLADR+ Status;*Day 1 dATP vs HLADR_CD4 significant!;  
PROC REG DATA=endo;  
   WHERE Time=1;  
*Pool;  
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*dATP;  
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   MODEL dATP=HLADR_CD8;  
TITLE Baseline Regression Analysis on endo;  
RUN;  

*Compare endo with HLADR+ Status;*Day 3 dATP vs HLADR_CD8 significant!;  
PROC REG DATA=endo;  
   WHERE Time=3;  
*Pool;  
   MODEL Pool=HLADR_CD4;  
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   MODEL Purine=HLADR_CD8;  
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   MODEL dCTP=HLADR_CD8;  
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   MODEL dGTP=HLADR_CD8;  
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MODEL TTP=HLADR_CD4;
MODEL TTP=HLADR_CD8;
*dATP;
MODEL dATP=HLADR_CD4;
MODEL dATP=HLADR_CD8;
TITLE Baseline Regression Analysis on endo;
RUN;

*Compare endo with HLADR+ Status;*Day 7 dATP vs HLADR_CD4/8 significant!;
PROC REG DATA=endo;
  WHERE Time=7;
  *Pool;
  MODEL Pool=HLADR_CD4;
  MODEL Pool=HLADR_CD8;
  *Purine;
  MODEL Purine=HLADR_CD4;
  MODEL Purine=HLADR_CD8;
  *Pyrimidine;
  MODEL Pyrimidine=HLADR_CD4;
  MODEL Pyrimidine=HLADR_CD8;
  *dCTP;
  MODEL dCTP=HLADR_CD4;
  MODEL dCTP=HLADR_CD8;
  *dGTP;
  MODEL dGTP=HLADR_CD4;
  MODEL dGTP=HLADR_CD8;
  *TTP;
  MODEL TTP=HLADR_CD4;
  MODEL TTP=HLADR_CD8;
  *dATP;
  MODEL dATP=HLADR_CD4;
  MODEL dATP=HLADR_CD8;
TITLE Baseline Regression Analysis on endo;
RUN;

*Compare endo with HLADR+ Status;*Day 20 dATP/TTP vs HLADR_CD4 significant!;
PROC REG DATA=endo;
  WHERE Time=20;
  *Pool;
  MODEL Pool=HLADR_CD4;
  MODEL Pool=HLADR_CD8;
  *Purine;
  MODEL Purine=HLADR_CD4;
  MODEL Purine=HLADR_CD8;
  *Pyrimidine;
  MODEL Pyrimidine=HLADR_CD4;
  MODEL Pyrimidine=HLADR_CD8;
  *dCTP;
  MODEL dCTP=HLADR_CD4;
  MODEL dCTP=HLADR_CD8;
  *dGTP;
  MODEL dGTP=HLADR_CD4;
  MODEL dGTP=HLADR_CD8;
  *TTP;
  MODEL TTP=HLADR_CD4;
  MODEL TTP=HLADR_CD8;
MODEL TTP=HLADR_CD8;
*dATP;
MODEL dATP=HLADR_CD4;
MODEL dATP=HLADR_CD8;
TITLE Baseline Regression Analysis on endo;
RUN;

*Compare endo with HLADR+ Status;*Day 30 dATP vs HLADR_CD4/8 and Purine vs HLADR_CD4 significant!;
PROC REG DATA=endo;
    WHERE Time=30;
    *Pool;
    MODEL Pool=HLADR_CD4;
    MODEL Pool=HLADR_CD8;
    *Purine;
    MODEL Purine=HLADR_CD4;
    MODEL Purine=HLADR_CD8;
    *Pyrimidine;
    MODEL Pyrimidine=HLADR_CD4;
    MODEL Pyrimidine=HLADR_CD8;
    *dCTP;
    MODEL dCTP=HLADR_CD4;
    MODEL dCTP=HLADR_CD8;
    *dGTP;
    MODEL dGTP=HLADR_CD4;
    MODEL dGTP=HLADR_CD8;
    *TTP;
    MODEL TTP=HLADR_CD4;
    MODEL TTP=HLADR_CD8;
    *dATP;
    MODEL dATP=HLADR_CD4;
    MODEL dATP=HLADR_CD8;
    TITLE Baseline Regression Analysis on endo;
RUN;

*Compare endo with HLADR+ Status;*Day 30 dATP vs HLADR_CD4/8 and Purine vs HLADR_CD4 significant!;
PROC REG DATA=endo;
    *Pool;
    MODEL Pool=HLADR_CD4;
    MODEL Pool=HLADR_CD8;
    *Purine;
    MODEL Purine=HLADR_CD4;
    MODEL Purine=HLADR_CD8;
    *Pyrimidine;
    MODEL Pyrimidine=HLADR_CD4;
    MODEL Pyrimidine=HLADR_CD8;
    *dCTP;
    MODEL dCTP=HLADR_CD4;
    MODEL dCTP=HLADR_CD8;
    *dGTP;
    MODEL dGTP=HLADR_CD4;
    MODEL dGTP=HLADR_CD8;
    *TTP;
    MODEL TTP=HLADR_CD4;
    MODEL TTP=HLADR_CD8;
    *dATP;
    MODEL dATP=HLADR_CD4;
    MODEL dATP=HLADR_CD8;

MODEL dATP=HLADR_CD4;
MODEL dATP=HLADR_CD8;
TITLE Baseline Regression Analysis on endo;

RUN;

*Compare endo values with age, weight;*weight vs TTP/dCTP/Pyrimidine/Pool Significant!;
PROC REG DATA=endo;
   *Pool;
      MODEL Pool=age;
      MODEL Pool=weight;
   *Purine;
      MODEL Purine=age;
      MODEL Purine=weight;
   *Pyrimidine;
      MODEL Pyrimidine=age;
      MODEL Pyrimidine=weight;
   *dCTP;
      MODEL dCTP=age;
      MODEL dCTP=weight;
   *dGTP;
      MODEL dGTP=age;
      MODEL dGTP=weight;
   *TTP;
      MODEL TTP=age;
      MODEL TTP=weight;
   *dATP;
      MODEL dATP=age;
      MODEL dATP=weight;
   TITLE Baseline Regression Analysis on endo;
RUN;

*Compare baseline values with BMI, eGFR;*No significant effects!;
PROC REG DATA=endo;
   WHERE Time=0;
   *Pool;
      MODEL Pool=BMI;
      MODEL Pool=eGFR;
   *Purine;
      MODEL Purine=BMI;
      MODEL Purine=eGFR;
   *Pyrimidine;
      MODEL Pyrimidine=BMI;
      MODEL Pyrimidine=eGFR;
   *dCTP;
      MODEL dCTP=BMI;
      MODEL dCTP=eGFR;
   *dGTP;
      MODEL dGTP=BMI;
      MODEL dGTP=eGFR;
   *TTP;
      MODEL TTP=BMI;
      MODEL TTP=eGFR;
   *dATP;
      MODEL dATP=BMI;
      MODEL dATP=eGFR;
   TITLE Baseline Regression Analysis on endo;
RUN;

*Summary of significant relationships at all time points: dATP vs HLADR and Pyrimidine vs Weight.;
PROC REG DATA=endo;
   MODEL dATP=HLADR_CD4;
   MODEL dATP=HLADR_CD8;
   MODEL TTP=Weight;
   Model dCTP=Weight;
RUN;

PROC REG DATA=endo;
   MODEL LndATP=HLADR_CD4;
   MODEL LndATP=HLADR_CD8;
   MODEL LnTTP=Weight;
   Model LndCTP=Weight;
RUN;

*controled with HIV_Status;
DATA endo;
   INFILE 'D:\Desktop\2dEndo SAS data sheet.csv' DSD FIRSTOBS=2;
   INPUT Id Subject Time HIV_Status TFV FTC dCTP dGTP TTP dATP HLADR_CD4 HLADR_CD8 Race Age Sex eGFR Weight BMI;
   RETAIN LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine Pool LnPurine LnPyrimidine LnPool;
   Purine=SUM(dGTP,dATP);
   Pyrimidine=SUM(TTP,dCTP);
   Pool=SUM(dGTP,dATP,TTP,dCTP);
   LnPool=LOG(Pool);
   IF TFV=0 THEN LnTFV=0;
      ELSE LnTFV=LOG(TFV);
   IF FTC=0 THEN LnFTC=0;
      ELSE LnFTC=LOG(FTC);
   LndCTP=LOG(dCTP);
   LndGTP=LOG(dGTP);
   LnTTP=LOG(TTP);
   LndATP=LOG(dATP);
   LnPurine=LOG(Purine);
   LnPyrimidine=LOG(Pyrimidine);
   *Define the interaction terms;
   HIVCD4=HLADR_CD4*HIV_Status;
   HIVCD8=HLADR_CD8*HIV_Status;
   HIVWeight=Weight*HIV_Status;
RUN;

PROC REG DATA=endo;
   MODEL LndATP=HLADR_CD4 HIV_Status HIVCD4 ;
   MODEL LndATP=HLADR_CD8 HIV_Status HIVCD8;
   MODEL LnTTP=Weight HIV_Status HIVWeight;
   Model LndCTP=Weight HIV_Status HIVWeight;
RUN;

*Only HIV status is affecting dATP level;
PROC REG DATA=endo;
   MODEL LndATP=HIV_Status;
   MODEL LndGTP=HIV_Status;
   MODEL LnTTP=HIV_Status;
   Model LndCTP=HIV_Status;
RUN;
*Mixed Model Analysis on HLADR vs dATP and Weight vs Pyrimidine.*

PROC MIXED DATA=endo COVTEST;
    CLASS Subject;
    MODEL LndATP=HLADR_CD4 HIV_Status / CHISQ S;
    RANDOM INTERCEPT / SUBJECT=subject;
RUN;

PROC MIXED DATA=endo COVTEST;
    CLASS Subject;
    MODEL LndATP=HLADR_CD8 HIV_Status / CHISQ S;
    RANDOM INTERCEPT / SUBJECT=subject;
RUN;

PROC MIXED DATA=endo COVTEST;
    CLASS Subject;
    MODEL LndCTP=Weight / CHISQ S;
    RANDOM INTERCEPT / SUBJECT=subject;
RUN;

PROC MIXED DATA=endo COVTEST;
    CLASS Subject;
    MODEL LnTTP=Weight / CHISQ S;
    RANDOM INTERCEPT / SUBJECT=subject;
RUN;

*Weight lost its significant effect after mixed model analysis;* *Analysis base on time;*

PROC REG DATA=endo;
    WHERE Time=0;
    MODEL LnTTP=Weight;
    Model LndCTP=Weight;
RUN;

PROC REG DATA=endo;
    WHERE Time=1;
    MODEL LnTTP=Weight;
    Model LndCTP=Weight;
RUN;

PROC REG DATA=endo;
    WHERE Time=3;
    MODEL LnTTP=Weight;
    Model LndCTP=Weight;
RUN;

PROC REG DATA=endo;
    WHERE Time=7;
    MODEL LnTTP=Weight;
    Model LndCTP=Weight;
RUN;

PROC REG DATA=endo;
    WHERE Time=20;
    MODEL LnTTP=Weight;
    Model LndCTP=Weight;
RUN;

PROC REG DATA=endo;
WHERE Time=30;
MODEL LnTTP=Weight;
Model LndCTP=Weight;
RUN;

*only at day 1 there is significant effect of pyrimidine and weight; *try multilevel analysis using mixed effect model;
PROC MIXED DATA=endo;
   CLASS Subject;
   MODEL LndCTP=Weight / SOLUTION;
   RANDOM INTERCEPT / SUBJECT=subject;
RUN;

*Still not significant;

*Below is the linear regression by time analysis;

DATA endo;
   INFILE 'D:\Desktop\2dEndo SAS data sheet.csv' DSD FIRSTOBS=2;
   INPUT Id Subject Time HIV_Status TFV FTC dCTP dGTP TTP dATP HLADR_CD4 HLADR_CD8 Race Age Sex eGFR Weight BMI;
   RETAIN LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine Pool LnPurine LnPyrimidine LnPool;
   Purine=SUM(dGTP,dATP);
   Pyrimidine=SUM(TTP,dCTP);
   Pool=SUM(dGTP,dATP,TTP,dCTP);
   LnPool=LOG(Pool);
   IF TFV=0 THEN LnTFV=0;
      ELSE LnTFV=LOG(TFV);
   IF FTC=0 THEN LnFTC=0;
      ELSE LnFTC=LOG(FTC);
   LndCTP=LOG(dCTP);
   LndGTP=LOG(dGTP);
   LnPurine=LOG(Purine);
   LnPyrimidine=LOG(Pyrimidine);
   HIVCD4=HLADR_CD4*HIV_Status;
   HIVCD8=HLADR_CD8*HIV_Status;
   HIVWeight=Weight*HIV_Status;
*Define the position of knot;
   knot1=MAX(time-1,0);
   knot2=MAX(time-2,0);
   knot3=MAX(time-3,0);
   knot4=MAX(time-4,0);
   knot5=MAX(time-5,0);
   knot6=MAX(time-6,0);
   knot7=MAX(time-7,0);
*Specified knot between certain time points;
   knot2750=MAX(time-2.750,0);
   knot1525=MAX(time-1.525,0);
   knot1550=MAX(time-1.550,0);
   knot1450=MAX(time-1.450,0);
   knot1575=MAX(time-1.575,0);
   knot1350=MAX(time-1.350,0);
   knot19=MAX(time-1.9,0);
   knot18=MAX(time-1.8,0);
knot17=MAX(time-1.7,0);
knot16=MAX(time-1.6,0);
knot15=MAX(time-1.5,0);
knot14=MAX(time-1.4,0);
knot13=MAX(time-1.3,0);
knot12=MAX(time-1.2,0);
knot11=MAX(time-1.1,0);
knot29=MAX(time-2.9,0);
knot28=MAX(time-2.8,0);
knot27=MAX(time-2.7,0);
knot26=MAX(time-2.6,0);
knot25=MAX(time-2.5,0);
knot24=MAX(time-2.4,0);
knot23=MAX(time-2.3,0);
knot22=MAX(time-2.2,0);
knot21=MAX(time-2.1,0);
knot31=MAX(time-3.1,0);
knot32=MAX(time-3.2,0);
knot33=MAX(time-3.3,0);
knot34=MAX(time-3.4,0);
knot35=MAX(time-3.5,0);
knot36=MAX(time-3.6,0);
knot37=MAX(time-3.7,0);
knot38=MAX(time-3.8,0);
knot39=MAX(time-3.9,0);
*defining categorical variable of Time;
t=Time;
RUN;

*Searching for variables; *knots are 1.55(dCTP) 1.4(TTP) 1.35(dGTP) and 2.9(dATP) based on AIC;
*For dCTP regression:
PROC MIXED DATA=endo;
   CLASS t;
   MODEL LndCTP=Time knot1550 / S CHISQ;
   RANDOM INTERCEPT Time knot1550 / Type=UN SUBJECT=Subject G V VCORR;
RUN;

**************************************************************************
*effect of weight: Significant!!!!;
PROC MIXED DATA=endo;
   CLASS t;
   MODEL LndCTP=Weight Time knot1550 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR S;
RUN;
PROC MIXED DATA=endo; *significant effect after adding weight slope interaction;
   CLASS t;
   MODEL LndCTP=Weight Time*weight Time knot1550 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;
PROC MIXED DATA=endo; *significant effect after adding weight slope interaction;
   CLASS t;
MODEL LndCTP = Weight Time * weight Time knot1550 / S CHISQ;
RANDOM INTERCEPT / Type = UN SUBJECT = Subject G V VCORR;
RUN;

*effect of sex: not Significant;
PROC MIXED DATA = endo;
   CLASS t Sex;
   MODEL LndCTP = Sex Time knot1550 / S CHISQ;
   RANDOM INTERCEPT / Type = UN SUBJECT = Subject G V VCORR;
RUN;

PROC MIXED DATA = endo;  *no significant effect on slope;
   CLASS t HIV_Status;
   MODEL LndCTP = HIV_Status Time knot1550 / S CHISQ;
   RANDOM INTERCEPT / Type = UN SUBJECT = Subject G V VCORR;
RUN;

*For TTP regression:;
PROC MIXED DATA = endo;
   CLASS t (ref = '0');
   MODEL LnTTP = Time knot14 / S CHISQ;
   RANDOM INTERCEPT Time knot14 / Type = UN SUBJECT = Subject G V VCORR;
RUN;

*effect of weight: not significant:();
PROC MIXED DATA = endo;
   CLASS t;
   MODEL LnTTP = Weight Weight * Time Time knot14 / S CHISQ;
   RANDOM INTERCEPT / Type = UN SUBJECT = Subject G V VCORR;
RUN;

*effect of HIV: not significant:();
PROC MIXED DATA = endo;
   CLASS t HIV_Status;
   MODEL LnTTP = HIV_Status Time knot14 / S CHISQ;
   RANDOM INTERCEPT / Type = UN SUBJECT = Subject G V VCORR;
RUN;

PROC MIXED DATA = endo;
   CLASS t HIV_Status;
   MODEL LnTTP = HIV_Status * Time HIV_Status Time knot14 / S CHISQ;
   RANDOM INTERCEPT / Type = UN SUBJECT = Subject G V VCORR;
RUN;

*For dGTP regression:;
PROC MIXED DATA = endo;
   CLASS t;
   MODEL LndGTP = Time knot1350 / S CHISQ;
   RANDOM INTERCEPT Time knot1350 / Type = UN SUBJECT = Subject G V VCORR;
RUN;

*HIV effects? not significant;
PROC MIXED DATA=endo;
   CLASS t HIV_Status;
   MODEL LndGTP=HIV_Status Time knot1350 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;
PROC MIXED DATA=endo;
   CLASS t HIV_Status;
   MODEL LndGTP=HIV_Status*Time HIV_Status Time knot1350 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;
PROC MIXED DATA=endo;
   CLASS t HIV_Status;
   MODEL LndGTP=HIV_Status Time knot1350 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;

*For dATP regression:;
PROC MIXED DATA=endo;
   CLASS t;
   MODEL LndATP=Time knot29 / S CHISQ;
   RANDOM INTERCEPT Time knot29 / Type=UN SUBJECT=Subject G V VCORR;
RUN;

*effect of HIV on intercept: not significant:((p=0.07);
PROC MIXED DATA=endo;
   CLASS t HIV_Status;
   MODEL LndATP=HIV_Status Time knot29 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;
PROC MIXED DATA=endo;
   CLASS t HIV_Status;
   MODEL LndATP=HIV_Status HIV_Status*Time Time knot29 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;
PROC MIXED DATA=endo;
   CLASS t HIV_Status;
   MODEL LndATP=Time knot29 HIV_Status*knot29 / S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;

*Stratified by HIV_Status;*Found significant on HIV positive subject only!!!;
PROC MIXED DATA=endo;
   MODEL LndATP=HLADR_CD4 HLADR_CD4*HIV_Status/ S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;
PROC MIXED DATA=endo;
   WHERE HIV_Status=1;
   MODEL LndATP=HLADR_CD4/ S CHISQ;
   RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;

PROC MIXED DATA=endo;
    WHERE HIV_Status=0;
    MODEL LndGTP=HLADR_CD4/ S CHISQ;
    RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;

*Plot the difference between HIV+ and HIV-;
PROC MEANS DATA=endo;
    CLASS Time HIV_Status;
    VAR dCTP dGTP TTP dATP LnTFV LnFTC LndCTP LndGTP LnTTP LnATP Purine Pyrimidine LnPurine LnPyrimidine HLADR_CD4 HLADR_CD8;
    OUTPUT OUT=endomean MEAN (dCTP dGTP TTP dATP LnTFV LnFTC LndCTP LndGTP LnTTP LnATP Purine Pyrimidine LnPurine LnPyrimidine HLADR_CD4 HLADR_CD8) = MEANdCTP MEANdGTP MEANtTP MEANdATP MEANLnTFV MEANLnFTC MEANLndCTP MEANLndGTP MEANLnTTP MEANLndATP MEANPurine MEANPyrimidine MEANLnPurine MEANLnPyrimidine MEANHLADR_CD4 MEANHLADR_CD8;
RUN;

PROC SGPANEL DATA=endomean;
    PANELBY HIV_Status;
    SERIES X=Time Y=MEANLndATP;
    TITLE 'LndATP change HIV- vs HIV+';
RUN;
PROC SGPANEL DATA=endomean;
    PANELBY HIV_Status;
    SERIES X=Time Y=MEANHLADR_CD4;
    TITLE 'HLADR_CD4 change HIV- vs HIV+';
RUN;
PROC SGPANEL DATA=endomean;
    PANELBY HIV_Status;
    SERIES X=Time Y=MEANHLADR_CD8;
    TITLE 'HLADR_CD8 change HIV- vs HIV+';
RUN;
PROC SGPANEL DATA=endomean;
    PANELBY HIV_Status;
    SERIES X=Time Y=MEANLndGTP;
    TITLE 'LndGTP change HIV- vs HIV+';
RUN;
PROC SGPANEL DATA=endomean;
    PANELBY HIV_Status;
    SERIES X=Time Y=MEANLnTTP;
    TITLE 'LnTTP change HIV- vs HIV+';
RUN;
PROC SGPANEL DATA=endomean;
    PANELBY HIV_Status;
    SERIES X=Time Y=MEANLndCTP;
    TITLE 'LndCTP change HIV- vs HIV+';
RUN;
PROC SGPLOT DATA=endomean;
    WHERE HIV_Status=0;
    SERIES X=Time Y=MEANLndATP;
    TITLE 'LndATP in HIV-negative subjects';
RUN;
**PROC SGPLOT** DATA=endomean;
WHERE HIV_Status=1;
SERIES X=Time Y=MEANLndATP;
TITLE 'LndATP in HIV-Positive subjects';
RUN;

**PROC SGPLOT** DATA=endomean;
WHERE HIV_Status=0;
SERIES X=Time Y=MEANHLADR_CD4;
TITLE 'HLADR_CD4 in HIV-negative subjects';
RUN;

**PROC SGPLOT** DATA=endomean;
WHERE HIV_Status=1;
SERIES X=Time Y=MEANHLADR_CD4;
TITLE 'HLADR_CD4 in HIV-Positive subjects';
RUN;

**PROC SGPLOT** DATA=endomean;
WHERE HIV_Status=0;
SERIES X=Time Y=MEANHLADR_CD8;
TITLE 'HLADR_CD8 in HIV-negative subjects';
RUN;

**PROC SGPLOT** DATA=endomean;
WHERE HIV_Status=1;
SERIES X=Time Y=MEANHLADR_CD8;
TITLE 'HLADR_CD8 in HIV-Positive subjects';
RUN;

*If increase the magnitude of HLADR_CD4 in HIV_Negative;*

**DATA** endo;
INFILE 'D:\Desktop\2dEndo SAS data sheet.csv' DSD FIRSTOBS=2;
INPUT Id Subject Time HIV_Status TFV FTC dCTP dGTP TTP dATP HLADR_CD4 HLADR_CD8 Race Age Sex eGFR Weight BMI;
RETAIN LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine Pool LnPurine LnPyrimidine LnPool;
Purine=SUM(dGTP,dATP);
Pyrimidine=SUM(TTP,dCTP);
Pool=SUM(dGTP,dATP,TTP,dCTP);
LnPool=LOG(Pool);
IF TFV=0 THEN LnTFV=0;
ELSE LnTFV=LOG(TFV);
IF FTC=0 THEN LnFTC=0;
ELSE LnFTC=LOG(FTC);
LndCTP=LOG(dCTP);
LndGTP=LOG(dGTP);
LnTTP=LOG(TTP);
LndATP=LOG(dATP);
LnPurine=LOG(Purine);
LnPyrimidine=LOG(Pyrimidine);
HLADR_CD410=10*HLADR_CD4;
HLADR_CD810=10*HLADR_CD8;
t=Time;
RUN;

**PROC MIXED** DATA=endo;
WHERE Time GT 0;
MODEL LndATP=HLADR_CD4 HIV_Status HLADR_CD4*HIV_Status/ S CHISQ;
RANDOM INTERCEPT / Type=UN SUBJECT=Subject G V VCORR;
RUN;

*Linear Regression by HIV_Status;
DATA endo;
  INFILE 'D:\Desktop\2dEndo SAS data sheet.csv' DSD FIRSTOBS=2;
  INPUT Id Subject Time HIV_Status TFV FTC dCTP dGTP TTP dATP HLADR_CD4 HLADR_CD8 Race Age Sex eGFR Weight BMI;
  RETAIN LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine Pool LnPurine LnPyrimidine LnPool;
  Purine=SUM(dGTP,dATP);
  Pyrimidine=SUM(TTP,dCTP);
  Pool=SUM(dGTP,dATP,TTP,dCTP);
  LnPool=LOG(Pool);
  IF TFV=0 THEN LnTFV=0;
   ELSE LnTFV=LOG(TFV);
  IF FTC=0 THEN LnFTC=0;
   ELSE LnFTC=LOG(FTC);
  LndCTP=LOG(dCTP);
  LndGTP=LOG(dGTP);
  LnTTP=LOG(TTP);
  LndATP=LOG(dATP);
  LnPurine=LOG(Purine);
  LnPyrimidine=LOG(Pyrimidine);
  HIVCD4=HLADR_CD4*HIV_Status;
  HIVCD8=HLADR_CD8*HIV_Status;
  HIVWeight=Weight*HIV_Status;

*Define the position of knot;
  knot1=MAX(time-1,0);
  knot2=MAX(time-2,0);
  knot3=MAX(time-3,0);
  knot4=MAX(time-4,0);
  knot5=MAX(time-5,0);
  knot6=MAX(time-6,0);
  knot7=MAX(time-7,0);

*Specified knot between certain time points;
  knot2750=MAX(time-2.750,0);
  knot1525=MAX(time-1.525,0);
  knot1550=MAX(time-1.550,0);
  knot1450=MAX(time-1.450,0);
  knot1575=MAX(time-1.575,0);
  knot1350=MAX(time-1.350,0);
  knot19=MAX(time-1.9,0);
  knot18=MAX(time-1.8,0);
  knot17=MAX(time-1.7,0);
  knot16=MAX(time-1.6,0);
  knot15=MAX(time-1.5,0);
  knot14=MAX(time-1.4,0);
  knot13=MAX(time-1.3,0);
  knot12=MAX(time-1.2,0);
  knot11=MAX(time-1.1,0);
  knot29=MAX(time-2.9,0);
  knot28=MAX(time-2.8,0);
  knot27=MAX(time-2.7,0);
knot26=MAX(time-2.6,0);
knot25=MAX(time-2.5,0);
knot24=MAX(time-2.4,0);
knot23=MAX(time-2.3,0);
knot22=MAX(time-2.2,0);
knot21=MAX(time-2.1,0);
knot31=MAX(time-3.1,0);
knot32=MAX(time-3.2,0);
knot33=MAX(time-3.3,0);
knot34=MAX(time-3.4,0);
knot35=MAX(time-3.5,0);
knot36=MAX(time-3.6,0);
knot37=MAX(time-3.7,0);
knot38=MAX(time-3.8,0);
knot39=MAX(time-3.9,0);

*defining categorical variable of Time;
t=Time;
RUN;

DATA endo;
  INFILE 'D:\Desktop\2dEndo SAS data sheet.csv' DSD FIRSTOBS=2;
  INPUT Id Subject Time HIV_Status TFV FTC dCTP dGTP TTP dATP HLADR_CD4 HLADR_CD8 Race Age Sex eGFR Weight BMI;
  RETAIN LnTFV LnFTC LndCTP LndGTP LnTTP LndATP Purine Pyrimidine Pool LnPurine LnPyrimidine LnPool;
  Purine=SUM(dGTP,dATP);
  Pyrimidine=SUM(TTP,dCTP);
  Pool=SUM(dGTP,dATP,TTP,dCTP);
  LnPool=LOG(Pool);
  IF TFV=0 THEN LnTFV=0;
      ELSE LnTFV=LOG(TFV);
  IF FTC=0 THEN LnFTC=0;
      ELSE LnFTC=LOG(FTC);
  LndCTP=LOG(dCTP);
  LndGTP=LOG(dGTP);
  LnTTP=LOG(TTP);
  LndATP=LOG(dATP);
  LnPurine=LOG(Purine);
  LnPyrimidine=LOG(Pyrimidine);
  HIVCD4=HLADR_CD4*HIV_Status;
  HIVCD8=HLADR_CD8*HIV_Status;
  HIVWeight=Weight*HIV_Status;

  *Transform HLADR values;
  IF HLADR_CD4=0 THEN LnHLADR_CD4=-0.693;
      ELSE LnHLADR_CD4=LOG(HLADR_CD4);
  IF HLADR_CD8=0 THEN LnHLADR_CD8=-0.693;
      ELSE LnHLADR_CD8=LOG(HLADR_CD8);

  *Define the position of knot;
knot1=MAX(time-1,0);
knot1_35=MAX(time-1.35,0);
knot1_4=MAX(time-1.4,0);
knot1_55=MAX(time-1.55,0);
knot2_9=MAX(time-2.9,0);
knot2=MAX(time-2,0);
knot3=MAX(time\(-3, 0\));
knot4=MAX(time\(-4, 0\));
knot5=MAX(time\(-5, 0\));
knot6=MAX(time\(-6, 0\));
knot4_5=MAX(time\(-4.5, 0\));
knot7=MAX(time\(-7, 0\));
knot8=MAX(time\(-8, 0\));
knot9=MAX(time\(-9, 0\));
knot10=MAX(time\(-10, 0\));
knot11=MAX(time\(-11, 0\));
knot12=MAX(time\(-12, 0\));
knot13=MAX(time\(-13, 0\));
knot14=MAX(time\(-14, 0\));
knot15=MAX(time\(-15, 0\));
knot16=MAX(time\(-16, 0\));
knot17=MAX(time\(-17, 0\));
knot18=MAX(time\(-18, 0\));
knot19=MAX(time\(-19, 0\));
knot20=MAX(time\(-20, 0\));
knot21=MAX(time\(-21, 0\));
knot22=MAX(time\(-22, 0\));
knot23=MAX(time\(-23, 0\));
knot24=MAX(time\(-24, 0\));
knot25=MAX(time\(-25, 0\));
knot26=MAX(time\(-26, 0\));
knot27=MAX(time\(-27, 0\));
knot28=MAX(time\(-28, 0\));
knot29=MAX(time\(-29, 0\));
*defining categorical variable of Time;
t=Time;
RUN;
PROC PRINT DATA=endo;
  VAR Subject Time LnHLADR_CD4 LnHLADR_CD8;
RUN;
PROC MEANS DATA=endo;
  CLASS Time HIV_Status;
  VAR LnHLADR_CD4 LnHLADR_CD8;
  OUTPUT OUT=endomean MEAN (LnHLADR_CD4 LnHLADR_CD8) = MEANLnHLADR_CD4 MEANLnHLADR_CD8;
RUN;
PROC SGPANEL DATA=endomean;
  PANELBY HIV_Status;
  SERIES X=Time Y=MEANLnHLADR_CD4;
  TITLE 'LnHLADR_CD4 change HIV- vs HIV+';
RUN;
PROC SGPANEL DATA=endomean;
  PANELBY HIV_Status;
  SERIES X=Time Y=MEANLnHLADR_CD8;
  TITLE 'LnHLADR_CD8 change HIV- vs HIV+';
RUN;
*Linear regression on HLA-D;
PROC MIXED DATA=endo;
  WHERE HIV_Status=0;
  CLASS t (REF='0');
MODEL LnHLADR_CD8=Time / S CHISQ;
RANDOM INTERCEPT / SUBJECT=Subject G V VCORR;
RUN;

PROC MIXED DATA=endo;
   WHERE HIV_Status=0;
   CLASS t (REF='0');
   MODEL LnHLADR_CD4=Time knot155 / S CHISQ;
   RANDOM INTERCEPT / SUBJECT=Subject G V VCORR;
RUN;

**Normality check;
PROC UNIVARIATE DATA=endo;
   CLASS HIV_Status;
   VAR LnHLADR_CD8;
   OUTPUT OUT=normality skewness = skewness kurtosis = kurtosis ;
RUN;
PROC PRINT DATA=normality;
RUN;

PROC UNIVARIATE DATA=endo;
   CLASS HIV_Status;
   VAR LnHLADR_CD8;
   OUTPUT OUT=normality skewness = skewness kurtosis = kurtosis ;
RUN;
PROC PRINT DATA=normality;
RUN;

PROC SGPLOT DATA=endo;
   HISTOGRAM LnHLADR_CD4;
   BY HIV_Status;
   DENSITY LnHLADR_CD4;
   TITLE 'HISTOGRAM of LOG TRANSFORMED HLADR_CD4';
RUN;
PROC SGPLOT DATA=endo;
   HISTOGRAM LnHLADR_CD8;
   BY HIV_Status;
   DENSITY LnHLADR_CD8;
   TITLE 'HISTOGRAM of LOG TRANSFORMED HLADR_CD8';
RUN;

*Immune activation status modeling;*
*For HLADR_CD4 regression;
PROC MIXED DATA=endo COVTEST;
   WHERE Time^=0 and Time^=1.35 and Time^=1.4 and Time^=1.55
       and Time^=2.9 and Subject^=8345;
   *WHERE Subject^=6560 and Subject^=7567 and Subject^=9657;
   *WHERE Subject^=8345;
   CLASS id HIV_Status t;
   MODEL LnHLADR_CD4=Time knot5 HIV_Status / S CHISQ
   OUTPRED=pred1r OUTPREDM = pred1f;
   REPEATED t / TYPE=UN SUBJECT=Id;
   *RANDOM INTERCEPT / SUBJECT=Id G V VCORR;
RUN;
PROC SORT DATA=pred1f;
BY Time;
RUN;
GOPTIONS RESET=ALL VSIZE=13 HSIZE=9;
symbol1 c=blue  v=square  h=.5  i=j  w=7;
symbol2 c=red   v=triangle h=.5 i=j  w=7;
symbol3 c=blue  v=dot    h=.5       r=21;
symbol4 c=red   v=star   h=.5       r=19;
axis1 order=(-1 to 4.5 by 1) label=(a=90 'Predicted and Observed LnHLADR_CD4');
axis2 order=(0 to 30 by 10) label=(a=90 'Time (days)');

PROC GPLOT data=pred1f;
  plot pred*time=HIV_Status / vaxis=axis1;
  plot2 LnHLADR_CD4*Time = id / vaxis=axis1;
RUN;
QUIT;

*For HLADR_CD8 regression;
PROC MIXED DATA=endo COVTEST;
  WHERE Time^=0 and Subject^=6560;
  *WHERE Subject^=6560 and Subject^=7567 and Subject^=9657;
  CLASS id HIV_Status t;
  MODEL LnHLADR_CD8=Time HIV_Status / S CHISQ OUTP=pred1r OUTPM = pred1f;
  *REPEATED t / TYPE=AR(1) SUBJECT=Id;
  RANDOM INTERCEPT / SUBJECT=id G V VCORR;
RUN;
PROC SORT DATA=pred1f;
  BY Time;
RUN;
GOPTIONS RESET=ALL VSIZE=13 HSIZE=9;
symbol1 c=blue  v=square  h=.5  i=j  w=7;
symbol2 c=red   v=triangle h=.5 i=j  w=7;
symbol3 c=blue  v=dot    h=.5       r=21;
symbol4 c=red   v=star   h=.5       r=19;
axis1 order=(-1 to 4.5 by 1) label=(a=90 'Predicted and Observed LnHLADR_CD8');
axis2 order=(0 to 30 by 10) label=(a=90 'Time (days)');

PROC GPLOT data=pred1f;
  plot pred*time=HIV_Status / vaxis=axis1;
  plot2 LnHLADR_CD8*Time = id / vaxis=axis1;
RUN;
QUIT;

*endogenous dNTP modeling;

****For dCTP regression;
PROC MIXED DATA=endo COVTEST;
  CLASS id HIV_Status t;
  MODEL LndCTP=Time knot1_55/ S CHISQ OUTP=pred1r OUTPM = pred1f;
  RANDOM INTERCEPT / SUBJECT=id G V VCORR;
RUN;
PROC SORT DATA=pred1f;
  BY Time;

RUN;
GOPTIONS RESET=ALL VSIZE=13 HSIZE=9;
symbol1 c=black v=square h=.5 i=j w=7;
symbol2 c=black v=triangle h=.5 i=j w=7;
symbol3 c=blue v=dot h=.5 r=21;
symbol4 c=red v=star h=.5 r=19;
axis1 order=(5.5 to 7.5 by 1) label=(a=90 'Predicted and Observed LnCTP');
axis2 order=(0 to 30 by 10) label=(a=90 'Time (days)');
PROC GPLOT data=pred1f;
   plot pred*time=HIV_Status / vaxis=axis1;
   plot LndCTP*Time = id / vaxis=axis1;
RUN;
QUIT;

***For TTP regression;
PROC MIXED DATA=endo COVTEST;
   CLASS id HIV_Status t;
   MODEL LnTTP=Time knot1_4 / S CHISQ OUTP=pred1r OUTPM = pred1f;
   RANDOM INTERCEPT / SUBJECT=id G V VCORR;
RUN;
PROC SORT DATA=pred1f;
   BY Time;
RUN;
GOPTIONS RESET=ALL VSIZE=13 HSIZE=9;
symbol1 c=black v=square h=.5 i=j w=7;
symbol2 c=black v=triangle h=.5 i=j w=7;
symbol3 c=blue v=dot h=.5 r=21;
symbol4 c=red v=star h=.5 r=19;
axis1 order=(4 to 7.5 by 0.5) label=(a=90 'Predicted and Observed LnTTP');
axis2 order=(0 to 30 by 10) label=(a=90 'Time (days)');
PROC GPLOT data=pred1f;
   plot pred*time=HIV_Status / vaxis=axis1;
   plot LndCTP*Time = id / vaxis=axis1;
RUN;
QUIT;

***For dGTP regression;
PROC MIXED DATA=endo COVTEST;
   CLASS id HIV_Status t;
   MODEL LndGTP=Time knot1_35 / S CHISQ OUTP=pred1r OUTPM = pred1f;
   RANDOM INTERCEPT / SUBJECT=id G V VCORR;
RUN;
PROC SORT DATA=pred1f;
   BY Time;
RUN;
GOPTIONS RESET=ALL VSIZE=13 HSIZE=9;
symbol1 c=black v=square h=.5 i=j w=7;
symbol2 c=black v=triangle h=.5 i=j w=7;
symbol3 c=blue v=dot h=.5 r=21;
symbol4 c=red v=star h=.5 r=19;

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axis1 order=(4.5 to 7 by 0.5) label=(a=90 'Predicted and Observed LndGTP');
axis2 order=(0 to 30 by 10) label=(a=90 'Time (days)');

PROC GPLOT data=pred1f;
  plot pred*time=HIV_Status / vaxis=axis1;
  plot2 LndGTP*Time = id / vaxis=axis1;
RUN;
QUIT;

****For dATP regression;
****without baseline values;
PROC MIXED DATA=endo COVTEST;
  WHERE Time>0;
  CLASS id HIV_Status t;
  MODEL LndATP=Time knot2_9 HIV_Status/ S CHISQ OUTP=pred1r
            OUTPM = pred1f;
  RANDOM INTERCEPT / SUBJECT=id G V VCORR;
RUN;
PROC SORT DATA=pred1f;
  BY Time;
RUN;
GOPTIONS RESET=ALL VSIZE=13 HSIZE=9;
symbol11 c=blue  v=square   h=.5 i=j  w=7;
symbol12 c=red   v=triangle h=.5 i=j  w=7;
symbol13 c=blue   v=dot   h=.5   r=21;
symbol14 c=red  v=star   h=.5   r=19;
axis1 order=(4 to 6 by 0.5) label=(a=90 'Predicted and Observed LndATP');
axis2 order=(0 to 30 by 10) label=(a=90 'Time (days)');

PROC GPLOT data=pred1f;
  plot pred*time=HIV_Status / vaxis=axis1;
  plot2 LndATP*Time = id / vaxis=axis1;
RUN;
QUIT;

****with baseline values;
PROC MIXED DATA=endo COVTEST;
  CLASS id HIV_Status t;
  MODEL LndATP=Time knot2_9 HIV_Status/ S CHISQ OUTP=pred1r
            OUTPM = pred1f;
  RANDOM INTERCEPT / SUBJECT=id G V VCORR;
RUN;
PROC SORT DATA=pred1f;
  BY Time;
RUN;
GOPTIONS RESET=ALL VSIZE=13 HSIZE=9;
symbol11 c=blue  v=square   h=.5 i=j  w=7;
symbol12 c=red   v=triangle h=.5 i=j  w=7;
symbol13 c=blue   v=dot   h=.5   r=21;
symbol14 c=red  v=star   h=.5   r=19;
axis1 order=(4 to 6 by 0.5) label=(a=90 'Predicted and Observed LndATP');
axis2 order=(0 to 30 by 10) label=(a=90 'Time (days)');

PROC GPLOT data=pred1f;
plot pred*time=HIV_Status / vaxis=axis1;
plot2 LndATP*Time = id / vaxis=axis1;
RUN;
QUIT;

*****IPERGAY simulation analysis;
DATA Sim;
    INFILE 'c:\Users\chenxin\Desktop\IPERGAY.csv' DSD FIRSTOBS=2;
    INPUT ID TIME IND AMT PBMC ENDO RATIO;
RUN;

PROC UNIVARIATE data=Sim noprint;
    var RATIO;
    CLASS TIME;
    output out=results pctlpre=P_ pctlpts= 5 to 100 by 5;
RUN;
PROC PRINT data=results;
RUN;
APPENDIX C

Complete R Code
C.1 Data Visualization

```r
endo <- read.csv(file.choose(), header=T, na.strings = ".")

attach(endo)

# Making Basic Plots with Raw data
library(ggplot2)
endo$HIV <- as.factor(endo$HIV)

#plot dATP
ggplot(endo, aes(Time, dATP, colour=HIV, group=Subject)) +
  scale_colour_manual(values=c("dodgerblue4", "firebrick3"), name="HIV Status",
                     breaks=c("1", "0"), labels=c("Positive","Negative")) +
  geom_line(size=1) +
  theme_bw() +
  theme(text = element_text(size=15)) +
  labs(title="Change of dATP Level") +
  xlab("Time (days)") +
  ylab("dATP (fmol per million cells)")

#plot dCTP
ggplot(endo, aes(Time, dCTP, colour=HIV, group=Subject)) +
  scale_colour_manual(values=c("dodgerblue4", "firebrick3"), name="HIV Status",
                     breaks=c("1", "0"), labels=c("Positive","Negative")) +
  geom_line(size=1) +
  theme_bw() +
  theme(text = element_text(size=15)) +
  labs(title="Change of dCTP Level") +
  xlab("Time (days)") +
  ylab("dCTP (fmol per million cells)")

#plot dGTP
ggplot(endo, aes(Time, dGTP, colour=HIV, group=Subject)) +
  scale_colour_manual(values=c("dodgerblue4", "firebrick3"), name="HIV Status",
                     breaks=c("1", "0"), labels=c("Positive","Negative")) +
  geom_line(size=1) +
  theme_bw() +
  theme(text = element_text(size=15)) +
  labs(title="Change of dGTP Level") +
  xlab("Time (days)") +
  ylab("dGTP (fmol per million cells)")

#plot TTP
ggplot(endo, aes(Time, TTP, colour=HIV, group=Subject)) +
  scale_colour_manual(values=c("dodgerblue4", "firebrick3"), name="HIV Status",
                     breaks=c("1", "0"), labels=c("Positive","Negative")) +
  geom_line(size=1) +
  theme_bw() +
  theme(text = element_text(size=15)) +
  labs(title="Change of TTP Level") +
  xlab("Time (days)") +
  ylab("TTP (fmol per million cells)")

#Making Mean and SE Plots with Log transformed Data.

#log transform data
endo$logdATP <- log(dATP)
endo$logdCTP <- log(dCTP)
endo$logdGTP <- log(dGTP)
endo$logTTP <- log(TTP)

#function of summary statistics
library(doBy)
summarySE <- function(data=NULL, measurevar, groupvars=NULL, na.rm=FALSE, conf.interval=.95, .drop=TRUE) {
  library(plyr)

  # New version of length which can handle NA's: if na.rm==T, don't count them
  length2 <- function(x, na.rm=FALSE) {
    if (na.rm) sum(!is.na(x))
    else length(x)
  }
  
  # function calculates the mean of a variable conditional on groups
  function.groups <- function(x, group) {
    mean(x[is.na(group)==F], na.rm=na.rm)
  }

  # function calculates the standard error of the mean
  function.groups.SE <- function(x, group) {
    se <- sqrt(mean(((x-mean(x[is.na(group)==F], na.rm=na.rm))^2), na.rm=na.rm)/length2(x, na.rm=na.rm))
    se
  }

  # function calculates the function of the first two summary statistics
  function.summary <- function(x, group) {
    data.frame(summary3 = function.groups.SE(x, group))
  }

  # apply function over summarySE
  data.frame <- eval(simplify2array(aggregate(data, groupvars, function.summary, na.rm=na.rm)))

  # add group column
  data.frame$Group <- names(group)

  # function calculates the confidence interval
  function.conf <- function(x, group) {
    ci <- t.test(x[is.na(group)==F], na.rm=na.rm)$conf.int
    ci
  }

  # function calculates the function of the first two summary statistics
  function.summarySE <- function(x, group) {
    data.frame(summary3 = function.conf(x, group))
  }

  # apply function over summarySE
  data.frame <- eval(simplify2array(aggregate(data, groupvars, function.summarySE, na.rm=na.rm)))

  # add group column
  data.frame$Group <- names(group)

  # calculate the confidence interval
  data.frame$CI <- ifelse(na.rm, range(ci), range(c(NA, ci), na.rm=na.rm))

  # return the data frame
  data.frame
}

summarySE(endo$logdATP, group=endo$HIV)
summarySE(endo$logdCTP, group=endo$HIV)
summarySE(endo$logdGTP, group=endo$HIV)
summarySE(endo$logTTP, group=endo$HIV)
```
# This does the summary. For each group's data frame, return a vector with
# N, mean, and sd
datac <- ddply(data, groupvars, .drop=drop,
  .fun = function(xx, col) {
    c(N = length2(xx[[col]]), na.rm=na.rm),
    mean = mean (xx[[col]], na.rm=na.rm),
    sd = sd (xx[[col]], na.rm=na.rm)
  })
}, measurevar

# Rename the "mean" column
datac <- rename(datac, c("mean" = measurevar))
datac$se <- datac$sd / sqrt(datac$N)  # Calculate standard error of the mean

# Confidence interval multiplier for standard error
# Calculate t-statistic for confidence interval:
# e.g., if conf.interval is .95, use .975 (above/below), and use df=N-1
ciMult <- qt(conf.interval/2 + .5, datac$N-1)
datac$ci <- datac$se * ciMult
return(datac)

#Making data summary
dATPc <- summarySE(endo, measurevar="logdATP", groupvars=c("Time","HIV"))
dCTPc <- summarySE(endo, measurevar="logdCTP", groupvars=c("Time","HIV"))
dGTPc <- summarySE(endo, measurevar="logdGTP", groupvars=c("Time","HIV"))
TTPc <- summarySE(endo, measurevar="logTTP", groupvars=c("Time","HIV"))

#Plotting
Code

#Plot logdATP
ggplot(dATPc, aes(x=Time, y=logdATP, colour=HIV)) +
  geom_errorbar(aes(ymin=logdATP-se, ymax=logdATP+se), width=1.2, size=1) +
  geom_line(size=1.2) + scale_colour_manual(values=c("dodgerblue4",
  "firebrick3"), name="HIV Status", breaks=c("1", "0"),
  labels=c("Positive","Negative")) + geom_point(size=5, shape=21, fill="white")
+ theme_bw() + theme(text = element_text(size=15)) + labs(title="Change of logdATP Level (Mean, SE)"), xlab("Time (days)"), ylab("logdATP (fmol per million cells)")

#Plot logdCTP
ggplot(dCTPc, aes(x=Time, y=logdCTP, colour=HIV)) +
  geom_errorbar(aes(ymin=logdCTP-se, ymax=logdCTP+se), width=1.2, size=1) +
  geom_line(size=1.2) + scale_colour_manual(values=c("dodgerblue4",
  "firebrick3"), name="HIV Status", breaks=c("1", "0"),
  labels=c("Positive","Negative")) + geom_point(size=5, shape=21, fill="white")
+ theme_bw() + theme(text = element_text(size=15)) + labs(title="Change of logdCTP Level (Mean, SE)"), xlab("Time (days)"), ylab("logdCTP (fmol per million cells)")

#Plot logdGTP
ggplot(dGTPc, aes(x=Time, y=logdGTP, colour=HIV)) +
  geom_errorbar(aes(ymin=logdGTP-se, ymax=logdGTP+se), width=1.2, size=1) +
  geom_line(size=1.2) + scale_colour_manual(values=c("dodgerblue4",
  "firebrick3"), name="HIV Status", breaks=c("1", "0"),
  labels=c("Positive","Negative")) + geom_point(size=5, shape=21, fill="white")
+ theme_bw() + theme(text = element_text(size=15)) + labs(title="Change of logdGTP Level (Mean, SE)"), xlab("Time (days)"), ylab("logdGTP (fmol per million cells)")
#Plot logTTP

```r
ggplot(TTPc, aes(x=Time, y=logTTP, colour=HIV)) +
geom_errorbar(aes(ymin=logTTP-se, ymax=logTTP+se), width=1.2, size=1) +
geom_line(size=1.2) +
geom_line(aes(y=logTTP), size=1)
```

```r
+ scale_colour_manual(values=c("dodgerblue4", "firebrick3"), name="HIV Status", breaks=c("1", "0"),
labels=c("Positive", "Negative")) + geom_point(size=5, shape=21, fill="white")
```

```r
+ theme_bw() +
theme(text = element_text(size=15))
```

```r
+ labs(title="Change of logTTP Level (Mean, SE)") +
xlab("Time (days)") +
ylab("logdATP (fmol per million cells)"
```

#Making Summary Plot for endo method paper clinical application section

```r
endosum <- read.csv(file.choose(), header=T, na.strings = ".")
```

```r
ggplot(endosum, aes(x = dNTP, y = Median)) +
geom_bar(stat="identity", fill="dodgerblue4", position="stack") +
geom_errorbar(aes(ymin=1Q1, ymax=1Q3)) +
ggtitle("Bar Plot of Median and Interquartile Range") +
theme_bw() +
theme(panel.grid.major = element_blank()) +
ylab("dNTP (fmol per million cells)") +
```

```r
theme(text = element_text(size=15))
```
C.2 Code for visual predictive check

#######################################################################
## predcheck.R [17MAY2007]   IDS (c) 2008
## - PDx-Pop  -
## Description: Creates plots of simulated 95th, 90th, 50th, 10th, and
## 5th quantiles vs. observed data
## The PDx-Pop interface automatically completes the following 3
## arguments:
#######################################################################
## import data (includes simulated and observed data) = sim.data

runpath <- ("c:/pdxpop51/1812-tfvlink/")
# 22MAY2008 added graphics devices for Linux & Darwin
runno <- ("pc-TFV_link")
pdxpath <- ("C:/pdxpop51")
label <- ("TFV-DP")

# 22MAY2008 added graphics devices for Linux & Darwin

if ("Windows"==as.list(Sys.info()[1])){
  system(paste(pdxpath,"/predtable.exe ",runno,sep=""), , wait = TRUE)}
if ("Windows"!=as.list(Sys.info()[1])){
  system(paste(pdxpath,"/predtable.p ",runno,sep=""), , wait = TRUE)}
cat("PDx-Pop Predictive Check Report for Run ",runno,"
")
cat("predcheck.R [10MAY2011]\n")
require(graphics)
require(utils)
require(grDevices)
require(stats)
InFileName <- paste(runpath, runno,".txt", sep = "")
sim.data <- NULL
plot.data <- NULL
sim.data <- read.table(header = T, file = InFileName, skip=1)

# Remove DV == 0
sim.data<-sim.data[sim.data$EVID==0, ]

# select DATA BY DOSE or TRT

#sim.data <- sim.data[sim.data$HIV==0, ] #sub-setting by treatment
cat("Summary of Simulated Data:
")
print(summary(sim.data))
cat("No. of simulated observations = ",length(sim.data[,1]),"
")
# Order data frame by TIME

sim.data <- sim.data[order(sim.data$TIME), ]  #

# Add vector for binning observation times

spltime <- unique(sim.data$TIME)
if (is.element("NTIM", names(sim.data))) {spltime <- unique(sim.data$NTIM)}

# calculate boundary times for fixed design study

if (!is.element("mdpt", names(sim.data))){
sim.data$mdpt <- rep(0, length(sim.data$TIME))
bndtime <- rep(0, length(spltime)+1)
for(j in 1:length(spltime)){
  if(j == 1) { bndtime[j]<- spltime[j]/2 }
  else bndtime[j]<-spltime[j-1]+(spltime[j]-spltime[j-1])/2
}
  bndtime[length(spltime)+1] <- max(spltime[length(spltime)]+(spltime[length(spltime)]-spltime[length(spltime)-1])/2, ceiling(max(sim.data$TIME)))

ilast <- 0.0
#i<-bndtime[3]

  for(i in bndtime) { # bin boundaries

    if(i == bndtime[1]) {
      vec <- which(sim.data$TIME <= bndtime[1])
sim.data$mdpt[c(vec)] <- bndtime[1]
    }
    if(i != bndtime[1]) {
      vec <- which(sim.data$TIME <= i & sim.data$TIME > ilast)
sim.data$mdpt[c(vec)] <- spltime[ which(bndtime==i)-1]
    }

    ilast <- i
  }

}  

if ("Windows"==as.list(Sys.info())[1]) windows()
if ("Linux"==as.list(Sys.info())[1]) X11()
if ("Darwin"==as.list(Sys.info())[1]) quartz()
um <- 0
denom <- 0
times <- unique(sim.data$mdpt)
CIupper.90 <- double(length(times))
CIlower.90 <- double(length(times))
Clupper.80 <- double(length(times))
Clower.80 <- double(length(times))
ci.50 <- double(length(times))

## matrix sum is a matrix that summarizes number of obs that fall outside CI
matrix.sum <- matrix(0, 3, length(times))
for(i in 1:length(times)) {
  ob <- sim.data$OBS[sim.data$mdpt==times[i]]
dv <- sim.data$DV[sim.data$mdpt == times[i]]
  ci.90 <- quantile(dv, probs = c(0.05, 0.95))  ##90% CI
  CIupper.90[i] <- ci.90[2]
  Clower.90[i] <- ci.90[1]
  ci.80 <- quantile(dv, probs = c(0.1, 0.9))  ##80% CI
  CIupper.80[i] <- ci.80[2]
  Clower.80[i] <- ci.80[1]
  ci.50[i] <- median(dv)
  temp1 <- which(ob > ci.90[2])
  temp2 <- which(ob < ci.90[1])
  matrix.sum[1, i] <- length(temp1)
  matrix.sum[2, i] <- length(temp2)
  num <- num + matrix.sum[1, i] + matrix.sum[2, i]
  matrix.sum[3, i] <- length(ob)
  denom <- denom + matrix.sum[3, i]
}

# Generate min and max values for x-axis and y-axis
ymin <- min(c(Clower.90, Clower.80, ci.50, sim.data$OBS))
ymax <- max(c(Clupper.90, Clupper.80, ci.50, sim.data$OBS))
xmin <- min(sim.data$TIME)
xmax <- max(sim.data$TIME)  #

# Plot data
plot(0, 0, log="y", type = "n", ylim = c(0.5, 100), xlim = c(xmin, 1), xlab = "Time (days)", ylab = "TFV-DP (fmol/million cells)"
points(sim.data$TIME, sim.data$OBS, pch = 1, cex = 1)
lines(smooth.spline(times, Clower.90, df=100), lty = 1)
lines(smooth.spline(times, Clupper.90, df=100), lty = 1)  #
lines(smooth.spline(times, CIlower.80, df=100), lty = 5)
lines(smooth.spline(times, Clupper.80, df=100), lty = 5)
lines(smooth.spline(times, CIlower.90, df=100), lty = 8)  #
legend(0.50*1, 0.95*100, c("50th Quantile","10th-90th Quantiles","5th-95th Quantiles"),lty=c(8,5,1),bty="n")

mtext(outer=T, paste("VPC TFV plasma-pbm model (first dose)"," vs. Simulated 95th, 90th, 50th, 10th, and 5th Quantiles")
side=3, line=-1)

#export graph
export.graph(Name = "GSD2",
  Graph = ",
  ExportType = "WMF",
  FileName = paste(runpath, runno,".WMF", sep = ""),
  sep="")
#  OutputFile = "",
#  ColorBits = "",
#  QFactor = "",
#  Progressive = F,
#  NumPasses = "",
#  Height = "",
#  Width = "",
#  Units = "")
APPENDIX D

Complete NONMEM Code
$ERROR$

; exponential error model
DELI=0
IF (F.LE.0.0001) DELI=1
IPRE=F
W= IPRE +DELI
IRES= DV-IPRE
IWRE=IRES/W
Y = W*EXP(ERR(1))

; proportional error model
DELI=0
IF (F.LE.0.0001) DELI=1
IPRE=F
W= IPRE +DELI
IRES= DV-IPRE
IWRE=IRES/W
; Y = F + W*ERR(1)

$EST NOSBT NOOBT NOTBT METHOD=1 INTERACTION PRINT=5 MAX=9999 SIG=3
MSFO=tfv_plasma.MSF

$THETA
(0, 1440) ;[CL(L/DAY)]
(0, 370) ;[V2(L)]
(0, 5470, ) ;[Q(L/DAY)]
(0, 854, 2000) ;[V3(L)]
(0, 77.6, 200) ;[KA(1/DAY)]

$OMEGA
0.1 ;[P] omega(1,1)
0.1 ;[P] omega(2,2)
0.1 ;[P] omega(3,3)
0.1 ;[P] omega(4,4)

$SIGMA
0.04 ;[P] sigma(1,1)

$TABLE ID TIME EVID TAD CWRES IRES IWRE IPRE CSS HL CL V2 Q V3 KA HIV
RACE AGE SEXM EGFR WTKG BMI ONEHEADER NOPRINT FILE=TFV_PLASMA.tab

$TABLE ID KA CL V2 Q V3 HIV RACE AGE SEXM EGFR WTKG BMI ONEHEADER
NOPRINT FILE=TFV_PLASMA.tab

$TABLE ID ETA1 ETA2 ETA3 FIRSTONLY NOAPPEND NOPRINT FILE=TFV_PLASMA.eta

$COV PRINT=E
D.2 FTC plasma PK model

;Model Desc: 2 compart oral
;Project Name: 1812-ftcplasma
;Project ID: NO PROJECT DESCRIPTION

;Project ID: NO PROJECT DESCRIPTION

$PROB RUN# FTC_PLASMA
$INPUT C=DROP PID=DROP ID AMT TIME DV PBMC=DROP HIV RACE AGE SEXM EGFR WTKG BMI MDV EVID
$DATA EMTRICITABINE.CSV IGNORE=C
$SUBROUTINES ADVAN4 TRANS4
$PK
  TVCL=THETA(1)
  CL=TVCL*EXP(ETA(1))
  TVV2=THETA(2)+SEXM*THETA(6)
  V2=TVV2*EXP(ETA(2))
  TVQ=THETA(3)
  Q=TVQ;*EXP(ETA(3))
  TVV3=THETA(4)
  V3=TVV3*EXP(ETA(3))
  TVKA=THETA(5)
  KA=TVKA;*EXP(ETA(4))
  S2=V2
 ;CODING TIME AFTER DOSE
 IF (AMT.GT.0) THEN
 TDOS=TIME
 TAD=0.0
ENDIF
 IF (AMT.EQ.0) TAD=TIME-TDOS
 ;CODING AUC AND CSS
 AUC=200000/CL
 CSS=AUC/1
 ;CODING T1/2
 HLBeta=0.693/(0.5*((Q/V2+Q/V3+CL/V2)-SQRT((Q/V2+Q/V3+CL/V2)**2-4*Q/V3*CL/V2)))
 HLaAlpha=0.693/(0.5*((Q/V2+Q/V3+CL/V2)+SQRT((Q/V2+Q/V3+CL/V2)**2-4*Q/V3*CL/V2)))
$ERROR
  DEL=0
  IF (F.LE.0.0001) DEL=1
  IPRE=F
  W= IPRE +DEL
  IRES= DV-IPRE
  IWRE=IRES/W
  Y = W*EXP(ERR(1))
$EST INTERACTION NOTBT NOSBT NOOBT METHOD=1 PRINT=5 MAX=9999 SIG=3
MSFO=FTC_plasma.MSF
$THETA
  (0, 437) ;[CL]
  (0, 100) ;[BASE V2]
  (0, 10) ;[Q]
  (0, 50) ;[V3]
$OMEGA$ $\text{BLOCK}(2)$
\begin{align*}
0.1 & \text{;[P]} \omega(1,1) \\
0.01 & \text{;[A]} \omega(1,2) \\
0.1 & \text{;[P]} \omega(2,2) \\
\end{align*}

$OMEGA$
\begin{align*}
0.001 & \text{;[A]} \omega(1,3) \\
0.01 & \text{;[A]} \omega(2,3) \\
0.1 & \text{;[P]} \omega(3,3) \\
\end{align*}

$SIGMA$
\begin{align*}
0.1 & \text{;[P]} \sigma(1,1) \\
\end{align*}

$TABLE$ $ID$ $TIME$ $TAD$ $EVID$ $CWRES$ $IRES$ $IWE$ $IPRE$ $CSS$ $HLbeta$ $HLalpha$ $CL$ $V2$ $Q$
\begin{align*}
V3 & \text{KA} \\
\end{align*} $HIV$ $RACE$ $AGE$ $SEXM$ $EGFR$ $WTKG$ $BMI$ $ONEHEADER$ $NOPRINT$
FILE=$FTC\_PLASMA$.tab
$TABLE$ $ID$ $TIME$ $CSS$ $HLbeta$ $HLalpha$ $CL$ $V2$ $Q$ $V3$ $KA$ $ONEHEADER$ $NOPRINT$
FILE=$PATAB\_FTC\_PLASMA$
$TABLE$ $ID$ $ONEHEADER$ $NOPRINT$ FILE=$COTAB\_FTC\_PLASMA$
$TABLE$ $ID$ $ONEHEADER$ $NOPRINT$ FILE=$CATAB\_FTC\_PLASMA$
$TABLE$ $ID$ $IPRE$ $IRES$ $IWE$ $ONEHEADER$ $NOPRINT$ FILE=$SDTAB\_FTC\_PLASMA$
$TABLE$ $ID$ $CSS$ $HLbeta$ $HLalpha$ $CL$ $V2$ $Q$ $V3$ $KA$ $FIRSTONLY$ $NOAPPEND$ $NOPRINT$
FILE=$FTC\_PLASMA$.par
$TABLE$ $ID$ $ETA1$ $ETA2$ $ETA3$ $FIRSTONLY$ $NOAPPEND$ $NOPRINT$ FILE=$FTC\_PLASMA$.eta
$COV$ $PRINT=E$
D.3 TFV plasma–PBMC link PK model

;Model Desc: tfv_seqparametercut
;Project Name: 1812-tfvlink
;Project ID: NO PROJECT DESCRIPTION

;Project ID: NO PROJECT DESCRIPTION

$PROB RUN# TFV_link
$INPUT C=DROP SID=DROP ID AMT TIME PLASMA=DROP DV HIV Race Age Sex eGFR WTKG BMI MDV ICL IV2 IQ IV3 IKA CMT EVID $DATA TENOFOVIR_SEQLINK.CSV IGNORE=C $SUBROUTINES ADVAN13 TRANS1 TOL=6 $MODEL
NCOMP=6
COMP = (DEPOT, DEFDOS)
COMP = (CENTRAL)
COMP = (RESPONSE, DEFOBS)
COMP = (PHERIPH)
COMP = (PRECUR)
COMP = (REABS)

$PK
CL2  = ICL
V2   = IV2
KA2  = IKA
Q    = IQ
V4   = IV3
PIN  = THETA(1)
POUT = CL2/V2
KIN  = THETA(1)*EXP(ETA(1))
CP50 = THETA(2)
KOUT = THETA(3)*EXP(ETA(2))
R    = THETA(4)
S2   = V2
K20  = CL2/V2
K24  = Q/V2
K42  = Q/V4

IF (AMT.GT.0) THEN
TDOS=TIME
TAD=0.0
ENDIF
IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
DADT(1) = -KA2*A(1)
DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
C2 = A(2)/V2
DADT(5) = PIN*C2-A(5)*POUT
CP=A(5)
KR=KOUT*R/100
DADT(3) = KIN*C2/(1+CP/CP50)-KOUT*A(3)+KR*A(6)
DADT(4) = -K42*A(4) + K24*A(2)
DADT(6) = KR*A(3) - KR*A(6)
$ERROR
CTOL=A(5)
CRE=A(6)
KA3=KIN*(A(2)/V2)/(1+A(5)/CP50)
R=KOUT*R/100
KEL=KOUT*(1-R/100)
KSUM=R+R+KEL
HL=0.693/(0.5*(KSUM-SQRT(KSUM**2-4*R*KEL)))

DEL=0
IF (F.LE.0.0001) DEL=1
IPRE=F
W= IPRE +DEL
IRES= DV-IPRE
IWRE=IRES/W
Y = F + W*ERR(1)

$EST NOSBT NOOBT NOTBT METHOD=1 INTERACTION PRINT=50 MAX=9999 NSIG=2
SIGL=6 MSFO=TFV_link.MSF NOABORT

$THETA
(0, 1.4);[PIN]
(0, 6.6);[CT50]
(0, 0.4) ;[KOUT]
(0, 5.8);[R]

$OMEGA
0.24 ;[P] omega(1,1)
0.32 ;[P] omega(2,2)

$SIGMA
0.1 ;[P] sigma(1,1)

$TABLE ID TIME TAD IPRE IRES IWRE CWRES HL CTOL CRE KA2 K20 K24 PIN
POUT KIN CP50 KOUT KA3 HIV Race Age Sex eGFR WTKG BMI ONEHEADER NOPRINT
FILE=TFV_link.tab
$COV PRINT=E UNCONDITIONAL
$TABLE ID ONEHEADER NOPRINT FILE=COTABTFV_link
$TABLE ID ONEHEADER NOPRINT FILE=CATABTFV_link
$TABLE ID TIME IPRE IRES IWRE CWRES ONEHEADER NOPRINT
FILE=SDTABTFV_link
$TABLE ID CTOL CRE PIN POUT KIN KOUT CP50 FIRSTONLY NOAPPEND NOPRINT
FILE=TFV_link.par
$TABLE ID ETA1 ETA2 FIRSTONLY NOAPPEND NOPRINT FILE=TFV_link.eta
D.4 FTC plasma–PBMC link PK model

;Model Desc: FTC_seqparametercut
;Project Name: 1812-ftclink
;Project ID: NO PROJECT DESCRIPTION

;Project ID: NO PROJECT DESCRIPTION

$PROB RUN# FTC_link
$INPUT C=DROP SID=DROP ID AMT TIME PLASMA=DROP PBMC=DROP DV HIV Race
Age Sex eGFR WTKG BMI
MDV ICL IV2 IQ IV3 IKA CMT
$DATA EMTRICITABINE_SEQLINK.CSV IGNORE=C
$SUBROUTINES ADVAN13 TRANS1 TOL=6
$MODEL
  NCOMP=6
  COMP = (DEPOT, DEFDOS)
  COMP = (CENTRAL)
  COMP = (RESPONSE, DEFOBS)
  COMP = (PHERIPH)
  COMP = (PRECUR)
  COMP = (REABS)
$PK
  CL2  = ICL
  V2   = IV2
  KA2  = IKA
  Q    = IQ
  V4   = IV3
  TIN  = THETA(1)+THETA(5)*HIV
  TOUT = CL2/V2
  KIN  = (THETA(1)+THETA(5)*HIV)*EXP(ETA(1))
  CT50 = THETA(2)
  KOUT = THETA(3)*EXP(ETA(2))
  R    = THETA(4)
  S2   = V2
  K20  = CL2/V2
  K24  = Q/V2
  K42  = Q/V4

  IF (AMT.GT.0) THEN
    TDOS=TIME
    TAD=0.0
  ENDIF
  IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
  DADT(1) = -KA2*A(1)
  DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
  C2 = A(2)/V2
  RR=R/100
  KR=KOUT*RR
  DADT(5) = C2*TIN-A(5)*TOUT
  CT=A(5)
  DADT(3) = KIN*C2/(1+CT/CT50)-KOUT*A(3)+KR*A(6)
  DADT(4) = -K42*A(4) + K24*A(2)
\[
DADT(6) = KR*A(3) - KR*A(6)
\]

$ERROR$
\[
CTOL=A(5)
\]
\[
CRE=A(6)
\]
\[
KA3=KIN*(A(2)/\sqrt{2})*(1+A(5)/CT50)
\]
\[
R=KOUT*R/100
\]
\[
KEL=KOUT*(1-R/100)
\]
\[
KSUM=R+R+KEL
\]
\[
HL=0.693/(0.5*(KSUM-SQRT(KSUM**2-4*R*KEL)))
\]

\[
DEL=0
\]
\[
IF (F.LT.0.0001) DEL=1
\]
\[
IPRE=F
\]
\[
W= IPRE + DEL
\]
\[
IRES= DV-IPRE
\]
\[
IWRE=IRES/W
\]
\[
Y = F + W*ERR(1)
\]

$EST NOSBT NOOBT NOTBT METHOD=1 INTERACTION PRINT=50 MAX=9999 NSIG=2$

SIGNAL=6 MSFO=FTC_link.MSF NOABORT

$\theta$
\[
(0, 68.3); [\text{TIN}]
\]
\[
(0, 2460); [\text{CT50}]
\]
\[
(0, 1.7); [\text{KOUT}]
\]
\[
(0, 14.4); [\text{R}]
\]
\[
(0, 13)
\]

$\omega$
\[
0.03; [\text{P}] \ \omega(1,1)
\]
\[
0.05; [\text{P}] \ \omega(2,2)
\]

$\sigma$
\[
0.1; [\text{P}] \ \sigma(1,1)
\]

$\Sigma$
\[
\text{TIME TAD IPRE IRES IWRE CWRES HL CTOL CRE KA2 K20 K24 TIN TOUT KIN CT50 KOUT KA3 HIV Race Age Sex eGFR WTKG BMI ONEHEADER NOPRINT FILE=FTC_link.tab}$
$\Sigma$
\[
\text{COV PRINT=E UNCONDITIONAL}$

$\Sigma$
\[
\text{TIME IPRE IRES IWRE CWRES ONEHEADER NOPRINT FILE=COTAFTC_link}$
$\Sigma$
\[
\text{COV PRINT=E UNCONDITIONAL}$

$\Sigma$
\[
\text{TIME IPRE IRES IWRE CWRES ONEHEADER NOPRINT FILE=COTAFTC_link}$
$\Sigma$
\[
\text{TIME CTOL CRE TIN TOUT KIN KOUT CT50 FIRSTONLY NOAPPEND NOPRINT FILE=FTC_link.par}$
$\Sigma$
\[
\text{ETA1 ETA2 FIRSTONLY NOAPPEND NOPRINT FILE=FTC_link.eta}$
D.5 TFV-DP–dATP link PKPD model

; Model Desc: TFVSEQENDOA
; Project Name: 1812-endoa
; Project ID: NO PROJECT DESCRIPTION

; Project Name: 1812-endoa
; TFV-DP LINK dATP

$PROB RUN# TFVLINKA
$INPUT C SID ID TIME AMT ICL IV2 IKA IQ IV3 ITIN IKIN IKOUT ICT50 IR DV
HLADR_CD4 HLADR_CD8 HIV RACE AGE SEX eGFR WTKG BMI MDV EVID OBASE
$DATA TFVSEQENDOA.csv IGNORE=C

$SUBROUTINES ADVAN13 TOL=6
$MODEL
  NCOMP=7
  COMP = (DEPOT, DEFDOS)
  COMP = (CENTRAL)
  COMP = (PBMC)
  COMP = (PHERIPH)
  COMP = (TOL)
  COMP = (REABS)
  COMP = (ENDOT, DEFOBS)

$PK
  CL2  = ICL
  V2   = IV2
  KA2  = IKA
  Q    = IQ
  V4   = IV3
  TIN  = ITIN
  TOUT = CL2/V2
  KIN  = IKIN
  CT50 = ICT50
  KOUT = IKOUT
  R    = IR
  S2   = V2
  K20  = CL2/V2
  K24  = Q/V2
  K42  = Q/V4

  BPOP   = THETA(1)
  IIV    = THETA(2)
  RV     = THETA(3)
  BIDV   = BPOP*EXP(ETA(2)*IIV)
  IIVW   = IIV**2/(IIV**2+RV**2)
  RVW    = RV**2/(IIV**2+RV**2)
  BRV    = EXP(ETA(1)*RV*IIVW)
  IBASE  = (BPOP*RVW + OBASE*IIVW)*BRV
  K10    = THETA(5)
  K0     = IBASE*THETA(5)
  A_0(7) = K0/K10
EC50 = EXP(THETA(4))*EXP(ETA(3))

IF (AMT.GT.0) THEN
  TDOS=TIME
  TAD=0.0
ENDIF
IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
  DADT(1) = -KA2*A(1)
  DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
  C2 = A(2)/V2
  DADT(5) = TIN*C2-A(5)*TOUT
  CTOL=A(5)
  KR=KOUT*R/100
  DADT(3) = KIN*C2/(1+CTOL/CT50)-KOUT*A(3)+KR*A(6)
  DADT(4) = -K42*A(4) + K24*A(2)
  DADT(6) = KR*A(3) - KR*A(6)
  DADT(7) = K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)

$ERROR
IF (TIME.EQ.0) THEN
  IPRE=IBASE
ELSE
  IPRE=A(7)
ENDIF
DEL=0
IF (F.LE.0.0001) DEL=1
W= IPRE +DEL
IRES= DV-IPRE
IWRE=IRES/W
Y = IPRE*EXP(EPS(1)*RV)

KT=K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)
PBMC=A(3)
PLASMA=A(2)/V2
EFFECT=( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)

$EST NOSBT NOOBT NOTBT METHOD=1 INTERACTION PRINT=50 MAX=9999 NSIG=2
SIGL=6 MSFO=TFVLINKA.msf
$THETA
  (0, 160) ;[R0]
  (0, 0.22) ;[IIV]
  (0, 0.26);[RV]
  (0, 6.9) ;[EC50]
  1 FIXED ;[K10]

$OMEGA
  1 FIXED ;[P] omega(1,1)
  1 FIXED ;[P] omega(2,2)
  1.7 ;[P] omega(3,3)

$SIGMA
  1 FIXED ;[P] sigma(1,1)
$TABLE ID TIME OBASE IBASE K0 K10 EC50 HIV RACE AGE SEX eGFR WTKG BMI ONEHEADER NOPRINT FILE=TFVLINKA.tab
;$TABLE ID TIME EVID KT PBMC PLASMA EFFECT HIV ONEHEADER NOPRINT NOTITLE FORMAT=,1PE11.4 FILE=ENDOAM3LINKPBMC.tab
$TABLE K0 K10 EC50 ETA1 ETA2 ETA3 FIRSTONLY NOAPPEND NOPRINT FILE=TFVLINKA.eta
;parameter table;parameter table;parameter table;parameter table;parameter table;parameter table
$TABLE WTKG AGE BMI EGFR ONEHEADER NOPRINT FILE=COTABTFVLINKA ;continuous covariates table;continuous covariates table;continuous covariates table;continuous covariates table;continuous covariates table;continuous covariates table
$TABLE HIV SEX RACE ONEHEADER NOPRINT FILE=CATABTFVLINKA ;categorical covariates table;categorical covariates table;categorical covariates table;categorical covariates table;categorical covariates table;categorical covariates table
$TABLE ID DV TIME PRED IPRE WRES IWRE RES IRES ONEHEADER NOPRINT FILE=SDTABTFVLINKA; sandard table file; sandard table file; sandard table file; sandard table file; sandard table file; sandard table file
$COV PRINT=E UNCONDITIONAL
D.6 TFV-DP–dGTP link PKPD model

; Model Desc: TFVSEQENDOG
; Project Name: 1812-endog
; TFV-DP LINK dGTP

$PROB RUN# TFVLINKG
$INPUT C SID ID TIME AMT ICL IV2 IKA IQ IV3 ITIN IKIN IKOUT ICT50 IR DV HLADR CD4 HLADR CD8 HIV RACE AGE SEX eGFR WTKG BMI MDV EVID OBASE
$DATA TFVSEQENDOG.csv IGNORE=C

; FOR MODELING M3 SIMULATION DATA
;$INPUT C=DROP SID=DROP ID TIME AMT ICL IV2 IKA IQ IV3 ITIN IKIN IKOUT ICT50 IR DV MDV EVID
;$DATA C:\pdxpop51\1812-ENDO\pc-ENDOGM3LINKPBMC.csv IGNORE=C

$SUBROUTINES ADVAN13 TOL=6
$MODEL
   NCOMP=7
   COMP = (DEPOT, DEFDOS)
   COMP = (CENTRAL)
   COMP = (PBMC)
   COMP = (PHERIPH)
   COMP = (TOL)
   COMP = (REABS)
   COMP = (ENDOT, DEFOBS)

$PK
   CL2  = ICL
   V2   = IV2
   KA2  = IKA
   Q    = IQ
   V4   = IV3
   TIN  = ITIN
   TOUT = CL2/V2
   KIN  = IKIN
   CT50 = ICT50
   KOUT = IKOUT
   R    = IR
   S2   = V2
   K20  = CL2/V2
   K24  = Q/V2
   K42  = Q/V4

   BPOP   = THETA(1)
   ;IF (TIME.EQ.0) THEN ;take out for simulation
   ;OBASE = DV ;take out for simulation
   ;ENDIF ;take out for simulation

   IIV    = THETA(2)
   RV     = THETA(3)
   BIDV   = BPOP*EXP(ETA(2)*IIV)
   IIVW   = IIV**2/(IIV**2+RV**2)
   RVW    = RV**2/(IIV**2+RV**2)
   BRV    = EXP(ETA(1)*RV*IIVW)
;OBASE = BPOP*EXP(ETA(2)*IIV)*EXP(ETA(1)*RV); include for simulation
IBASE = (BPOP*RVW + OBASE*IIVW)*BRV
K10 = THETA(5)
K0 = IBASE*THETA(5)
A_0(7) = K0/K10
EC50 = EXP(THETA(4))
GAM = THETA(6)

IF (AMT.GT.0) THEN
TDOS=TIME
TAD=0.0
ENDIF
IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
DADT(1) = -KA2*A(1)
DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
C2 = A(2)/V2
DADT(5) = TIN*C2-A(5)*TOUT
CTOL=A(5)
KR=KOUT*R/100
DADT(3) = KIN*C2/(1+CTOL/CT50)-KOUT*A(3)+KR*A(6)
DADT(4) = -K42*A(4) + K24*A(2)
DADT(6) = KR*A(3) - KR*A(6)
DADT(7) = K0 * ( 1 - (1/(1+TIME**GAM)) * A(3) / ( EC50 + A(3) ) ) - K10*A(7)

$ERROR
IF (TIME.EQ.0) THEN
IPRE=IBASE
ELSE
IPRE=A(7)
ENDIF

DEL=0
IF (F.LE.0.0001) DEL=1
W= IPRE +DEL
IRES= DV-IPRE
IWRE=IRES/W

Y = IPRE*EXP(EPS(1)*RV)

KT=K0 * ( 1 - (1/(1+TIME**GAM)) * A(3) / ( EC50 + A(3) ) )
PBM=A(3)
PLASMA=A(2)/V2
EFFECT=( 1 - (1/(1+TIME**GAM)) * A(3) / ( EC50 + A(3) ) )

$EST NOSBT NOOBT NOTBT METHOD=1 INTERACTION PRINT=50 MAX=9999 NSIG=2 SIGL=6 MSFO=TFVLINKG.msf
$THETA
(0, 240) ;[R0]
(0, 0.20) ;[IIV]
(0, 0.27);[RV]
(0, 4) ;[EC50]
1 FIXED ;[K10]
(0, 0.93) ; [GAM]

$OMEGA
1 FIXED ; [P] omega(1,1)
1 FIXED ; [P] omega(2,2)

$SIGMA
1 FIXED ; [P] sigma(1,1)

$TABLE ID TIME OBASE IBASE K0 K10 EC50 EFFECT HIV RACE AGE SEX eGFR
WTKG BMI ONEHEADER NOPRINT FILE=TFVLINKG.tab
$TABLE ID TIME EVID KT PBMC PLASMA EFFECT HIV ONEHEADER NOPRINT
NOTITLE FORMAT=,1PE11.4 FILE=ENDOGM3LINKPBMC.tab
$TABLE ID ETA1 ETA2 FIRSTONLY NOAPPEND NOPRINT FILE=TFVLINKG.eta

$TABLE K0 K10 EC50 ETA1 ETA2 ONEHEADER NOPRINT FILE=PATABTFVLINKG
;parameter table;parameter table;parameter table;parameter table;parameter
 table;parameter table;parameter table;parameter table;parameter table
$TABLE WTKG AGE BMI EGFR ONEHEADER NOPRINT FILE=COTABTFVLINKG
;continuous covariates table;continuous covariates table;continuous
covariates table;continuous covariates table;continuous covariates
table;continuous covariates table;continuous covariates table
$TABLE HIV SEX RACE ONEHEADER NOPRINT FILE=CATABTFVLINKG ;categorical
covariates table;categorical covariates table;categorical covariates
table;categorical covariates table;categorical covariates
table;categorical covariates table;categorical covariates table
$TABLE ID DV TIME PRED IPRE WRES IREG RES IRES ONEHEADER NOPRINT
FILE=SDTABTFVLINKG; sandard table file; sandard table file; sandard
table file; sandard table file; sandard table file; sandard table file;
sandard table file; sandard table file

$COV PRINT=E UNCONDITIONAL
D.7 FTC-TP–dCTP link PKPD model

;Model Desc: ENDOCM3LINKPBMC
;Project Name: 1812-endoc
;FTC-TP LINK dCTP

$PROB RUN# FTCLINKC
$INPUT C SID ID TIME AMT ICL IV2 IKA IQ IV3 ITIN IKIN IKOUT ICT50 IR DV HLADR_CD4 HLADR_CD8 HIV RACE AGE SEX eGFR WTKG BMI MDV EVID OBASE
$DATA FTCSEQENDOC.csv IGNORE=C

$SUBROUTINES ADVAN13 TOL=6
$MODEL
   NCOMP=7
   COMP = (DEPOT, DEFDOS)
   COMP = (CENTRAL)
   COMP = (PBMC)
   COMP = (PHERIPH)
   COMP = (TOL)
   COMP = (REABS)
   COMP = (ENDOT, DEFOBS)

$PK
   CL2  = ICL
   V2   = IV2
   KA2  = IKA
   Q    = IQ
   V4   = IV3
   TIN  = ITIN
   TOUT = CL2/V2
   KIN  = IKIN
   CT50 = ICT50
   KOUT = IKOUT
   R    = IR
   S2   = V2
   K20  = CL2/V2
   K24  = Q/V2
   K42  = Q/V4
   BPOP  = THETA(1)
   IIV  = THETA(2)
   RV  = THETA(3)
   BIDV = BPOP*EXP(ETA(2)*IIV)
   IIVW = IIV**2/(IIV**2+RV**2)
   RVW = RV**2/(IIV**2+RV**2)
   BRV = EXP(ETA(1)*RV*IIVW)
   IBASE = (BPOP*RVW + OBASE*IIVW)*BRV
   K10  = THETA(5)
   K0   = IBASE*THETA(5)
   A_0(7) = K0/K10
   EC50  = EXP(THETA(4))*EXP(ETA(3))

IF (AMT.GT.0) THEN
   TDOS=TIME
   TAD=0.0
ENDIF
IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
DADT(1) = -KA2*A(1)
DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
C2 = A(2)/V2
DADT(5) = TIN*C2-A(5)*TOUT
CTOL=A(5)
KR=KOUT*R/100
DADT(3) = KIN*C2/(1+CTOL/CT50)-KOUT*A(3)+KR*A(6)
DADT(4) = -K42*A(4) + K24*A(2)
DADT(6) = KR*A(3) - KR*A(6)
DADT(7) = K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)

$ERROR
IF (TIME.EQ.0) THEN
IPRE=IBASE
ELSE
IPRE=A(7)
ENDIF

DEL=0
IF (F.LE.0.0001) DEL=1
W= IPRE +DEL
IRES= DV-IPRE
IWRE=IRES/W
Y = IPRE*EXP(EPS(1)*RV)

KT=K0 * ( 1- A(3) / ( EC50 + A(3) ) )
PBMC=A(3)
PLASMA=A(2)/V2
EFFECT=( 1- A(3) / ( EC50 + A(3) ) )

$EST NOSBT NOOBT NOTBT METHOD=1 INTERACTION PRINT=50 MAX=9999 NSIG=2
SIGL=6 MSFO=FTCLINKC.msf
$THETA
(0, 770) ;[R0]
(0, 0.20) ;[IIV]
(0, 0.23);[RV]
(0, 11) ;[EC50]
1 FIXED ;[K10]

$OMEGA
1 FIXED ;[P] omega(1,1)
1 FIXED ;[P] omega(2,2)
0.68 ;[P] omega(3,3)

$SIGMA
1 FIXED ;[P] sigma(1,1)

$TABLE ID TIME OBASE IBASE K0 K10 EC50 HIV RACE AGE SEX eGFR WTKG BMI ONEHEADER NOPRINT FILE=FTCLINKC.tab
;TABLE ID TIME EVID KT PBMC PLASMA EFFECT HIV ONEHEADER NOPRINT NOTITLE FORMAT=,1PE11.4 FILE=ENDOCM3LINKPBMC.tab
$TABLE ID ETA1 ETA2 ETA3 FIRSTONLY NOAPPEND NOPRINT FILE=FTCLINKC.eta

$TABLE K0 K10 EC50 ETA1 ETA2 ETA3 ONEHEADER NOPRINT FILE=PATABFTCLINKC
 ;parameter table; ;parameter table;
$TABLE WTKG AGE BMI EGFR ONEHEADER NOPRINT FILE=COTABFTCLINKC
 ;continuous covariates table;continuous covariates table
$TABLE HIV SEX RACE ONEHEADER NOPRINT FILE=CATABFTCLINKC ;categorical
 covariates table; categorical covariates table
$TABLE ID DV TIME PRED IPRE WRES I&WRE RES IRES ONEHEADER NOPRINT
 FILE=SDTABFTCLINKC; standard table file; standard table file

$COV PRINT=E UNCONDITIONAL
D.8 FTC-TP–TTP link PKPD model

;Model Desc: ENDOTM3LINKPBMC
;Project Name: 1812-endot
;FTC-TP LINK TTP

$PROB RUN# FTCLINKTT
$INPUT C SID ID TIME AMT IV2 IKA IQ IV3 ITIN IKIN IKOUT ICT50 IR DV
HLADR_CD4 HLADR_CD8 HIV RACE AGE SEX eGFR WTKG BMI
MDV EVID OBAS -
$DATA FTCSEQENDOT.csv IGNORE=C

$SUBROUTINES ADVAN13 TOL=6
$MODEL
  NCOMP=7
  COMP = (DEPOT, DEFDOS)
  COMP = (CENTRAL)
  COMP = (PBMC)
  COMP = (PHERIPH)
  COMP = (TOL)
  COMP = (REABS)
  COMP = (ENDOT, DEFOBS)

$PK
  CL2  = ICL
  V2   = IV2
  KA2  = IKA
  Q    = IQ
  V4   = IV3
  TIN  = ITIN
  TOUT = CL2/V2
  KIN  = IKIN
  CT50  = ICT50
  KOUT = IKOUT
  R    = IR
  S2   = V2
  K20  = CL2/V2
  K24  = Q/V2
  K42  = Q/V4
  
  BPOP = THETA(1)
  IIV  = THETA(2)
  RV   = THETA(3)
  BIDV = BPOP*EXP(ETA(2)*IIV)
  IIVW = IIV**2/(IIV**2+RV**2)
  RVW  = RV**2/(IIV**2+RV**2)
  BRV  = EXP(ETA(1)*RV*IIVW)
  ;OBASE = BPOP*EXP(SQRT(IIV**2+RV**2)*ETA(1)); include for simulation
  IBASE = (BPOP*RVW + OBASE*IIVW)*BRV
  K10  = THETA(5)
  K0   = IBASE*THETA(5)
  A_0(7) = K0/K10
  EC50  = EXP(THETA(4))*EXP(ETA(3))
IF (AMT.GT.0) THEN
TDOS=TIME
TAD=0.0
ENDIF
IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
DADT(1) = -KA2*A(1)
DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
C2 = A(2)/V2
DADT(5) = TIN*C2-A(5)*TOUT
CTOL=A(5)
KR=KOUT*R/100
DADT(3) = KIN*C2/(1+CTOL/CT50)-KOUT*A(3)+KR*A(6)
DADT(4) = -K42*A(4) + K24*A(2)
DADT(6) = KR*A(3) - KR*A(6)
DADT(7) = K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)

$ERROR
IF (TIME.EQ.0) THEN
IPRE=IBASE
ELSE
IPRE=A(7)
ENDIF
DEL=0
IF (F.LE.0.0001) DEL=1
W= IPRE +DEL
IRES= DV-IPRE
IWRE=IRES/W
Y = IPRE*EXP(EPS(1)*RV)
KT=K0 * ( 1- A(3) / ( EC50 + A(3) ) )
PBMC=A(3)
PLASMA=A(2)/V2
EFFECT= A(3) / ( EC50 + A(3) )

$EST NOSBT NOOBT NOTBT METHOD=1 INTERACTION PRINT=50 MAX=9999 NSIG=2
SIGL=6 MSFO=FTCLINKTT.msf

$THETA
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(0, 0.38) ;[IIV]
(0, 0.36) ;[RV]
(0, 9.8) ;[EC50]
1 FIXED ;[K10]

$OMEGA
1 FIXED ;[P] omega(1,1)
1 FIXED ;[P] omega(2,2)
1.0 ;[P] omega(3,3)

$SIGMA
1 FIXED ;[P] sigma(1,1)
$TABLE ID TIME OBASE IBASE K0 K10 EC50 HIV RACE AGE SEX eGFR WTKG BMI ONEHEADER NOPRINT FILE=FTCLINKTT.tab
;$TABLE ID TIME EVID KT PBMC PLASMA EFFECT HIV ONEHEADER NOPRINT NOTITLE FORMAT=,1PE11.4 FILE=ENDOTM3LINKPBMC.tab
$TABLE ID ETA1 ETA2 ETA3 FIRSTONLY NOAPPEND NOPRINT FILE=FTCLINKTT.eta
$TABLE K0 K10 EC50 ETA1 ETA2 ETA3 ONEHEADER NOPRINT FILE=PATABFTCLINKTT ;parameter table;parameter table;parameter table;parameter table
$TABLE WTKG AGE BMI EGFR ONEHEADER NOPRINT FILE=COTABFTCLINKTT ;continuous covariates table;continuous covariates table
$TABLE HIV SEX RACE ONEHEADER NOPRINT FILE=CATABFTCLINKTT ;categorical covariates table;categorical covariates table
$TABLE ID DV TIME PRED IPRE WRES IWRK RES IRES ONEHEADER NOPRINT FILE=SDTABFTCLINKTT; sandard table file; sandard table file
$COV PRINT=E UNCONDITIONAL
D.9 TFV:dATP IPERGAY trial simulation

;Project Name: 1812-endoa
;TFV-DP LINK dATP

$PROB RUN# TFVIPERGAY
$INPUT C SID ID TIME AMT II ADDL DV MDV
$DATA IPERGAYTFVsimulation.csv IGNORE=C

$SUBROUTINES ADVAN13 TOL=6
MODEL
  NCOMP=7
  COMP = (DEPOT, DEFDOS)
  COMP = (CENTRAL)
  COMP = (PBMC)
  COMP = (PHERIPH)
  COMP = (TOL)
  COMP = (REABS)
  COMP = (ENDOT, DEFOBS)

$PK
  CL2  = THETA(1)*EXP(ETA(1))
  V2   = THETA(2)*EXP(ETA(2))
  KA2  = THETA(3)*EXP(ETA(3))
  Q    = THETA(4)*EXP(ETA(4))
  V4   = THETA(5)*EXP(ETA(5))
  TIN  = THETA(6)
  TOUT = CL2/V2
  KIN  = THETA(6)*EXP(ETA(6))
  CT50 = THETA(7)*EXP(ETA(7))
  KOUT = THETA(8)*EXP(ETA(8))
  R    = THETA(9)*EXP(ETA(9))
  S2   = V2
  K20  = CL2/V2
  K24  = Q/V2
  K42  = Q/V4

  BPOP   = THETA(10)
  IBASE  = THETA(10)*EXP(ETA(10))
  K10    = 1
  K0     = IBASE*1
  A_0(7) = K0/K10
  EC50   = EXP(THETA(11))*EXP(ETA(11))

  IF (AMT.GT.0) THEN
    TDOS=TIME
    TAD=0.0
  ENDIF
  IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
  DADT(1) = -KA2*A(1)
  DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
  C2 = A(2)/V2
DADT(5) = TIN*C2-A(5)*TOUT
CTOL=A(5)
KR=KOUT*R/100
DADT(3) = KIN*C2/(1+CTOL/CT50)-KOUT*A(3)+KR*A(6)
DADT(4) = -K42*A(4) + K24*A(2)
DADT(6) = KR*A(3) - KR*A(6)
DADT(7) = K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)

$ERROR
IF (TIME.EQ.0) THEN
IPRE=IBASE
ELSE
IPRE=A(7)
ENDIF
IND=IREP
DEL=0
IF (F.LE.0.0001) DEL=1
W= IPRE +DEL
IRES= DV-IPRE
IWRE=IRES/W
Y = IPRE*EXP(EPS(1))
KT=K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)
PBMCA=A(3)
PLASMA=A(2)/V2
EFFECT=( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)
REDUCTION=(IBASE-A(7))/IBASE
ENDO=A(7)
RATIO=A(3)/A(7)

$THETA
 1410;[CL2]
 390;[V2]
 80.1;[KA2]
 5390;[Q]
 877;[V4]
 1.4;[KIN]
 6.55;[CT50]
 0.228;[KOUT]
 5.82;[R]
 155 ;[R0]
 6.93 ;[EC50]

$OMEGA
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 0.288 ;[P] omega(2,2)
 0 FIXED ;[P] omega(3,3)
 0.0693 ;[P] omega(4,4)
 0 FIXED ;[P] omega(5,5)
 0.238 ;[P] omega(6,6)
 0 FIXED ;[P] omega(7,7)
 0.316 ;[P] omega(8,8)
 0 FIXED ;[P] omega(9,9)
0.220 ; [P] omega(10,10)
1.70 ; [P] omega(11,11)

$SIGMA
  0.258 ; [P] sigma(1,1)

$SIM (2014) ONLY SUB=1000

; $ESTIMATION MAXEVAL=0

$TABLE ID TIME IND AMT EVID PBMC ENDO RATIO REDUCTION ONEHEADER NOPRINT
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D.10 FTC:dCTP IPERGAY trial simulation

;Model Desc: FTCLINKCSIMULATION
;Project Name: 1812-pred
;Project ID: NO PROJECT DESCRIPTION

;Project Name: 1812-endoa
;FTC LINK dCTP

$PROB RUN# FTCIPERGAY
$INPUT C SID ID TIME AMT II ADDL DV MDV
$DATA IPERGAYFTCsimulation.csv IGNORE=C

$SUBROUTINES ADVAN13 TOL=6
$MODEL
    NCOMP=7
    COMP = (DEPOT, DEFDOS)
    COMP = (CENTRAL)
    COMP = (PBMC)
    COMP = (PHERIPH)
    COMP = (TOL)
    COMP = (REABS)
    COMP = (ENDOT, DEFOBS)

$PK

IF (ICALL.EQ.4.AND.NEWIND.NE.2) THEN
    CALL RANDOM (2, R)
    IF (R.LE.0.5) SEXM=0
    ELSE IF (R.GT.0.5) SEXM=1
ENDIF

;IF (ICALL.EQ.4.AND.NEWIND.NE.2) THEN
;    CALL RANDOM (3, R)
;    IF (R.LE.0.5) HIV=0
;    ELSE IF (R.GT.0.5) HIV=1
;ENDIF

CL2  = THETA(1)*EXP(ETA(1))
V2   = (THETA(2)+24.3*SEXM)*EXP(ETA(2))
KA2  = THETA(3)*EXP(ETA(3))
Q    = THETA(4)*EXP(ETA(4))
V4   = THETA(5)*EXP(ETA(5))
TIN  = THETA(6)+0*31.3
TOUT = CL2/V2
KIN  = (THETA(6)+0*31.3)*EXP(ETA(6))
CT50 = THETA(7)*EXP(ETA(7))
KOUT = THETA(8)*EXP(ETA(8))
RR   = THETA(9)*EXP(ETA(9))
S2   = V2
K20  = CL2/V2
K24  = Q/V2
K42  = Q/V4
BPOP = THETA(10)
IBASE = THETA(10)*EXP(ETA(10))
K10 = 1
K0 = IBASE*1
A_0(7) = K0/K10
EC50 = EXP(THETA(11))*EXP(ETA(11))

IF (AMT.GT.0) THEN
TDOS=TIME
TAD=0.0
ENDIF
IF (AMT.EQ.0) TAD=TIME-TDOS

$DES
DADT(1) = -KA2*A(1)
DADT(2) = KA2*A(1) - K20*A(2) + K42*A(4) - K24*A(2)
C2 = A(2)/V2
DADT(5) = TIN*C2-A(5)*TOUT
CTOL=A(5)
KR=KOUT*RR/100
DADT(3) = KIN*C2/(1+CTOL/CT50)-KOUT*A(3)+KR*A(6)
DADT(4) = -K42*A(4) + K24*A(2)
DADT(6) = KR*A(3) - KR*A(6)
DADT(7) = K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)

$ERROR
IF (TIME.EQ.0) THEN
IPRE=IBASE
ELSE
IPRE=A(7)
ENDIF

IND=IREP
DEL=0
IF (F.LE.0.0001) DEL=1
W= IPRE +DEL
IRES= DV-IPRE
IWRE=IRES/W

Y = IPRE*EXP(EPS(1))

KT=K0 * ( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)
PBMC=A(3)
PLASMA=A(2)/V2
EFFECT=( 1- A(3) / ( EC50 + A(3) ) ) - K10*A(7)
REDUCTION=(IBASE-A(7))/IBASE
ENDO=A(7)
RATIO=A(3)/A(7)

$THETA
482;[CL2]
99.4;[V2]
55.7;[KA2]
141;[Q]
166;[V4]
41.6;[KIN]
3320;[CT50]
1.6;[KOUT]
16.0;[RR]
771;[R0]
10.7;[EC50]

$OMEGA
0.0942 ;[P] omega(1,1)
0.0319 ;[P] omega(2,2)
0 FIXED ;[P] omega(3,3)
0 FIXED ;[P] omega(4,4)
0.0335 ;[P] omega(5,5)
0.0358 ;[P] omega(6,6)
0 FIXED ;[P] omega(7,7)
0.0561 ;[P] omega(8,8)
0 FIXED ;[P] omega(9,9)
0.195 ;[P] omega(10,10)
0.681 ;[P] omega(11,11)

$SIGMA
0.229 ;[P] sigma(1,1)

$SIM (2014) (201402 UNIFORM) (201403 UNIFORM) ONLY SUB=1000

$TABLE ID TIME IND AMT EVID PBMC ENDO RATIO REDUCTION ONEHEADER NOPRINT
FILE=FTCIPERGAY.tab
APPENDIX E

Individual Pharmacokinetics and Pharmacodynamics Parameters from NONMEM
# E.1 TFV plasma PK model

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