

DISSERTATION

PHARMACOKINETIC AND PHARMACODYNAMIC EVALUATION OF HIV-1
PRE-EXPOSURE PROPHYLAXIS CANDIDATES IN HUMANIZED MICE

Submitted by

Milena Veselinovic

Graduate Degree Program in Cell and Molecular Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2014

Doctoral Committee:

Advisor: Ramesh Akkina

Tawfik Aboellail
Mercedes Gonzalez-Juarrero
Olve Peersen

Copyright by Milena Veselinovic 2014

All rights received

ABSTRACT

PHARMACOKINETIC AND PHARMACODYNAMIC EVALUATION OF HIV-1 PRE-EXPOSURE PROPHYLAXIS CANDIDATES IN HUMANIZED MICE

In the absence of a vaccine, alternative preventative approaches against HIV-1 are needed. In pre-exposure prophylaxis (PrEP) approach, antiretroviral drugs, broadly neutralizing antibodies (bnAb) or other biological molecules are administered orally or topically for the prevention of HIV-1 infection. For successful PrEP design pharmacokinetic (PK) and pharmacodynamic (PD) studies are needed to define protective levels of antiretrovirals in mucosal tissues. The RAG-hu mice used here represent a small animal model in which human immune system is reconstituted by haematopoietic stem cells (HSC) in the immunodeficient BALB/c- Rag1^{-/-} γ c^{-/-} and BALB/c- Rag2^{-/-} γ c^{-/-} mice. This model was previously shown to be suitable for HIV-1 mucosal transmission and protection studies. In the experiments presented here we evaluated the utility of RAG-hu mice for the study of PK-PD aspects of antiretroviral drugs in the context of PrEP.

The PK studies focused on tissue distribution of the RT inhibitor Tenofovir (TFV), the integrase inhibitor Raltegravir (RAL) and the entry inhibitor Maraviroc (MVC) following single and combinatorial oral application. Drug kinetics were examined systemically in blood plasma, and in vaginal, rectal and colonic mucosal tissues, which are the sites of HIV-1 transmission and initial viral spread. Antiretrovirals were applied in human equivalent doses to achieve steady state kinetics. Data obtained from single oral applications verified favorable PrEP profile of TFV. While results showed that RAL and MVC represent promising PrEP candidates, the data suggest that the PrEP doses would need to be higher than therapeutic ones in order to allow for once a day dosing. In combinatorial TFV/RAL and TFV/MVC oral application studies, increase in the active form of TFV (Tenofovir diphosphate, TFV-DP) accompanied by agonistic effect for

the second drug in combination was observed, which can be characterized as highly favorable for PrEP applications. This is the first report on combinatorial PK of TFV, RAL and MVC in mucosal tissues which informs further testing of TFV/MVC and TFV/RAL PrEP approaches in non-human primates (NHP) and in clinical settings. For topical PrEP potential, PK profiles of TFV, RAL and MVC were also evaluated in vaginal mucosa following topical application of gel formulations. With all three drugs, one to two log higher concentrations were achieved in vaginal mucosa compared to oral application reflecting previous findings in humans. Intracellular concentrations of TFV-DP in humanized mice corresponded to the levels observed previously in human vaginal mucosa.

In PD studies, the protective effect of topical PrEP with single drug and combinatorial TFV, RAL and the RT inhibitor UC781 gels was evaluated against mucosal HIV-1 transmission. High level of protection was seen with combinatorial microbicide gels – 80% (4/5) protection by TFV/UC781 gel and 87.5% (7/8) protection by TFV/RAL gel, indicating their suitability for further testing in preclinical trials. In another PD study, protective efficacy of bnAb VRC01 was examined against mucosal transmission of HIV-1 in the form of topical PrEP. The VRC01 gel (1 mg/ml) conferred protection in 77.7% (7/9) animals, while the combination of b12, 4E10, 2F5 and 2G12 bnAb which target different epitopes on the HIV-1 envelope conferred complete protection (5/5 animals) against HIV-1 mucosal transmission. These data suggest that bnAb could be effective agents for topical PrEP against HIV-1.

In summary, these proof of concept PK and PD studies validated RAG-hu mouse model for preclinical evaluation of new anti-HIV-1 drugs and bnAb for oral and topical PrEP, thus providing data for further NHP studies and human clinical trials.

ACKNOWLEDGEMENTS

I owe my sincere gratitude to Dr Akkina for his valuable mentorship and generous support during my graduate studies in his laboratory. I am also grateful to all former and present members of my lab - graduate students, postdoctoral fellows and undergraduates for their friendship, support and help over the years. I thank the committee members Dr Gonzalez-Juarrero, Dr Peersen and Dr Aboellail for their helpful suggestions and feedback regarding my research and academic work at Colorado State University as a doctoral student.

I thank my family and my husband for their love, support and encouragement through these four years of my graduate studies and throughout my life in general, as none of my achievements would be possible without them. I only hope to go further and make them even more proud, as new exciting opportunities await in the near future.

TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
CHAPTER 1.....	1
Introduction	1
1.1 Discovery of HIV-1.....	1
1.2 The Origin of HIV-1 and genealogy data.....	3
1.3 Structure of HIV-1.....	5
1.3.1. Structural HIV-1 proteins	8
1.3.2. Regulatory HIV-1 proteins	10
1.3.3. Accessory HIV-1 proteins.....	11
1.3.4. Regulation of gene expression from the proviral genome.....	12
1.4 HIV-1 life cycle.....	13
1.5 HIV-1 infection in humans.....	17
1.5.1. HIV-1 Transmission and early infection.	18
1.5.2. Acute HIV-1 syndrome and establishment of a viral set point.	20
1.5.3. Immune response – failure of the immune system against HIV-1.	21
1.5.4. HIV-1 induced immune dysfunction - immune activation, exhaustion and immunosenescence.	25
1.5.5. Chronic phase of HIV-1 infection and transition to AIDS.....	27
1.5.6. HIV-1 latency.	29
1.6 Animal models for HIV-1 research	31
1.6.1. Nonhuman primate (NHP) models.....	32
1.6.2. Humanized mouse models.....	34
1.7 Therapeutic approaches for HIV-1 infection.....	39
1.7.1. HIV-1 vaccine approach	39
1.7.2. HIV-1 Antiretrovirals	40
1.7.3. Gene therapy approaches.....	43
1.8 HIV-1 prevention.....	45
1.8.1. Topical PrEP – microbicide approach.....	47
1.8.2. Oral PrEP approach	50
1.8.3. BnAb microbicide approach	52
CHAPTER 2.....	55
Topical gel formulation of broadly neutralizing anti-HIV-1 monoclonal antibody VRC01 confers protection against HIV-1 vaginal challenge in a humanized mouse model	55

2.1 Summary	55
2.2 Introduction.....	55
2.3 Materials and methods	58
2.4 Results	61
2.5 Discussion	65
CHAPTER 3.....	68
HIV-1 Pre-Exposure Prophylaxis: Mucosal Tissue Drug Distribution of RT Inhibitor Tenofovir and Entry Inhibitor Maraviroc in a Humanized Mouse Model	68
3.1 Summary	68
3.2 Introduction.....	68
3.3 Materials and Methods	71
3.4 Results	74
3.5 Discussion	84
CHAPTER 4.....	90
Mucosal Tissue Drug Distribution of HIV-1 Integrase Inhibitor Raltegravir in a Humanized Mouse Model	90
4.1 Summary	90
4.2 Introduction.....	91
4.3 Materials and methods	92
4.4 Results	95
4.5 Discussion	101
CHAPTER 5.....	108
Combinatorial Pre-Exposure Prophylaxis:	108
Mucosal Tissue Drug Distribution of HIV-1 RT Inhibitor Tenofovir, Integrase Inhibitor Raltegravir and Entry Inhibitor Maraviroc Following Combinatorial Oral Application in a Humanized Mouse Model.....	108
5.1 Introduction.....	108
5.2 Materials and methods	109
5.3 Results	112
5.3.1. Pharmacokinetics of combinatorial Raltegravir and Tenofovir oral application.....	112
5.3.2. Pharmacokinetics of combinatorial Maraviroc and Tenofovir oral application	121
5.3.3. Pharmacokinetics of combinatorial Raltegravir and Maraviroc oral application.....	130
5.3.4. Summary of the comparison between plasma and tissue ARV kinetics following single and dual drug application.....	136
5.4 Discussion	138
CHAPTER 6.....	142
Topical PrEP: Pharmacokinetic and Pharmacodynamic (PK-PD) Modeling	142
In Humanized Mice	142

6.1 Pharmacokinetic study – mucosal tissue exposure of antiretrovirals TFV, MVC and RAL following topical application (microbicide gel formulations) in a humanized mouse model ...	142
6.1.1. Introduction	142
6.1.2. Materials and methods	144
6.1.3. Results	145
6.1.4 Discussion.....	147
6.2 Pharmacodynamic (protection) study of antiretrovirals TFV, RAL and UC781 following topical application (gel formulations) in a humanized mouse model.....	149
6.2.1. Introduction	149
6.2.2. Materials and methods	150
6.2.3. Results	152
6.2.4. Discussion.....	157
CHAPTER 7	160
Summary and Future Directions	160
REFERENCES	164

CHAPTER 1

Introduction

1.1 Discovery of HIV-1

The discovery of human immunodeficiency virus (HIV-1) and its identification as the causative agent of AIDS were one of the major achievements of virology in the 20th century. In the year 1981, an increase in rare opportunistic infections, like pulmonary *Pneumocystis carinii*, and occurrence of rare neoplasms such as Kaposi sarcoma, was first reported. The article “*Pneumocystis Pneumonia*–Los Angeles” by a group of physicians at University of California in Los Angeles (UCLA), published in Center for Disease Control (CDC) “Morbidity and Mortality Weekly Report” journal in 1981, was the first account of AIDS pathology in the literature [1]. As the number of reports with unusual clinical manifestations and unknown origin continued to increase, the Centers for Disease Control (CDC) formed a Task Force on Kaposi’s sarcoma and Opportunistic Infections, followed by Epidemiology Working Group on Kaposi’s sarcoma by National Cancer Institute (NCI) and National Institutes of Health (NIH) Working Group establishment. Soon after, the disease was identified as infectious in origin, certain groups such as intravenous drug users, homosexuals and hemophiliacs were shown to be at high risk and the first case of blood-transfusion associated AIDS was documented in a newborn [2-6].

In 1983, the team of researchers from the Pasteur Institute in Paris, led by Dr. Luc Montagnier reported the isolation of a new retrovirus Lymphadenopathy-associated virus (LAV) claiming that the agent is associated, but not identifying it directly as a causative agent of AIDS [7]. The virus was isolated from a lymph node of an AIDS patient with generalized lymphadenopathy, a hallmark of the pre-AIDS disease stage seen in many clinical cases at the time. The same year, the first large longitudinal studies of high risk populations, namely the Multicenter AIDS Cohort Study (MACS) and the San Francisco Men’s Health Study (SFMHS),

as well as international collaborative project SIDA (Syndrome d'immunodéficience Acquise) based in Kinshasa, Zaire, were launched. In 1984, it was announced that Dr. Robert Gallo and his team at the NCI identified the retrovirus causing AIDS and named it Human T-cell Lymphotropic virus-type III (HTLV-III), documenting the findings in a series of publications [8-12]. Simultaneously, the team of Dr. Levy at UCSF published an analysis of the lymphocytotropic retrovirus (AIDS-associated retrovirus, ARV) isolated from AIDS-affected homosexual population, indicating a close association between the isolated retrovirus and AIDS [13]. In both cases the origins of viral isolates were combined blood cells from AIDS patients.

Following isolation of the virus, further scientific advances included complete genetic sequencing of HTLV-III and LAV, as well as the identification of CD4 on T helper lymphocytes as the entry receptor [14-17]. HTLV-III and LAV were soon recognized as the same virus, and named by International Committee on Taxonomy of viruses as Human Immunodeficiency Virus (HIV-1) in 1986 [18]. In 1985 the first AIDS antibody test, which was Enzyme-linked immunosorbent assay (ELISA)-based, was developed and the first studies on the antiretroviral Azidothymidine (AZT) were started, which led to the FDA approval of AZT as the first agent for AIDS treatment in 1987 [19]. The World Health Organization (WHO) launched its Global Program on AIDS in 1987, and that same year the first Western blot-based HIV-1 diagnostic test was approved by the FDA [20, 21].

In these early years, HIV-1/AIDS went from a rare pathology seen in a certain social groups to a global pandemic and the full extent of this pandemic was only recognized as diagnostic tools became available and the criteria for diagnosis were better defined. Based on the epidemiological reports from the early 1980s, HIV-1 appeared suddenly in the population, causing a widespread epidemic in just a couple of years [4, 5, 22]. The emergence and the scale of the epidemic raised numerous questions regarding the origin of the HIV-1, which will be briefly discussed in the following subchapter.

1.2 The Origin of HIV-1 and genealogy data

As for many infectious agents, AIDS as a disease emerged in the population and spread before the scientists were able to identify the underlying cause. The sudden onset, as seen according to the epidemiological data predominantly from the US, in addition to the severity of the disease prompted the questions regarding the source of the virus. We now know that hidden in the natural animal reservoir, HIV-1 has evolved over time and finally jumped to another species. Unfortunately, in this case the new host was the human species. This represents one of the remarkable aspects of the emergence and the origin of HIV-1. In case of zoonotic infectious diseases the tropism switch happened and enabled the viruses to infect multiple species, including humans, but rarely did the virus emerge as almost 100% fatal in the human population, posing a global danger and causing a pandemic in a relatively shorter amount of time, as did HIV-1.

HIV-1 originated from recurrent transmissions of different strains of simian immunodeficiency virus (SIV) from multiple nonhuman primate (NHP) species to humans in West and Central regions of Africa [23, 24]. Thus, the initial HIV-1 infections happened in the form of zoonoses. The exposure events suspected to allow for the transmission include hunting and preparing of primate meat (bushmeat), as well as illegal trading of NHP. The existing HIV-1 groups (lineages) are M (main, major), N (non-M, non-O), O (outlier) and recently discovered P lineage, while HIV-1 type 2 virus has been classified into groups A to H [23]. The HIV-1 lineage M has been the cause of the global disease spread and pandemic, and this predominant type of HIV-1 developed from the chimpanzee (*Pan troglodytes troglodytes*) virus - SIV_{cpz} found in natural animal reservoirs in Central Africa [24]. The initial spread that preceded global epidemic was probably through the Congo River basin to urban Kinshasa, from where infection spread further, enabled and facilitated by increasing interconnectivity of the African continent and the rest of the world. HIV-1 group N originated from a different subset of SIV_{cpz} compared to the M

lineage, while HIV-1 group O and group P are thought to have evolved from SIV_{gor} strains in wild-living, natural gorilla (*Gorilla gorilla gorilla*) reservoirs in West Africa [23, 25]. The oldest known human samples infected with HIV-1 are serum and a lymph node biopsy sample from Kinshasa dating back to 1959 and 1960, respectively, indicating the existence of the infection in the population several decades before the start of the epidemic in the 1980s [26, 27]. The time of different HIV-1 lineage emergence has been modeled by specific computerized molecular clock programs, which are able to track back the most recent common ancestors (tMRCAs) between HIV-1 and corresponding SIV strains and define evolutionary rates of HIV-1 [25, 28, 29]. The molecular clock programs, enhanced in their abilities by the recent progress in genome sequencing, have defined probable years of origin for HIV-1 group M within the first two decades of the 20th century, and similar findings are available for the O lineage, while the N group is thought to have emerged through a later transmission event in the second half of the last century [28, 29].

The NHP hosts are able to efficiently control SIV since infections are lifelong and do not cause immunopathology or result in lethal immunodeficiency as in humans. Thus, the analysis of the defense mechanisms that NHP developed through centuries of serving as hosts for multiple SIV strains is an intriguing aspect of SIV/NHP studies [30, 31]. Due to a high degree of homology between HIV-1 and originating SIV strains, these efficient viral control mechanisms could potentially be transferred to humans, as a form of an immunomodulatory therapy against HIV-1 [31, 32]. The study of parental SIV_{cpz} and SIV_{gor} strains can also offer clues regarding the evolution of HIV-1 and further our knowledge regarding potential future directions of HIV-1 evolution in human population [28]. The viral evolution continues, for HIV-1 in human population as the virus diversifies into multiple subtypes, as well as for SIV in natural animal NHP reservoirs, and it is most probable that additional new cross-species transmissions will occur. The newly identified recombinant form of HIV-1 named A3/CRF02, which is a fusion of the two dominant circulating strains in Western Africa, has been shown to progress to AIDS faster than

any of the over 60 different epidemic strains circulating globally [33]. This recent finding emphasizes the need for surveillance and monitoring of HIV-1 subtypes in human population as well as in natural reservoirs in order to develop the methods of predicting the next emerging viral form, which usually has higher fitness and accelerated disease progression, as in the case of HIV-1 A3/02.

As indicated above, HIV-1 has evolved from its SIV ancestors into a virus capable of infecting humans; moreover, it evolved into a virus able to efficiently evade human immune responses and finally a virus that establishes a life-long latent infection within its human host. In the next several subchapters the structure and the life cycle of HIV-1 will be described, with the emphasis on the mechanisms that enable HIV-1 to be such a successful, immunoevasive and resistant human pathogen.

1.3 Structure of HIV-1

Based on the viral taxonomy classification HIV-1 belongs to the family *Retroviridae*, subfamily *Lentivirinae* and to the genus *Lentivirus*. As in other members of the *Retroviridae* family, the HIV-1 genome consists of a single positive-sense strand RNA and belongs to Group VI of (+)ssRNA-RT viruses with DNA intermediate within the Baltimore classification [34-36].

The HIV-1 virion is spherical in shape and has a 120nm diameter. Each virion carries two copies of the genomic RNA. The RNA genome is stabilized by the nucleocapsid (p7) and is surrounded by a cone-shaped capsid which consists of approximately 2000 copies of the viral protein p24 [37]. The capsid is surrounded on the outside by the matrix protein (p17) shell and the HIV-1 viral envelope, which is formed from the host cellular lipid bilayer membrane during the budding event from the surface of the infected cell. Host proteins, such as actin and major histocompatibility complex (MHC) antigens, are still present in the viral envelope of the newly formed virions together with viral Gag and Pol proteins. The glycosylation pattern on the surface

of the viral particle is also inherited from the host, enabling one form of HIV-1 molecular mimicry and immune evasion, which will be discussed later in more detail with regard to the humoral immune response to HIV-1 infection. Viral envelope proteins are organized into heterodimer subunits embedded into the lipid bilayer, consisting of the surface glycoprotein (gp) gp120 noncovalently attached to the transmembrane glycoprotein gp41. The gp120/gp41 subunits form trimers visible in electron micrographs as sparsely dispersed spikes on the surface of the viral envelope [38, 39]. Both gp120 and gp41 have crucial roles in the viral entry, which will be described in the next chapter. In addition, as the most exposed part of the virus, the envelope and in particular gp120 represent the most variable regions of HIV-1. Based on the degree of variability gp120 has been divided into distinct more conserved core and surface-exposed variable loops (V1 to V5) [38-40]. Single point or multiple complex mutations in V loops, and in particular V3 loop, drive viral antigenic diversity and escape from the antibody response of the host, and will be described in more detail in the humoral response section [40]. Specific mutation patterns in the V3 region of the gp120 have also been defined as events leading to the co-receptor switch from CCR5 usage early in the course of the disease to CXCR4 co-receptor usage later with progression to AIDS [41].

The HIV-1 genome contains nine genes which encode for 19 different viral proteins (Fig 1). In order to be able to encode for multiple proteins with a relatively small genome, HIV-1 has a complex genome structure and multiple overlapping open reading frames (ORFs). Overlapping genes (such as *env*, *tat* and *rev*) are transcribed from different ORFs within the same genomic region [42, 43]. The genome also contains 7 distinct regulatory elements, namely long terminal repeats (LTRs), trans-activation response element (TAR), *rev* response element (RRE), *psi* element (PE), poly(T) slippery site (SLIP), cis-acting response sequences (CRS) and inhibitory RNA sequence (INS) [42, 43].

When integrated into the human host cellular genome, the size of HIV-1 provirus is approximately 9.8 kilobases [42]. LTRs are located at both ends of the provirus, and 5' and 3'

LTRs have important roles in controlling the proviral gene expression as an RNA Polymerase II promoter, and in transcriptional termination and proviral transcript polyadenylation, respectively [44]. The central part of the genome, flanked on both sides by LTRs [43], encodes for 4 different groups of viral proteins with their overlapping ORFs (Fig 1.1):

1. Main structural proteins – Gag and Env
2. Main catalytic polyprotein – Pol
3. Regulatory proteins – Tat and Rev
4. Accessory proteins - Vpu, Vpr, Vif, and Nef

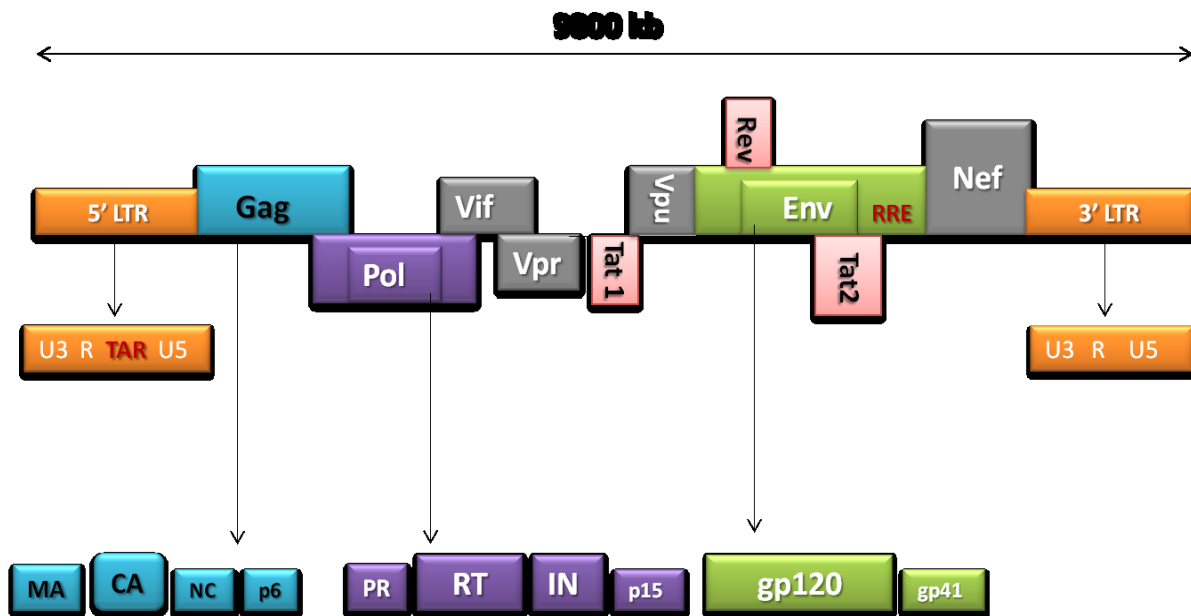


Figure 1.1. HIV-1 genome and proteins. Structural proteins cleaved from Gag polyprotein: MA-matrix (p17), CA-capsid (p24), NC-nucleocapsid (p7) and p6. Enzymes cleaved from Pol polyprotein: PR-protease (p10), RT-reverse transcriptase (p50), IN-integrase (p31) and p15 (RNaseH). Envelope proteins cleaved from Env polyprotein (gp160): external gp120 and transmembrane gp41. Regulatory genes: Tat (splicing variants Tat1/2) and Rev. Accessory protein genes: Vif, Vpr, Vpu and Nef. Regulatory elements: LTR- long terminal repeat (U3, R and U5 regions); TAR - trans-activation response element; RRE - Rev response element.

1.3.1. Structural HIV-1 proteins

Gag. Gag precursor protein is generated by transcription of the GAG gene and translation from the unspliced viral mRNA, and has a molecular weight of 55 KDa (p55). Post-translation, the N-terminus is modified by myristoylation, which allows for the association with the cytoplasmic side of the cellular membrane, and subsequent recruitment of two viral RNA genome copies, and additional viral and host cell proteins [45]. This complex formation results in the budding event of the newly formed viral particle from the surface of the infected cell. Within the new virion, p55 is cleaved into four different structural proteins, namely matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7) and p6 [46]. The MA protein remains attached to the viral lipid bilayer, as it is formed by the cleavage of the p55 N-terminus which is in contact with the viral lipid bilayer-forming cellular membrane. The role of p17 is to stabilize the structure of the viral particle by forming the matrix protein shell. Additionally, a subset of MA proteins are incorporated into the pre-integration complex (PIC), which contains viral cDNA, and mediates transport of the PIC into the nucleus through the recognition of a nuclear transport motif within the MA protein [47]. This event is crucial for the ability of HIV-1 to infect nondividing cells and it represents an important advantage in the process of establishing both active infection and latent HIV-1 reservoirs [48]. The capsid protein (p24) forms a conically shaped HIV-1 core by multimerization of approximately 2000 p24 subunits. The NC protein recognizes a packaging signal (four stem-loop structures) on the 5' end of the viral RNA, and mediates incorporation of the RNAs into HIV-1 virions [49]. The NC protein has also been implicated in the facilitation of the reverse transcription of the viral RNA [50]. Finally, the p6 protein enables Gag-Vpr protein interactions and subsequent Vpr incorporation into the immature virion, which has a role in the late stages of the budding and virion release [51].

Gag-Pol precursor. The Gag-Pol precursor is generated by ribosomal frame shifting, triggered by a specific RNA motif located in the distal region of the Gag RNA. It is estimated that

the shift happens 5% of the time and translation continues within the pol reading frame without interruption [52]. The resulting Pol polypeptide is cleaved during maturation by the viral protease apart from Gag and then into four distinct proteins, namely protease (PR, p10), reverse transcriptase (RT, p50), RNase H (p15), and integrase (IN, p31). HIV-1 protease functions as a dimer, belongs to the class of aspartyl proteases, and cleaves Gag and Gag-Pol polypeptide precursors into multiple functional proteins during virion maturation, as previously described. The HIV-1 protease enzyme is one of three main targets of antiretrovirals developed for HIV-1 therapy thus far. The RT is the enzyme responsible for the process of reverse transcription, in which a double-stranded DNA copy of the viral RNA genome is made. This process represents a distinct feature of retroviruses. One of the crucial characteristics of HIV-1 RT, which has both RNA- and DNA-dependent polymerase activities, is the lack of the proofreading activity, which results in the highly error prone replication and extremely high HIV-1 mutation rates [53]. The RNase H protein is also required for reverse transcription, as it removes the original RNA template to allow for the generation of a second, complementary DNA strand following reverse transcription. HIV-1 integrase mediates integration of proviral DNA into the host genome after the formation of PIC and its entry into the nucleus [54]. Proviral genome integration represents another distinct feature of retroviruses and a detailed overview of the mechanisms will be given in the next chapter, as protease, reverse transcriptase and more recently integrase represent major targets of anti-HIV-1 therapeutics.

Env. The Env protein is translated from a singly spliced viral mRNA and is glycosylated during the transport from the endoplasmic reticulum through the Golgi apparatus with the addition of 25-30 complex N-linked glycans. The 160 KDa precursor Env protein (gp160) is cleaved into gp120 and gp41 by host proteases and embedded as a trimer of gp120/gp41 heterodimers into the lipid bilayer of the viral envelope [55]. The role of the envelope proteins in viral entry and immune escape mechanisms will be discussed in the next couple of subchapters. A recently developed class of antiretrovirals called fusion inhibitors target gp41 and have shown

promising results thus far. Additionally, because of its display on the surface of virions, a large portion of the host humoral immune response is directed against Env, which contains multiple neutralizing antibody epitopes. The role of the Env in shaping the humoral immune response and recent advances such as the discovery of broadly neutralizing antibodies against Env, will be discussed further in the second chapter.

1.3.2. Regulatory HIV-1 proteins

The viral regulatory proteins Tat and Rev are essential for HIV-1 replication [56].

Tat. Two distinct forms of this transcriptional transactivator are translated from the same viral mRNA, which is either fully spliced to form the 72 amino acid Tat protein or incompletely spliced to form a longer, 102 amino acid Tat protein. Tat acts by binding to the transactivation response element (TAR) on the 5' end of the viral RNA, enhancing the basal level of transcription from the LTR by at least 1000-fold [57]. The mechanism of Tat transcriptional activation will be described in detail in the viral cycle subchapter.

Rev. This 19kDa regulatory factor binds to the Rev response element (RRE) in the second intron region of the HIV-1 genome, inducing a switch from early to late phase of HIV-1 gene expression from the integrated proviral DNA [56]. The Rev-induced switch facilitates nuclear export of unspliced and incompletely spliced HIV-1 RNAs which would otherwise be retained in the nucleus. This process is regulated by a negative feedback loop triggered thorough extensive accumulation of aberrant RNA and a decrease in RNA splicing, which ultimately reduce Rev protein levels. Rev is required for HIV-1 replication and generation of late viral products and infectious virion progeny; nevertheless, it has not been targeted by any of the currently approved therapeutic approaches.

1.3.3. Accessory HIV-1 proteins

Accessory proteins are not essential for HIV-1 replication and viral production *in vitro* in the immortalized T-cell lines. However, each HIV-1 accessory protein plays an important role in evading the host cell response to HIV-1 infection and promotes HIV-1 virulence *in vivo* [58-60].

Nef. The NEF gene is located at the 3' end of the HIV-1 genome, overlapping partially with the 3' LTR and is one of first transcribed, early HIV-1 genes. Nef increases HIV-1 virulence by multiple mechanisms, including downregulation of CD4 and MHC class I molecules from the surface of infected cells [61]. By independent mechanisms, which are still being analyzed, Nef increases the infectivity of the HIV-1 virions, which have decreased fusion ability and reduced rate of the proviral DNA synthesis in the absence of Nef. *In vivo*, in long-term non-progressors and in cases of SIV infection using Nef-defective mutants, it has been shown that the disease progression is delayed [62].

Vpr. This accessory protein is crucial for the infection of nondividing cells by HIV-1, as it tethers the PIC to the nuclear pore and enables the transport of PIC to the nucleus [63]. In addition, Vpr induces cell cycle arrest in the G2 phase by dysregulation of the p34 cdc2/cyclin B complex [63]. It is also a LTR-transcription enhancer [63].

Vpu. The Vpu protein is expressed from the Env encoding mRNA, but its translation is attenuated compared to Env due to inefficient translation initiation codon. By triggering the ubiquitin-mediated degradation of CD4 molecules from the complex with Env in the endoplasmic reticulum, Vpu enhances the process of virion assembly by increasing the availability of Env molecules [58]. Vpu also enhances the release of virions through the formation of channel-like structures by oligomerization in the plasma membrane [64].

Vif. This accessory protein is necessary for HIV-1 replication in primary cells (CD4+ T cells, macrophages) and in certain T cell lines (non-permissive cells), due to the incomplete synthesis of the proviral DNA in the absence of Vif. Interaction of Vif with the cellular restriction

factor APOBEC3G helps block the antiviral activity of APOBEC3G and viral replication is enhanced [58]. This interaction and viral inhibition is considered a part of the innate antiviral response, and represents an attractive target for future therapeutic approaches [65].

1.3.4. Regulation of gene expression from the proviral genome

The HIV-1 gene expression is under complex control from both viral and host cellular factors, which act at the transcriptional or posttranscriptional level [56]. All HIV-1 genes can be divided into early and late genes. Transcription of early genes *tat*, *rev* and *nef* is Rev-independent, and these proteins are the first ones to accumulate within infected host cells. Expression of the late genes *gag*, *pol*, *env*, *vpr*, *vpu* and *vif* requires functional Rev to mediate nuclear export of unspliced or incompletely spliced viral mRNAs to the cytoplasm for translation. The regulation at the transcriptional level is through the only promoter in the HIV-1 genome, located in the 5' LTR. The 5' LTR itself is comprised of the U3 and U5 regulatory regions, in between which is the R region, where the transcription is initiated. Cellular transcription factors, both constitutive and inducible, initiate transcription from the viral 5' LTR and in this process most transcripts are short. This abortive transcription is under the control of the downstream element within the LTR, called the inducer of short transcripts (IST). Nevertheless, the process is leaky and a low number of complete transcripts that encode the Tat protein allow the Tat/TAR axis to enhance the level of viral transcription by more than a 1000-fold.

The primary transcript (approximately 9kb long) is differentially spliced into more than 30 species of mRNA, which are translated into the array of HIV-1 proteins [56, 66]. Three main types of HIV-1 mRNAs are unspliced mRNAs (generating Gag and Gag-Pol precursor proteins), incompletely spliced mRNAs (splice donor site nearest to the 5' end of the genome, which can generate Env, Vpr, Vpu, Vif and shorter Tat form) and completely spliced mRNAs (both HIV-1 genome introns spliced, these can generate Rev, Nef and longer Tat form). Alternatively, the

primary transcript (unspliced mRNA) is unmodified and incorporated into the newly formed viral particles, as the viral RNA genome [67].

1.4 HIV-1 life cycle

The HIV-1 life cycle consists of six main phases (Fig 1.2):

1. Binding and entry into the target cell
2. Reverse transcription of the viral RNA genome into cDNA
3. Integration of the proviral DNA into the host genome
4. Transcription of viral proteins from the proviral DNA
5. Assembly and maturation of the viral particles
6. Release of the new infectious virions



Figure 1.2. HIV-1 life cycle and stages targeted by currently approved antiretrovirals.

Binding and entry into the target cell. HIV-1 binding to the cellular surface is mediated by the viral Env glycoproteins gp120 and gp41. Initial step is the binding of gp120 to the CD4 receptor (immunoglobulin superfamily member), which represents the only cellular receptor capable of this binding. This limits the tropism of the virus to CD4-expressing cells, such as monocytes/macrophages and T cells, although there are exceptions from this rule such as brain microglia and dendritic cells [68-71]. The gp120 CD4 binding site (CD4bs) is formed by three elements of the gp120 core (inner, outer domain and the bridging sheet) which are in contact with N-termini of the four immunoglobulin domains of the CD4 receptor. The CD4bs is a highly conserved region of gp120 and represents a target for the generation of broadly neutralizing

antibodies (bnAb) to its conserved epitopes. One of the new generation bnAb, VRC01, was tested in a topical gel protection study presented here and has shown considerable level of protection against mucosal HIV-1 challenge in humanized mice (Chapter 2). The binding of gp120 to CD4 induces a conformational change - the movement of the gp120 V1/V2 variable loops, unmasking the chemokine co-receptor-binding site, allowing subsequent binding to the chemokine co-receptor [72-74]. The chemokine co-receptors belong to the 7-transmembrane G-protein coupled receptor family, and HIV-1 Env binds to either CCR5 on monocytes, macrophages and memory T cells or to CXCR4 on naïve and memory T cells. Initial HIV-1 infections are mostly CCR5-tropic (R5 tropic viruses), and the switch to CXCR4 (X4) tropic or R5X4 dual tropic viruses often arises later during infection as the disease progresses to AIDS [75]. The co-receptor binding event induces further conformational changes in Env, which allow gp41 to interact with the plasma membrane, inserting its hydrophobic N-terminus into the host membrane and initiating fusion of the viral Env and host membranes resulting in release of the viral core into the cytoplasm [76]. After the uncoating event and degradation of the viral core, the components necessary for the reverse transcription are released into the cytoplasm. In the event of direct cell-to-cell transfer of HIV-1, receptor and co-receptor binding by viral Env triggers formation of specific virological synapses, which enable cell-to-cell transmission and evasion of the immune response [77].

Reverse transcription of the viral RNA genome into cDNA. This is a multistep process in which single stranded RNA genome is converted into double stranded proviral DNA, which will be integrated into the host genome. RT lacks proofreading activity, leading to high mutation rate – on average 1-10 errors per genome and per round of replication, and resulting hypervariability of HIV-1 genome [53].

Integration of the proviral DNA into the host genome. Following reverse transcription, proviral DNA creates a complex with the IN, Vpr and MA viral proteins, as well as multiple cellular factors, to create the PIC. Transport of PIC to the nucleus is mediated by MA

and Vpr and enabled by the cellular microtubule system, which is partially “highjacked” by HIV-1 [78] [79]. Integrase is the main enzyme mediating the integration of the proviral DNA into the genome following PIC entry into the nucleus. Integration has three separate phases [54]. The first phase, which happens prior to PIC transport to the nucleus, is characterized by Integrase cleaving two nucleotides from each 3’ end of the linear viral DNA duplex (exonuclease activity of the integrase). Within the nucleus, Integrase utilizes its double-stranded endonuclease activity to cleave host DNA at the integration site. Host factors LEDGF/p75 enable tethering of the viral DNA to the host chromatin, and the ligase activity of Integrase generates a single covalent linkage at both ends of the proviral DNA, resulting in ligation of the 3’ overhang of the proviral DNA to the 5’ end of the host DNA [80]. The remaining unpaired flanking regions and gaps are repaired by the cellular DNA repair machinery, as the integrated DNA is not recognized as foreign. HIV-1 preferentially integrates into transcriptionally active regions of the genome and this preference is thought to be determined more by the chromatin condensation state and accessibility of the host genome, rather than the host DNA sequence itself [81]. Non-integrated proviral DNA is quickly degraded in the cytoplasm (hours to days) and does not represent a significant template for the generation of viral mRNAs and proteins [82]. Integrated viral DNA is either actively transcribed along with the host genes, enabling active HIV-1 replication, or it enters a dormant, non-transcribed state known as HIV-1 latency [83].

Transcription of viral proteins from the proviral DNA. This phase of the viral cycle as well as the mechanisms of its regulation have been described in the previous subchapter.

Assembly of immature viral particles, virion maturation and release of new virions. The main viral factor in the formation of the new virions is the Gag polyprotein. Gag is cleaved into four functional viral proteins - MA, CA, NC and p6, which are assembled together with the two full length RNA genome transcripts into the nascent viral particles [84]. Env glycoproteins are trafficked through endoplasmic reticulum and then Golgi by a Gag-independent mechanism, and then recruited to the plasma membrane based on a signaling motif within gp41. For the

budding process, HIV-1 proteins utilize the host ESCRT pathway (Endosomal Sorting Complexes Required for Transport), namely the ESCRT-I and ESCRT-III proteins [85]. The release of virions is facilitated by Vpu in the late stages of the replication cycle, through Vpu interaction with tetherin [86]. This interaction blocks the tetherin antiviral function, the blockage of the release of HIV-1 viral particles by binding (tethering) them to the plasma membrane. Finally, maturation of released virions is catalyzed by the viral protease, which cleaves Gag and Pol, inducing a condensation and stabilization of the genomic viral RNA. Together, these processes result in the formation of mature HIV-1 virions with the characteristic matrix shell, conical capsid and nucleocapsid structure which contains individual fully functional HIV-1 proteins. Mature virions are able to infect new cellular targets and propagate the infection further.

1.5 HIV-1 infection in humans

HIV-1 infection in humans is characterized by several distinct phases, which include viral transmission, acute phase, chronic (asymptomatic) phase and the development of Acquired Immune Deficiency Syndrome (AIDS) (Fig 1.3).

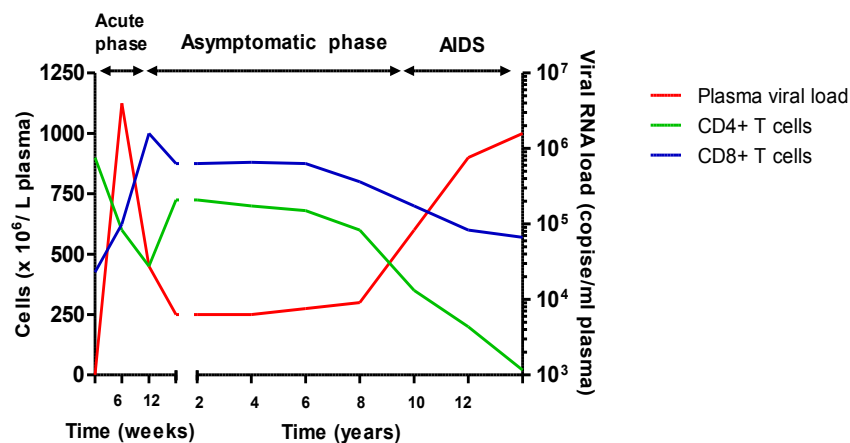


Figure 1.3. HIV-1 infection course in humans.

1.5.1. HIV-1 Transmission and early infection.

The primary route of HIV-1 transmission is sexual contact with an infected person, resulting in exposure to cell-free or cell-associated virus in the semen, cervicovaginal fluid or mucosal surfaces [87]. Other modes of transmission include vertical (mother to child) transmission and parenteral transmission (infection via injection drug use, accidental exposure in medical or laboratory settings, exposure of blood or blood products via transfusion). The focus of the research presented here is modeling and development of effective preventative strategies against mucosal HIV-1 transmission in a humanized mouse model; therefore, the mechanisms of this type of transmission will be discussed in detail. The fact that more than 90% of all HIV-1 transmissions globally are through mucosal route emphasizes the need for such preventative approach [87, 88].

For sexual transmission of HIV-1 the overall rate of infection per one unprotected event is estimated to be 0.08% for male to female and 0.04% for female to male transfer [89]. The rate of transmission increases proportionally with viral load, which is the number of HIV-1 particles per ml of human blood [90]. The rate of transmission is also increased in persons with conditions such as chronic mucosal infections, genital infections and the presence of other sexually transmitted diseases (STDs), which compromise integrity of the protective mucosal barrier in the genital tract and recruit higher numbers of immune cells to the mucosa, which can then be infected by the virus [87]. The female population is at higher risk of acquiring HIV-1 through sexual intercourse due to intrinsic anatomical, physiological and hormonal makeup, which enhances the probability of transmission [91]. One additional reason for higher infection rates is the composition of the vaginal microbial flora, which can represent a protective factor or an enhancing one, depending on the part of the menstrual cycle [92]. High progesterone levels and consequent alterations in the vaginal microbiota and mucosal barrier are thought to increase the probability of HIV-1 infection [92].

Despite decades of research, due to the rarity of early HIV-1 diagnosis in humans and inability to follow initial HIV-1 infection in patients, as well as the lack of adequate experimental models, the early events in the HIV-1 transmission and establishment of infection remain poorly defined. Nevertheless, NHP models have allowed for significant insight into the early events SIV or SHIV-1 infection *in vivo*, showing high level of analogy to HIV-1 [93, 94]. These *in vivo* studies are also complemented by *ex vivo* studies utilizing genital or rectal mucosa explants [95-97].

From the SIV studies, the first targeted cells in the mucosa were identified as partially activated CD4 T cells, which express the CCR5 co-receptor [98]. The initial productive replication happens in this cellular population within the first couple of days following exposure. The events preceding infection in this founder population of CD4 T cells are the least understood and include migration of the virus through the mucosal barrier and interactions of the virus with dendritic cells (DCs), such as resident Langerhans cells (LCs) and macrophages [88, 99]. It is thought that the formation of a virological synapse between DCs and CD4 T cells enables productive infection in the founder CD4 T cell population [100, 101]. Host cellular factors such as integrins and virus-harboring endosomal compartments have been implicated in synapse formation [100]. In addition to this first hypothesis of infection by virological synapse, a second hypothesis is that the virus is protected and carried across the epithelia by LCs, which migrate to the deeper mucosal layers to infect founder CD4 T cells [102]. Recent insights indicate that the virus might be using a combination of both mechanisms for the establishment of the early founder population.

The second step, also defined in NHP and later confirmed in humans, is the propagation of infection locally within the mucosa to fully activated CD4 T cells. Fully activated CD4 T cells are more susceptible to virus entry because they express higher levels of HIV-1 co-receptors than naive or weakly activated cells, and also have increased rates of replication and viral production due to the activation of signaling pathways and elevated expression of transcription

factors promoting transcription of the integrated provirus [98, 103]. The third step, which happens within the first week following exposure, is the migration of HIV-1 into the local draining lymph nodes [98, 103]. At this point the systemic infection is eminent and any prevention strategy has lost the chance to be effective. The fourth phase is dissemination into lymphoid tissues systemically, primarily the gut-associated lymphoid tissue (GALT). In this phase a dramatic decrease in CD4 T cells is seen, due to local depletion in the GALT, which represents the major lymphoid reservoir in the human body [104-106].

Despite the low rate of transmission per sexual contact/exposure, defined based on the large epidemiological studies, it is thought that the successful HIV-1 infection in humans can be established by a single viral particle transfer [107]. This fact emphasizes the importance of attacking the virus early during the founder “bottleneck”, while the opportunity to reduce the rate of replication to below $n=1$ still exists [103]. The probability of blocking a single viral particle from transfer through mucosal barrier seems less probable as an efficient strategy for HIV-1 prevention, especially in the context of already disrupted mucosa in vast percentage of population (infections, STDs, hormonal factors, etc.).

1.5.2. Acute HIV-1 syndrome and establishment of a viral set point.

Acute HIV-1 syndrome develops in most HIV-1 infected individuals within the first couple of weeks to a month following transmission. Clinical manifestations include fever, generalized lymphadenopathy, flu-like symptoms such as headaches, myalgias, anorexia, diarrhea and skin rashes, accompanied by high plasma viremia, which can reach millions of copies per ml of blood [108]. This dramatic increase in viral load is accompanied by a pronounced decrease in CD4 T cells, and has been connected to propagation of the HIV-1 infection in GALT. Massive CD4 depletion is a consequence of both direct viral killing of infected cells in the GALT and of the bystander cell death effect, defined as the triggering of apoptotic cell death in the uninfected

CD4 T cells and other immune cell populations such as CD8 T cells in the GALT [104-106].

Following the acute phase, HIV-1 viral load declines over several months and establishes a steady state viremia level, known as the viral set point [108]. Viral set point is a prognostic clinical parameter because it correlates well to the rate of the disease progression in untreated patients, with a lower viral set point associated with slower disease progression [109]. In this stage CD4 T cell levels also stabilize, rebounding from the pronounced decrease seen in the acute phase. During this stage, the virus is disseminated from its initial reservoirs such as GALT to peripheral lymphoid tissues, primarily lymph nodes, where it rapidly establishes stable and permanent viral reservoirs [110]. CD4 depletion is present in the lymph nodes but it is not as pronounced as in the initial spread in the GALT and these differences are an active area of research into HIV-1-induced pathogenesis [111].

1.5.3. Immune response – failure of the immune system against HIV-1.

The initial founder virus is thought to originate from a single viral particle or a single HIV-1 infected cell in the majority of transmissions [107]. During the early acute phase of HIV-1 infection, a surge in viral load and pronounced CD4 depletion are happening in the absence of an immune response. Studies of the early viral founder populations have shown that the envelopes of these viruses lack extensive glycosylation of gp120 and are highly susceptible to neutralization without the protective glycan shield [112]. This initial, low diversity HIV-1 population is an important target in therapeutic and recent preventative approaches. The acute phase lacking an adaptive immune response is followed by the development of HIV-1 specific T and B cells, and the viral population rapidly evolves and diversifies in response to the selective pressures of the adaptive immune system. The adaptive response is too narrow and starts too late in the infection, and HIV-1 is able to successfully escape and evolve into multiple neutralization-resistant forms, from the initial neutralization-sensitive viral population [113]. The

mechanisms of viral escape include both increased gp120 glycosylation, escape mutations generated by the error-prone RT that convert immunodominant viral peptide sequences into poorly recognized sequences, and conformational changes, such as expansion or contraction of the Env variable loops, which “bury” or hide the epitopes of broadly neutralizing antibodies and block the development of an efficient humoral response [39, 112]. Therefore, the three main protection strategies of the viral Env spike are defined as the glycan shield, variability of immunodominant epitopes and conformational masking of the conserved Env epitopes for neutralizing antibodies. It has been shown that the viral evolution involving these three mechanisms is followed to a certain extent by an evolution of the humoral response, but the resulting neutralizing potential in most cases remains low and is not able to efficiently target functionally restrained highly conserved epitopes in Env [114, 115]. The isolation of several broadly neutralizing antibodies from the “elite controllers” (HIV-1-infected individuals who do not progress to AIDS for decades in the absence of antiretroviral treatment) has revolutionized the approach of immunogen design for future vaccines by revealing the sensitive and highly conserved epitopes of HIV-1 Env that may be effectively targeted by antibodies, and demonstrating that a neutralizing antibody response can be generated *in vivo* and can control HIV-1 efficiently [116]. When tested *in vitro* and *in vivo* in NHP and humanized mouse models, these new bnAb were able to neutralize a high percentage of primary HIV-1 isolates, with the IC₅₀ in the micromolar or nanomolar range [117]. Four main classes of bnAb have been defined:

- 1) CD4 binding site (CD4bs) bnAb such as b12, VRC01, HJ16 and CH103
- 2) bnAb to the membrane-proximal external region (MPER) of gp41 such as 2F5, 4E10, 10E8 and Z13
- 3) bnAb to the Env glycans in the V3 region such as 2G12 and newer generation PGT121, PGT128, PGT135
- 4) the class of bnAb to the conformational, quaternary epitopes in the variable loops V1/V2, such as PG9, PG16 and PGT145

Following the discovery of multiple new generation bnAb from 2009 to 2012, current efforts are aimed at deciphering the path to generating bnAb-producing lineages of B cells *in vivo*, which is proving to be a rather difficult task. Most bnAb have atypical characteristics, such as extensive affinity maturation (somatic hypermutation), long (> 25 amino acid residues) complementarity-determining region CDR H3 on the heavy chain and some of them have substantial polyreactivity and autoreactivity properties [116]. Therefore, most potential bnAb producing B cell lineages are eliminated at one of the early stages in their development and natural development and maturation of a bnAb is observed only in a small number of HIV-1 infected individuals. Nevertheless, recent studies have been able to decipher some specifics of bnAb development in elite controllers, with the ultimate aim of developing such a response in a future vaccine strategy [115-118]. Another approach is gene-based continuous generation of bnAb from adenovirus vectors expressed in muscular tissue, so far tested in NHP and humanized mice [119, 120]. This approach seems effective but bears the burden of continuous exposure to bnAb, which could themselves trigger an immune or autoimmune response after prolonged presence. The topical application of bnAb in a gel form represents another potential preventative approach that has been tested *in vitro* and *in vivo*, including the publication outlined in the next chapter [121-126]. The excessive cost of producing bnAb in large quantities needed for this approach can be overcome by recently developed plant-based manufacturing technologies [127], which open the way to the development of feasible PrEP bnAb strategies in the near future.

Regarding the cellular, cytotoxic T lymphocyte (CTL) mediated immunity to HIV-1, there are indications that the initial HIV-1-specific CD8 T cell response contributes to reducing acute phase viral load and to establishing the viral set point. At later stages, a decline in CTL function is associated with loss of control over viral replication and disease progression. The failure of the CTL response to control viral replication can be summarized in two main phases: 1) early and unsuccessful CD8 T cell responses are narrow and focused on the immunodominant

epitopes, which facilitates viral escape, and 2) late CTL responses are inefficient due to the presence of HIV-1 escape mutants and the generalized immune dysfunction caused by CD4 depletion and exhaustion, driven by chronic immune activation [128]. The generation of effective CTL responses remains one of the goals in the development of anti-HIV-1 vaccine. A recent study in NHP demonstrated that a live-attenuated SIV vaccine is able to contain early SIV replication in the lymph nodes through induction of HIV-1-specific activated effector memory CD8⁺ T cells [129]. A second recent groundbreaking study in NHP has shown that the rhesus cytomegalovirus (rCMV)-based vectors expressing SIV antigens were able to offer protection against highly pathogenic challenge virus by induction of effector CD8⁺ cells and their persistence in mucosal tissues [130]. These studies emphasize the potential for development of an efficient antiviral CD8 T cell responses following vaccination and efforts to apply similar strategies in human vaccine trials are underway. The T cell specific vaccine trials to date and current strategies for designing a T cell based vaccine will be detailed later in the therapy subchapter.

To summarize, despite significant advances in our understanding of the humoral and cellular immune responses to HIV-1, which are effective at suppressing and controlling viral replication only in a small percentage of HIV-1 infected individuals, there are no approved immunomodulatory treatments for HIV-1 thus far. The development of an effective vaccine against HIV-1 has not yet been successful despite three decades of intensive research. Additionally, viral immune-evasion mechanisms, the immune dysfunction that develops during HIV-1 infection and the establishment of permanent HIV-1 reservoirs (HIV-1 latency), all contribute to the increased complexity of HIV-1 infection and undermine development of a vaccine or a complete cure for HIV-1. The latter two topics will be discussed in the next subchapters.

1.5.4. HIV-1 induced immune dysfunction - immune activation, exhaustion and immunosenescence.

In the acute phase of HIV-1 infection, massive depletion of CD4⁺ CCR5⁺ memory T cells occurs in the GALT, which serves as a major reservoir for this population of cells [104-106]. The depletion is thought to be the initial step in a systemic dysregulation of CD4 T cell function. HIV-1 infection also results in immunologic dysfunction of CD8⁺ T cells, B cells, natural killer (NK) cells and even nonlymphoid cell populations through increased cell turnover, elevated immune activation, accelerated differentiation, and a generalized disruption of homeostatic cellular responses [87, 131-133]. These multiple effects of HIV-1 infection on immune system phenotype and function result in quantitative alterations in immune cell populations, defects in immune system function, and the development of immunodeficiency.

In the GALT, cytopathic effects on CD4⁺ memory T cells are induced directly by HIV-1 infection and by indirect mechanisms termed the “bystander effect”, resulting in cell death of CD4⁺ memory T cells [134]. Another important factor, only recognized in the last couple of years, is the loss of the integrity of the mucosal barrier in the gut and the consequent microbial translocation to the systemic circulation [135]. The translocation contributes to the HIV-1-induced systemic activation and dysregulation and is a chronic feature of the infection, even in the treated patients. The specific mucosal depletion of the Th17 CD4⁺ T cells, which secrete IL-17 in the GALT, has been reported in NHP studies and in HIV-1-infected individuals, and is thought to be one of the mechanisms by which mucosal antibacterial barrier is disrupted [136]. In the peripheral lymphoid tissues, HIV-1 causes extensive germinal center and follicular hyperplasia in the lymph nodes in the acute phase of the infection, followed by the hypoplasia and collagen deposition during the chronic phase [137].

At the cellular level, the increased cellular turnover is seen in CD4 T cells and in other affected cell populations such as B cells, CD8 T cells and NK cells [131, 132]. The initiation of

cART decreases the rate of the turnover in all cell populations. In addition to the increased turnover which ultimately leads to the immune system exhaustion, there are qualitative alterations in the affected cellular populations. For the CD4 T cells, the depletion of the naïve and resting memory CD4⁺ pools is followed by the increase in the short-lived effector CD4 T cells in the chronic phase [138]. Similar expansion of the short-lived effector memory cells is seen also in the CD8⁺ pool [139]. Defective CD8⁺ T cells have decreased cytolytic abilities and show a decrease in cytokine secretion. The T cell skewing is accompanied by the similar alterations in the NK and B cellular pools [140, 141]. These profound changes are thought to be the consequence of the reduction in the regenerative potential due to the thymic dysfunction, permanent increase in the cellular turnover and differentiation and the generalized destruction of the lymph node architecture and function. The main driving force behind these functional and proliferative alterations is the chronic antigenic stimulation caused by the HIV-1 infection, which induces the state of chronic immune activation and ultimately leads to the immune system exhaustion and loss of the ability to regenerate or fully function. The cellular factors that play a role in the induction of the immune activation are bacterial lipopolysaccharide (LPS), type I interferon (IFN- α), proinflammatory cytokines (IL-1, IL-6 and IL-12) and proinflammatory chemokines (CXCL10) [131, 132, 135]. The disruption of the gut mucosal barrier and the consequent chronic bacterial LPS translocation into the circulation are considered the major driving force for the immune activation, although other mechanisms have been suggested. For the type I IFN, the induction of multiple IFN- α – stimulated genes (ISG) has been shown in the chronically infected individuals [142] [141].

Immune activation is characterized by the increase in the cellular activation markers, such as CD38 in CD8 T cells, in all immune cell populations. In addition, there is an increase in the cellular turnover (already described above), hypergammaglobulinemia (indicating an increased terminal differentiation of B cells) and increased susceptibility for the development of lymphoid malignancies, especially in B cells. Also, the increase in the apoptotic cell death is

seen in the activated CD4 T, CD8 T and B cells, probably through the ISGs and TRAIL/Fas signaling upregulation [143]. The progressive loss of proliferative capacity and effector functions has also been connected with the loss of the homing receptors for the lymph nodes (CD62L, CCR7, CXCR4, CXCR5) and the upregulation of the receptors for the homing to the inflamed extralymphoid tissues (CXCR3, CCR6, CD11c) [144, 145]. Additional cellular feature is the increase in the senescence and exhaustion markers on T and B cells such as the inhibitory receptor PD-1 [146].

1.5.5. Chronic phase of HIV-1 infection and transition to AIDS.

Chronic phase of the HIV-1 infection spans the period of 8 to 10 years between the acute disease phase and the development of AIDS [87, 133]. The main feature is a gradual decline in the CD4⁺ T cell number in the peripheral blood, caused by permanent state of immune activation and all its consequences described above. The primary criteria for the transition of the chronic state to AIDS is the decrease of CD4 T cell level below 200 cells per milliliter of blood. The median time for progression to AIDS in the untreated patients is estimated to be approximately 5-10 years. The direct symptoms of AIDS are non-specific and include fever, chills and sweating, progressive weakness and weight loss. Severe immunodeficiency, as the main characteristic of AIDS, leads to the array of the opportunistic infections caused by other viruses, bacteria and fungi. Immunodeficiency also enables uncontrolled oncogenesis with malignant transformation, and different types of cancer, especially the ones targeting the immune system (lymphomas), develop in the untreated AIDS patients. Once full-blown AIDS develops, without the treatment the survival time is less than a year [147].

With the development of multiple classes of antiretrovirals and highly active antiretroviral therapy (HAART), HIV-1 patients have a prolonged time before progression to AIDS [148]. With a longer life span of HIV-1 patients, who are on the life-long ARV therapy, the HIV-1-induced

pathology has been complemented with two major groups of co-morbidities. The first group of co-morbidities are the negative effects induced by the long term therapy and may involve renal, hepatic toxicity and other unwanted effects such as modifications of the lipid metabolism, as in the case of some protease and RT inhibitors [149]. The dysregulation of the lipid metabolism increases the risk of cardiovascular disease and more studies are addressing such chronic disease development and prevalence factors in the aging HIV-1-positive population [150, 151]. Another phenomenon seen with the extension in the life span of HIV-1 infected individuals is accelerated aging compared to the general population [152, 153]. Even though it is still early to definitely delineate the effects of actual aging from the effects of the virus, the state of permanent immune activation and the disruption of the mucosal barrier in the gut followed by the permanent bacterial translocation have been implicated in the elevated levels of proinflammatory markers and accelerated aging process, seen in many patients on HAART who show good control of the virus. The significance of the immunomodulatory and proinflammatory effects of HIV-1, which are not completely attenuated by HAART, still needs to be studied further and to be connected with accelerated aging and development of “end-organ diseases” such as cardiovascular disease, bone disease, metabolic and kidney abnormalities and neurocognitive dysfunction seen in the ART-treated HIV-1 patients [150, 151, 153, 154]. Additionally, in developing countries immune reconstitution inflammatory syndrome (IRIS) is common in HIV-1 infected patients. This syndrome develops in patients started on ART with low CD4 counts or with a secondary, opportunistic infection, and is characterized by strong and generalized immune response and extensive tissue damage. The underlying HIV-1-induced chronic immune activation and dysfunction, exacerbated by additional infection, has been implicated in the genesis of IRIS [111, 155].

The last group of HIV-1/AIDS related pathologies which are increasing in number are the co-infections with other viruses such as hepatitis B virus (HBV) and hepatitis C virus (HCV), with *Mycobacterium tuberculosis* in the case of HIV-1/TB co-infection and with *Plasmodium*

falciparum in malaria/HIV-1 co-infection [156]. The increase in co-infections is global and it is affecting the ability to efficiently treat HIV-1 as well as the existing co-infections. Therefore, co-infection studies represent an important area for the future multidisciplinary therapeutic and preventative global studies, especially for the developing world where their prevalence is the highest and rising [156].

1.5.6. HIV-1 latency.

Even though more than 30 different antiretrovirals have been approved for the treatment of HIV-1, the complete cure has not been found yet. The cART approach has prolonged patient survival and decreased morbidity, but it still implies live-long treatment and also residual immune activation and incomplete immune reconstitution in treated patients. The discontinuation in therapy leads to a rapid rebound in the viral load and return of the accompanying immune dysfunction. In addition, according to the epidemiological data in the developing world, there are 16 new infections for every 10 people started on cART. Moreover, in the low income countries, almost 50% of the infected do not have access to therapy. Underlying these global problems is the need for a complete, eradicating cure, and the main obstacle for developing such a strategy is HIV-1 latency.

HIV-1 latency is established early, within a couple days following transmission [110, 157]. The sparse but extremely stable HIV-1 viral reservoir is established within the resting memory T cells and remains transcriptionally silent until a reactivation event occurs. Initially, naïve CD4 cells are activated and differentiate into effector cells upon HIV-1 infection. The vast majority of effector CD4 T cells undergo cell death within a few weeks following infection, but a small percentage remains infected and enters a resting state as memory CD4 T cells, forming the viral reservoir. Recent study has shown that there are two subsets of memory CD4⁺ T cells – central memory (T_{CM}) and transitional memory (T_{TM}) cells. The former are found in patients

who control infection better and have normal CD4 levels, while the latter is the major cellular population within the reservoir in patients with low CD4 cells counts, suggesting that a different dynamics and composition of the reservoir can also be connected with disease pathology [110]. The resting memory T cells can persist in the inactive, quiescent state for decades and therefore form a long-lasting HIV-1 reservoir during the first days of infection. Several studies have shown that the HIV-1 infected cells are as rare as 1 in every 10^6 CD4+ resting memory T cells, and that these reservoirs do persist in patients on cART [110]. Based on the cellular half-life and the cellular turnover rate of CD4+ resting memory T cells, the estimation was made that it would take 73 years of therapy to completely eradicate the reservoir. Importantly, the Siliciano group has recently shown that a portion of these proviruses is in fact replication competent when latent state is reversed [158, 159]. Elegant studies involving different modes of reservoir activation and statistical modeling by the same group suggest that the reservoir size might have been underestimated and that it is approximately 60-fold larger than in original estimations [158, 160]. Current efforts are aimed at reservoir reactivation and eradication of latently infected cells, and include application of different histone-deacetylase (HDAC) inhibitors, such as valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA) in combination with protein kinase C (PKC) agonists prostratin and bryostatin [157]. Additional immune-boosting strategies, such as enhancement of the CTL response, might be needed in order to efficiently deplete the HIV-1 reservoir cells following activation [111, 157]. New methods that enable the quantification of viral reservoir are being developed, which will allow for more precise analysis of the effects of current eradication strategies and more detailed basic studies [110, 157]. This area of research has not had a major breakthrough yet, due to the inherent complexities of reactivating latent provirus without serious nonspecific side effects. For example, currently applied HDAC inhibitors have been shown to affect transcription of as many as 10% of cellular genes, bringing into question the initial anti-reservoir strategies [157]. In addition, the fact that the reservoir itself is not

understood completely is another barrier to developing efficient approaches to its activation [161].

As efforts to develop a sterilizing cure continue, from the perspective of the work presented here there is one additional conclusion. The importance of developing preventative approaches against HIV-1 mucosal transmission becomes even more obvious in the light of the described persistent viral reservoir formation once infection occurs. In addition, the outlined consequences of life-long therapy in HIV-1 infected patients further emphasize the need for a preventative approach. All the knowledge we currently have about the HIV-1 and the course of HIV-1 infection in humans, briefly outlined in the previous chapters, leads to the ultimate goal of designing efficient therapeutic and preventative approaches. The focus in the last chapters of this introduction will be the overview of animal models which have enabled *in vivo* studies of HIV-1 infection, and the overview of existing treatment and prophylactic strategies for HIV-1 as well as the ones being currently developed.

1.6 Animal models for HIV-1 research

The lack of a natural host for HIV-1 besides humans has led to a rare situation in which there are no animal models available to study an emerging disease. The solution in the early years of HIV-1 research was to turn to the natural predecessor of the HIV-1, the simian immunodeficiency virus (SIV), and to model and analyze HIV-1 infection in the context of its analogy with SIV. The nonhuman primate (NHP) models, in which different SIV and SHIV-1 (virus that has both SIV and HIV-1 genome parts) variants have been studied for several decades now, still represent a gold standard in the field, for basic research and for preventative studies. In recent years, the development of humanized mice, which harbor human immune system, has opened new possibilities for studying HIV-1 infection in the context of virus susceptible human cells being present. Both types of models are described below.

1.6.1. Nonhuman primate (NHP) models

The NHP models were the first ones to be utilized for the study of HIV-1 infection and the NHP research has contributed immensely to the understanding of the basic mechanisms of lentiviral infection, which can in part be translated to HIV-1 pathogenesis in humans [93, 94]. There are several available NHP models which include chimpanzees and macaques.

The SIV infection in NHP chimpanzee models does not naturally progress to AIDS. These animals represent an endangered species, which has made them an unsuitable model, although initial studies utilized NHP in the early years of the epidemic. The species-specific restriction factors in these animals such as TRIM α and APOBEC3G inhibit productive infection and these differences do represent an expanding field of study since analogous but not equally efficient restriction factors can also be found in humans [24, 32]. NHP chimpanzee models are not generally utilized in the studies of HIV-1 pathogenesis, therapeutic and preventative approaches or vaccine research.

Macaques have been utilized for *in vivo* studies with SIV and chimeric SIV/HIV-1 viruses [93]. The former originate in the accidental transmission of SIV from asymptomatic sooty mangabeys to macaques in several US primate research institutions. In addition to these pathogenic SIV_{sm} strains, a number of pathogenic SIV_{mac} (macaque origin) have been isolated, and have been used in vaccine and pathogenesis studies as the infection does cause simian AIDS [93, 94, 162]. The clinical course of the infection is also highly analogous to HIV-1 in humans both during the acute and chronic stage of immunopathology and onset of AIDS as well as in the CCR5 tropism of most pathogenic SIV strains. CD4 T cell decline is seen over time and GALT is the early and major site of this depletion as in HIV-1 infection. In addition to parenteral (intravenous inoculation) infections, the macaque models have been used to model vaginal and rectal infection and mucosal transmission of HIV-1 in humans [94, 162]. The initial high dose SIV challenges have been replaced with the more human transmission-analogous

repeat low-dose model (RLD) of exposure [94, 163]. The limitation of this model is that the Env of HIV-1 and SIV differ in the variable loop regions, which represent the primary targets for immune responses and generation of protective bnAb. Therefore, the efficacy studies for the HIV-1 B-cell based vaccines or immunogens cannot be studied in the SIV-macaque model, which is nevertheless valuable for pathogenesis and transmission studies.

Chimeric SIV/HIV-1 (SHIV-1) macaque infection has overcome some limitations of the SIV macaque models, by utilizing chimeric viruses in which a number of HIV-1 genes have been inserted into the SIV backbone [93, 163]. The choice of the genes inserted allows for designing the viruses with different HIV-1 Env proteins, which can then be utilized in vaccine studies. The insertion of several other genes, such as tat, rev and vpu enables further for these chimeras and infection to resemble that of HIV-1 itself. Highly pathogenic chimeric viruses which have been adapted for the propagation in macaques lead to the development of AIDS-like syndrome. The first limitation of this model is the rapid course of the disease, with maximal one year animal survival following infection. Although this rapid disease progression represents a cost and results benefit for short term experiments, it does not allow for long term studies. The second limitation is the X4 tropism of most highly pathogenic SHIV-1 strains developed to date. The course of the disease and the hallmarks of the most common - R5 infection type in humans cannot therefore be mimicked in these animals. The CD4 level decrease is present but, as for human X4 strains, it is due to the depletion of peripheral and not GALT CD4 T cells. The ultimate warning with regard to the usage of these animals in vaccine testing came from the recent human phase IIB efficacy study, the HVTN 502 (“STEP”) trial [164]. The CD8 T cell based vaccine candidate tested in this trial was assessed as highly promising in macaques, but proved to be not only non-protective but showed an enhancement of the HIV-1 acquisition in a subset of patients in the human trial. The increased vulnerability to the infection in some patients was correlated with pre-existing immunity to the adeno-viral vector itself and could not have been predicted in the SHIV-1 macaque study.

The recent development of R5 tropic SHIV-1 strains has enabled better modeling of the natural HIV-1 infection course in humans [94, 165]. Moreover, the development of the RLD exposure model of R5 SHIV-1 mucosal transmission will advance future protection studies in macaques [94]. Additional breakthroughs have been made with the introduction of not only B subtype env (dominant in the western world) but also the subtype C env (prevalent in some parts of Africa and the developing world) into the SHIV-1 constructs. These and future advances will allow for better mimicking of HIV-1 in SHIV-1 studies, which have already been extremely useful in many areas of basic HIV-1 research and antiviral regimens [166]. Nevertheless, the high cost and specific conditions (primate centers) in which the animals have to be housed, ethical considerations for the use of these higher species in research, as well as the differences between HIV-1 and SIV/SHIV-1 - at the genetic level (Vpx gene in SIV, Vpu in HIV-1 for example) and in the disease course duration (progression to AIDS within 6-12 months from the infection in macaques and in up to 10 years in humans), have prompted the need for a more suitable animal model for HIV-1 research. The humanized mice represent a small animal model in which HIV-1 itself in native form can be used to study infection and immunopathogenesis in the context of the human immune system, as described below.

1.6.2. Humanized mouse models

The recapitulation of the human immune system in different types of immunodeficient mice has been the basis of the development of the array of humanized mouse models [167-169]. The initially used athymic (nude) mice, had an efficient T cell depletion but B and NK cell immunity still persisted. The next immunodeficient strain to be used were severe combined immunodeficiency (SCID) mice, with the complete T and B cell depletion, as a result of the *Prkdc*^{-/-} (protein kinase DNA activated catalytic polypeptide) mutation that affects V(D)J recombination and the formation of the functional T and B cell receptors. However, the NK cells

in SCID mice were not affected by this mutation. The creation of non-obese diabetic (NOD)-SCID hybrid depleted innate immune responses generated by mouse NK cells and enabled further improvements in the human immune cell reconstitution. The latest improvement was the knockout of the IL-2 receptor common gamma chain gene (IL2-R γ c, knockout γ c^{-/-}), which efficiently disrupts the IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signaling and depletes mouse NK cell-derived immunity. Combining this mutation with previous immunodeficient mouse breeds was used to create multiple humanized mouse models, discussed below in more detail. In addition to the immunodeficient mice, the second component necessary for the generation of humanized mouse models are the human immune cells, which have a different origin depending on the model. The common point for all models is the need to precondition the mice prior to implantation of human cells or tissues. This is achieved by sublethal irradiation, which eradicates any residual mouse immunity which might interfere with implantation and development of human lineages. A short description of the early and newer generation humanized mice is given below.

Early humanized mouse models include:

- 1) Hu-PBL mice, created by intraperitoneal injections of human PBMCs into SCID mice. Alternatively, mice of (NOD)-SCID, NSG (NOD/SCID/ γ c^{-/-}) or NOG (NOD/Shi-scid/ γ c^{-/-}) genetic background can be used. These mice have good T cell levels but only short term experiments are possible, since implanted cells cannot persist beyond several weeks. There is no primary immune response and graft versus host disease develops in most animals.
- 2) SCID-hu mice, created by co-implantation of human fetal liver and thymus tissue under the kidney capsule in SCID or NOD-SCID mice, to create thy/liv organoid. The presence of human thymic tissue allows for maturation of T cells in the human background but the T cell reservoir is mostly in the organoid itself and not in the peripheral tissues. No

primary immune response can be developed and there is no multilineage haematopoiesis.

Even though these early models had significant limitations, they have contributed significantly to the understanding of HIV-1 pathogenesis [167-169] and have served as a basis for the development of newer generation humanized mice, which are listed below:

- 1) hu-HSC mice, created by intrahepatic injections of haematopoietic stem cells (HSCs, CD34+) into neonatal (up to 5 days old) NSG, NOG, Rag1^{-/-} γ c^{-/-} or Rag2^{-/-} γ c^{-/-} mice. In the latter immunodeficient mice the recombination activating genes 1 and 2 (Rag 1, Rag 2), which are essential for VDJ recombination and T cell receptor formation, are knocked down. Initial hu-HSC models were created by implanting adult mice of the NOD/SCID genetic background or more recently by implanting adult NSG or NOG mice. The adult models do show multilineage haematopoiesis; however, the T cell reconstitution is low. The switch to the newborn implantation has increased the level of human cell engraftment of all lineages, including the T cells. The advantages over the older generation humanized mouse models are the development of the primary immune response and human immune cell reconstitution in mucosal tissues, which allows for transmission studies [170-175]. The remaining drawbacks are the weak IgG response in comparison to IgM (weak class switching) and the lack of human HLA restriction, since the human immune system is reconstituted in the mouse thymic background.
- 2) BLT mice (acronym: bone marrow, liver, thymus), created by the implantation of human fetal liver and thymus under the kidney capsule, as in SCID-hu mice, but this procedure is followed by intravenous injections of the autologous HSCs the day after the implantation surgery. The genetic background in the early BLTs was NOD-SCID, and current models are made within the improved NSG, NOG, BALB/c-Rag1^{-/-} γ c^{-/-} or BALB/c- Rag2^{-/-} γ c^{-/-} knockout backgrounds. BLTs are currently the superior model, with

a strong human cellular and humoral immune response and high level of human reconstitution with B cells, T cells, NK cells, macrophages and dendritic cells. The crucial improvement for immunological studies is the human T cell HLA restriction of autologous CD34+ HSCs in the matching human thymus. The robust humanization in mucosal tissues allows for transmission studies [169, 175-179].

- 3) Hu-liver-HSC mice, which are created by the simultaneous implantation of HSCs and human hepatocytes. This model allows for HCV infection and generates human immune response. Importantly, hu-liver-HSC mice open the door for the study of the HIV-1/HCV co-infection, which has a high prevalence in human population and represents a significant global health problem. In addition, future pharmacokinetic and pharmacodynamics studies of the new antiretrovirals against both HIV-1 and HCV could be studied in this model in the context of human immune system and human liver, mimicking to the full extent drug exposure, drug metabolism and potential drug efficacy in humans [180, 181].

The humanized mouse models have been applied in various HIV-1 research areas. The basic research areas include HIV-1 immunopathogenesis and immune response alteration studies [167, 169], which have recently been expanded to additional studies of viral evolution and mechanisms of HIV-1 immune escape [182-186]. The improvements in the humanized models have also allowed the HIV-1 neuropathogenesis studies [187]. In addition, BLT model has enabled latency research, as one of the rare HIV-1 latency *in vivo* models which forms a CD4 resting T cell reservoir [188, 189]. Separate area of humanized mouse application in HIV-1 research are transmission studies, enabled by improved mucosal human engraftment of new generation models [124, 169, 175-179, 190, 191]. Importantly, humanized mice can support productive infection with CCR5 and CXCR4- tropic HIV-1 strains. Moreover, they exhibit human-like CD4 T cell depletion over time and GALT CD4 T cell depletion in the acute phase of the infection. Multiple therapeutic approaches, including application of different classes of

antiretrovirals, bnAb, specific aptamers, siRNAs, antiviral peptides, dendrimers, nanoformulated therapeutics and gene therapy approaches have been tested in humanized mice over the last decade [124, 167-169, 190-196]. The focus of one part of the research presented here are prevention studies, enabled by improved human engraftment of vaginal and rectal mucosa in the latest humanized mouse models [167, 178, 197]. Various agents, such as antiretrovirals, bnAb and siRNAs, have been already tested in this context in humanized mice [165, 169, 178]. Emerging field, to which a part of the research shown here hopes to contribute, is *in vivo* preclinical testing of new antiretroviral candidates, by analyzing their tissue distribution (pharmacokinetics, PK studies) and correlating it to the level of protection offered against the HIV-1 infection (pharmacodynamic, PD studies) [198].

There are ongoing and continuous efforts aimed at further improving the humanized mouse models, which would allow for the expansion of their applications for HIV-1 research [167, 168, 182, 199]. The first group of improvements include gene knock-in for the additional factors important in human haematopoiesis, such as human thrombopoietin (hTPO), which has resulted in enhanced engraftment and higher levels of myeloid cells. The knock-in of the IL-3 and granulocyte macrophage cytokine stimulating factor (GM-CSF) resulted in the improved myeloid lineage development and enhancement of macrophage repopulation of tissues, even alveolar macrophages in the lungs [200]. The knock-in of colony stimulating factor-1 (CSF-1) upregulated myeloid cell development, in particular monocyte/macrophage differentiation [200]. In addition, the knock-ins of IL-7, IL-15 and B cell activating factor (BAFF) into the humanized mice have resulted in the improved human T cell, NK cell and B cell development, respectively [167]. The second group of improvements is building further on the BLT model, which has already allowed T cell development in the context of human thymus. In this context the improvements are aimed at developing a humanized mouse with knock-ins of human HLAs class I and II, which would further improve “humanization” of the immune response in these animals. The third group of modifications is aimed at eliminating residual mouse immunity and

the recent transduction of SIRP α ligand CD47 into transplanted HSC or direct gene knock-in of SIRP α resulted in further depletion of mouse innate immunity [201].

1.7 Therapeutic approaches for HIV-1 infection

1.7.1. HIV-1 vaccine approach

Despite several decades of intensive research, there has been only limited success in the HIV-1 vaccine discovery thus far. From multiple vaccine trials to date, only one has shown limited success. The RV144 trial conducted in Thailand included four priming injections of a recombinant canarypox vector vaccine (ALVAC-HIV-1 [vCP1521]) and two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E), and showed moderate protection with 31% reduction in HIV-1 acquisition [202]. Multiple challenges exist in HIV-1 vaccine development process, which can be separated into two interconnected groups of factors. The first group is represented by virally-related obstacles, such as high mutation rate, genetic diversity of HIV-1 and viral immune evasion strategies which include conformational masking of conserved epitopes, glycan shield and other structural “tricks” that give HIV-1 constant advantage over the immune response [111, 203, 204]. In addition, immune activation, which constitutes a favorable component of immune response in other diseases, is enhanced, detrimental and only perpetuates inability of the immune system to clear the virus [87, 111]. Likewise, the primary targets of HIV-1 are CD4 T cells which are crucial for the shaping of almost every aspect of the adaptive immune response, and this cell reservoir is progressively depleted and dysfunctional during early stages of HIV-1 infection. Thus, any strategy that would involve CD4 T cell enhancement at early stages to clear the virus could actually enhance the infection by increasing the number of targets. Another major obstacle to developing an efficient vaccine strategy against HIV-1 is the establishment of the latent reservoir, which is by its nature,

as integrated genomic HIV-1 provirus in senescent cells, highly refractory to any vaccine intervention [157]. According to the recent studies, this reservoir is established within the first couple of days following transmission and an effective vaccine would have to overcome its rapid formation, in addition to actively propagating virus. The second obstacle to the development of HIV-1 vaccine is the lack of knowledge about the correlates of protective immunity in humans [205-207]. Should those be the plasma levels of protective bnAb, specific CTL response markers or indicators of some other anti-viral mechanisms, such as antibody-dependent cell-mediated cytotoxicity, or all of them together? Are these correlates the same for different globally predominant sybtypes or for different HIV-1 strains circulating globally? There is much that is not known regarding basic immunology behind HIV-1 infection and consequently about which mechanisms would trigger a protective response or block it. Recent report by Sui et al. describes an anti-SIV vaccine failure due to the inhibition of CD8 T cell responses by vaccine-induced myeloid-derived suppressor cells (MDSC), which was completely unexpected based on current knowledge of immune responses against HIV-1 [208]. Current efforts are therefore aimed at better understanding of HIV-1-induced immune dysregulation and early events in the infection, where a successful vaccine strategy would be targeted [111, 209, 210]. In addition, immune correlates from the RV144 trial are being analyzed in detail [205, 211]. Besides RV144 trial, the renewed optimism in the field was initiated with the recent discovery of multiple potent bnAb against HIV-1 and the development of new generation vaccine vectors which should increase the breadth of CTL responses in future vaccine candidates [116, 203-206].

1.7.2. HIV-1 Antiretrovirals

Prior to 1990s a few options existed for the antiretroviral treatment of HIV-1. The disease management was mostly focused on preventing and treating opportunistic infections and AIDS-related illnesses. In 1987 the first anti-HIV-1 drug AZT (Retrovir, GSK) was approved, and this

nucleoside analog RT inhibitor greatly improved clinical care and prognosis in HIV-1 patients, although with severe side effects due to initial high doses applied [212]. With the discovery of several antiretrovirals belonging to RT inhibitors and protease inhibitors in the mid-1990s, the therapy of HIV-1 was revolutionized [87, 148]. Combinatorial drug regimens applying both classes of ARVs transformed HIV-1 infection from a painful terminal disease to a chronic disease which can be managed long-term in most patients. Current list of ARVs is comprised of more than 25 agents which fall into 5 major groups. The initially discovered RT and protease inhibitors have been replaced with newer generation drugs, which are more potent antivirals with fewer side effects [87, 148, 149]. In addition, in recent years a new class of ARVs has been developed against the third major viral enzyme - the HIV-1 integrase. There are three antiretrovirals currently approved in this class – Raltegravir, Elvitegravir and Dolutegravir [148, 213]. Besides ARVs which target crucial parts of HIV-1 viral cycle, the earliest event, HIV-1 entry, has been targeted by the discovery of a fusion inhibitor (T-20, Fuzeon) and a CCR5 antagonist and entry inhibitor compound Maraviroc (Selzentry) [148, 213].

Accepted early on, the standard of HIV-1 care involves the administration of a combination of three different ARVs as highly-active antiretroviral therapy (HAART), recently renamed to combination antiretroviral therapy (cART). Current initial cART regimens include two nucleoside reverse transcriptase inhibitors (lamivudine or emtricitabine plus tenofovir, abacavir or zidovudine) with a non-nucleoside reverse transcriptase inhibitor, protease inhibitor, or integrase inhibitor [87]. This combinatorial approach was able to reduce HIV-1-related morbidity and mortality, and significantly improve survival rates and quality of life, as well as prolong the time to the development of AIDS. In most patients put on cART, there is a rapid suppression of the viral load to undetectable levels within a couple of months and subsequent significant immune system recovery, detected by the increase in the CD4 T cell levels [87]. Current triple regimens are aimed at inhibiting complete viral escape from the treatment by rapidly evolving HIV-1. However, the development of drug-resistant mutations still represents a major problem

and the emergence of specific escape mutants has been documented for all current classes of ARVs [214, 215]. A number of ARVs show rapid selection of highly-resistant single mutant, while others select several mutations which constitute an escape over longer period of time, and the adjustment of cART and screening for mutations are becoming a standard part of therapeutic approach. However, due to the suboptimal adherence to therapy in many patients and the fact that the generated mutants can be transmitted, there is an increase in the number of therapy-naïve patients which already carry the virus with resistance to one or more ARVs. This percentage of transmission of resistant HIV-1 variants has reached an alarming 10-17% level in high-income countries and is around 6% in the lower and middle-income countries with the increasing trends [87].

Besides the emergence of drug specific escape mutations, there are other concerns regarding cART as a life-long therapy. First of all, side effect and toxicity from the long-term therapy can develop over time, even though in a much more attenuated form compared to initial HIV-1 treatments [149]. In addition, despite the suppression of viral replication and partial immune reconstitution, residual levels of immune activation and dysfunction in patients on cART contribute to increased risk of cardiovascular disease, diabetes, lipid metabolism dysregulation and neurocognitive disorders [131, 132]. Permanent HIV-1 latency reservoirs represent a major obstacle to the sterilizing cure and thus far none of the cART approaches were able to tackle this problem in HIV-1 patients [110]. In addition, long-term adherence in patients represents another problem which contributes to poor disease control and generation of escape mutants. The new “single pill” formulations such as Stribild, Atripla and Complera are targeted at reducing the pill burden and increasing patient compliance [216].

The set point for the initiation of cART was initially 200 CD4 T cells per cubic mm of peripheral blood. Newer recommendations have increased this level to 350 CD4 T cells/mm³ blood and in 2013 World Health Organization has raised the criteria for initiation of cART to 500 CD4 T cells/mm³ blood [217]. These modifications are based on recent findings which indicate

that early initiation of therapy slows down disease progression, and further investigations are needed to confirm that there is actual benefit to this approach when CD4 levels are between 350 and 500 CD4 T cells/mm³ blood [218, 219].

Despite the drawbacks of cART listed above, the advent of this approach is one of the major medical achievements in human history. In that respect, recent studies have shown that the patients with the suppression of viral load and normal CD4 cell counts achieved on cART, can have near-normal life expectancy [220]. From the early days in which untreated patients were progressing to terminal AIDS within a year or less, this truly represents a major victory in the fight against HIV-1.

1.7.3. Gene therapy approaches

Gene therapy approaches for HIV-1 represent a growing field aimed at providing a “functional cure” for HIV-1, as opposed to the “sterilizing cure” which would eradicate the virus from the body completely. In this approach the aim is to block multiple points of the viral cycle, as in cART, but by application of different classes of transgenic RNA molecules as opposed to antiretrovirals [221, 222]. The current strategies are aimed at producing haematopoietic stem cells (HSC) or other classes of progenitor cells which would be either resistant to HIV-1 infection or able to produce anti-HIV-1 factors, or both of these. The ultimate goal therefore represents a creation of HIV-1-resistant immune system which would be continuously regenerated from HIV-1-resistant stem cell pool. In this way, virus would be continuously controlled without long-term cART and viral reservoirs would be suppressed.

The proof of concept for gene therapy approach is the only case of the HIV-1 cure documented thus far, the Berlin patient [223]. Following the allogeneic transplant of the haematopoietic and progenitor cell (HSPC) graft from a donor who was homozygous for 32 bp deletion in CCR5 gene, the immune system in this patient was reconstituted without the CCR5

co-receptor expression. Subsequently, with the cART discontinued this patient is successfully controlling the virus for years, without any detectable viral load and even detectable virus in multiple examined reservoir tissues. However, this approach would not be feasible in the form of the allogenic transplant on a global scale, but multiple approaches have been developed to achieve the same phenotype of CCR5 deletion, which would render CD4 T cells resistant to HIV-1. These include CCR5 knockdown by RNA interference, ribozymes or zinc finger nucleases (ZFN) [221]. Other major group of approaches includes the knockin of multiple host factors known to be restrictive to viral replication, such as tetherin, APOBEC3G and 3F, SAMHD1 and TRIM5 α [221, 222]. Transcriptional silencing approaches include RNA interference (RNAi) induced degradation of viral mRNAs and multiple strategies for disruption of the Tat/Rev viral axis [221, 222].

In parallel with these therapeutic approaches, multiple strategies are being developed for targeted delivery of therapeutic molecules to HSCs, progenitor precursors or directly to CD4 T cells, as the main cell pool being infected by HIV-1. These delivery methods include different constructs of adenoviral, adeno-associated viral and lentiviral vectors, as well as artificially designed nucleic acid aptamers and dendrimers [224]. Newly developed delivery methods increase the stability of the gene therapy molecules *in vivo* and boost antiviral effects as well as minimize the unspecific side effects.

The first gene therapy human study, published in NEJM this year by Tebas et al., used zinc-finger nucleases to disrupt the CCR5 gene in the autologous CD4 T cells which were then transfused back to HIV-1 patients [225]. This study demonstrated not only the proof of concept but also safety of this approach in humans, opening the door for further gene therapy clinical trials in the near future.

1.8 HIV-1 prevention

Multiple preventative approaches for HIV-1 have been developed and applied in the field since the emergence of the disease and none have been efficient in controlling the pandemic [87, 226, 227]. However, significant reduction in the infection rates has been achieved with the introduction of preventative educational programs and subsequent risk behavior reduction in general population. In addition, specific programs aimed at high risk groups, such as needle exchange for intravenous drug users, condom use promotion and treatment of other STDs in the sex worker and MSM population, have shown effectiveness to a certain level. In addition, the increased awareness regarding routes of HIV-1 transmission and higher number of people being tested globally have had the effect on the progression of the epidemic in some parts of the world. Nevertheless, some of the highly effective prevention approaches such as condom use and circumcision are not widely accepted in many parts of the world due to cultural and social specificities. The marginalization of high risk groups such as MSM, intravenous drug users or sex workers in many societies hampers the progress to a better control of the epidemic. Finally, low resource countries, especially in Africa, bare the highest burden of the epidemic from the very beginning and lack the infrastructure, funds and trained personnel to be able to handle the epidemic of such extent, with HIV-1 adult prevalence as high as 15%-30% in many southern African countries in 2012, according to the UNAIDS report from 2012.

Looking at the pandemic today, it is estimated that 35.3 million people were living with HIV-1 in 2012 (UNAIDS report 2012). Global incidence rate has decreased from 3.3 million in 2002 to 2.3 million in 2012, and it is thought that the introduction of cART had a major impact on this encouraging trend. Nevertheless, in the regions in which highest number of cases is due to MSM transmission, such as Western, Central Europe and both American continents, the incidence has stabilized despite of high cART implementation. Moreover, the number of AIDS-related deaths globally is still alarmingly high at 1.6 million in 2012, according to UNAIDS report.

The HIV-1 infection is one of the major health burdens globally, being the fifth leading cause of disability-adjusted life years (DALY) in general and the leading cause of DALY in 30-44 year old population in 2010 [228]. These sobering facts represent strong arguments for the development of new, more efficient preventative approaches, which would allow for better control and real attenuation of the HIV-1 pandemic.

In addition to the facts outlined above, which highlight poor control of the epidemic even though significant improvements have been made, there is one more specific angle which new preventative approaches should address, in order to be effective. In the high prevalence areas and especially in the most affected African countries, the incidence rates are exceedingly higher in young women compared to men, according to recent UNAIDS reports. Cultural specificities in addition to women being more biologically prone to mucosal HIV-1 transmission event have created such discrepancy. In addition, very often there is social complexity behind this higher incidence noted, such as sexual abuse, forced prostitution and inability to influence the male partner to proven protection methods which are male-controlled, such as condom use. Therefore, new prevention methods are needed and they should be able to empower women to protect themselves against HIV-1. The recent UNAIDS global statistics points to a fact that at this moment 50 young women are infected every hour by HIV-1 (UNAIDS 2013 report). To reduce this number, new approaches for HIV-1 prophylaxis have been initiated in recent years and the modeling of such approaches in humanized mice is the focus of the studies presented here. The new strategy is called pre-exposure prophylaxis (PrEP) and can be divided into two types. First type is topical, mucosal application of gels with antiretroviral agents – the topical PrEP. The second strategy is systemic, oral application of antiretroviral agents –oral PrEP. Both of these approaches will be discussed with the emphasis on the efforts made here in our humanized mouse model, which aim to contribute to further development of PrEP strategies.

1.8.1. Topical PrEP – microbicide approach

Initial microbicide studies tested various antiviral compounds, mostly aimed at disrupting the viral envelope and non-specific to HIV-1. Compounds tested, like nanoxynol-9, cellulose sulfate and C31 G gels were not only ineffective but were also toxic and increased the probability of HIV-1 transmission by disrupting vaginal mucosal barrier and causing local inflammation [229]. Another group of gels, such as Buffer gel, PRO 2000 and Carraguard gel, did not have toxicity issues but were shown to be ineffective in preventing HIV-1 infection in controlled clinical trials [229].

The breakthrough in the microbicide field happened with the idea of applying already approved therapeutic antivirals as preventative gel formulations [230, 231]. The drugs which target the preintegration steps in the viral cycle are considered more suitable for PrEP applications, and these include CCR5 antagonists, RT inhibitors and integrase inhibitors. Later stage targeted drugs, like protease inhibitors, would allow for integration event and could only be used as a supplemental agents in combinatorial approaches.

The most promising drug to date for microbicide application is Tenofovir (TFV), a nucleotide RT inhibitor. Since this drug targets the stage in the viral cycle prior to integration, it could theoretically prevent infection and formation of the reservoirs. The microbicide formulation of TFV has been tested in NHP against SHIV-1 as a 1% gel against low-dose repeated vaginal and rectal challenge and has shown high level of protection - 6/6 macaques for vaginal and 8/9 protected for rectal challenge [232, 233]. In BLT mice the 1% TFV gel has been shown as 88% protective if given 4h before and 4h after the vaginal challenge [179]. In the study presented here the protective effect of TFV 1% gel was analyzed against vaginal HIV-1 Bal challenge when applied 1h prior to exposure, to confirm data seen in the BLT study. This single drug group served as a control in the combinatorial gel study in which TFV was applied with either non-nucleoside RT inhibitor UC781 or with the integrase inhibitor Raltegravir (RAL). Both

combinatorial formulations showed high level of protection against mucosal challenge, confirming in RAG-hu mouse model the protective potential of microbicides against HIV-1 mucosal transmission. Based on the initial NHP studies and pharmacokinetic studies which showed high exposure in the genital and rectal mucosa, TFV was advanced to clinical trials. Thus far TFV remains the only drug for which PrEP microbicide efficacy against HIV-1 acquisition has been shown in a controlled clinical study. In the CAPRISA004 trial, 1% gel showed 39% protection when applied in a coitally-dependent manner, and these results were a big boost to the microbicide field [234]. However, in the subsequent VOICE clinical trial the same formulation was ineffective when applied as a once daily, non-coitally dependent microbicide [235]. The FACTS 001 trial which is repeating the set up in the CAPRISA004 study to reevaluate its effectiveness is ongoing [236]. Nevertheless, the described discrepancies have informed the field on the additional steps which need to be taken in order for PrEP to be more effective and consistent [236-239]. Two main goals have been identified –the increase in patient adherence and the establishment of correlations between the dose given, local mucosal concentration in the transmission site and protection offered. Current efforts in the field are aimed at resolving these issues and studies shown here, conducted in humanized mice, were aimed at profiling ARV kinetics in mucosal tissues and contributing to better understanding of the correlation between mucosal concentrations and protection against HIV-1 transmission.

In addition to TFV, microbicides currently investigated in clinical trials include TFV combination with emtricitabine (FTC). The TFV/FTC combination has already been approved as the first ever PrEP strategy agent by FDA for oral PrEP, while no microbicide formulations have been approved yet. Ongoing trials also include single dapivirine gel formulation and combinatorial gel with TFV [229, 240]. Dapivirine, the non-nucleoside RT inhibitor, has shown promising results in previous animal and initial phase I human studies regarding the toxicity and distribution of the drug in the vaginal tissue and cervicovaginal fluid, and additional phase I and II studies are ongoing [241, 242]. With HIV-1 being a highly mutating virus, it can be expected

that combinatorial approaches would have to be applied in order to overcome resistant mutations to first generation agents such as TFV, and to increase the level of protection. As outlined above, new microbicide candidate drugs, the non-nucleoside RT inhibitor UC781 and integrase inhibitor RAL have been tested as single drug gels and in combination with TFV in the RAG-hu mice studies presented here. In addition to these protection studies, complementary pharmacokinetic pilot study was conducted following gel application of TFV, RAL and another potential PrEP candidate, the entry inhibitor Maraviroc (MVC), to better define and correlate mucosal drug disposition to protection offered against mucosal HIV-1 infection.

Microbicide approach for PrEP could have multiple advantages over the oral PrEP approach. Based on the analysis of multiple clinical studies to date and field experience, low patient adherence represents the main obstacle for the transition of preventative approaches from the controlled clinical trials to real-life application. In this respect, microbicide application which would be coitally-dependent would potentially increase patient adherence, compared to continuous daily need to take the pill for oral PrEP [236]. In addition, in order to overcome the decrease in adherence with daily dosing, the long-lasting microbicide intra-vaginal rings (IVR) are being tested for prolonged drug release and topical protection [229, 236, 243]. The ring formulations currently in clinical trials include dapivirine and combinatorial dapivirine plus maraviroc IVR [229]. Furthermore, multipurpose prevention technologies (MPT), which would combine anti-HIV-1 drug(s) with the contraceptive and an agent against other STDs (genital herpes, Chlamydia and others) are currently under development [244]. The MPT would allow for multi-level protection urgently needed for female population in the most affected areas and would slow down the HIV-1 epidemic by multiple complementary mechanisms [244].

Further advantages of the microbicide approach include higher drug exposure in the mucosal tissues after topical compared to oral application. This has been shown for TFV in animal models and human studies [245]. For other ARVs there is limited data for topical application in NHP and most human studies to date were done following oral application [237,

246]. The humanized mouse studies shown here for TFV, MVC and RAL confirm the trend of higher exposures following topical compared to oral application, mimicking well the human kinetics [198].

Another advantage of microbicide approach is minimal systemic exposure with reduced toxicity and negative side effects [229, 245, 246]. Moreover, the lack of systemic exposure reduces the risk of mutant generation, which is a concern if current therapeutic agents are to be also utilized as PrEP [247]. The humanized mouse model could be applied in future studies which would inform on systemic exposure following topical application and the generation of mutants over time with PrEP applications, as a complement to the NHP studies. In addition, drug retention over time in mucosal tissues could be observed in humanized mice following microbicide application. These experiments would inform on the proper dosing schedules for topical PrEP in further NHP studies and clinical trials. Finally, multidrug approaches with novel PrEP candidates and their compatibility with first generation PrEP agents TFV and FTC could be explored in humanized mice in the combinatorial microbicide studies. The proof of concept studies shown here validate the model for future work in preclinical assessment of new microbicide PrEP candidates.

1.8.2. Oral PrEP approach

Oral PrEP approach has been tested in seven different clinical trials to date, and many are ongoing. In the iPrEx trial a 44% reduction in HIV-1 acquisition was shown for the TFV/FTC combinatorial pill in the MSM population [248]. In this study a direct link was also made between self-reported adherence and the reduction in the HIV-1 incidence, which was as high as 73% reduction for the group with >90% patient compliance. Importantly, the concentrations of the drugs in the parallel PK studies were correlated with the risk of HIV-1 infection, although only plasma and PBMC and not mucosal tissue levels were followed [248]. In the TDF2 trial which

included both male and female participants, the fixed combination TFV/FTC pill showed overall 64% protection against HIV-1 infection, but no conclusions about gender-specific protection were possible due to small study numbers [249]. In contrast to these two studies which reported positive results, the FEM-PrEP trial in heterosexual women with TFV/FTC pill was discontinued due to lack of protection [250]. Again, as in other previous PrEP studies which failed, the low adherence rate was the probable crucial factor in the failure of the trial. In the VOICE (MTN 003) trial, heterosexual women were randomized to receive oral daily TFV/FTC or TFV only, and this study did not show any reduction of the HIV-1 acquisition risk, again mainly to suboptimal patient compliance [236, 239]. In a recent Bangkok TFV study, once daily TFV pill showed HIV-1 risk reduction of 48.9% in the intravenous drug user population of both genders [251]. Two landmark trials in serodiscordant couples, the HPTN 052 and the Partners PrEP, reported significant reduction in the HIV-1 acquisition risk when two different strategies for oral PrEP were applied [252, 253]. The reduction in the sexual transmission risk was as high as 96% in the HPTN 052 trial, in which the HIV-1 positive partner was started on cART earlier than in the usual clinical practice. The earlier ART initiation was based on the CD4 T cell counts between 350-500 cells/mm³, as opposed to the usual below 200 or 300 CD4 T cell/mm³ initiation point. This study is the corner stone of the subsequent development of the “Test and Treat strategy”, in which earlier initiation of therapy represents a form of PrEP for the uninfected partner(s). In the Partner PrEP trial, daily TFV and TFV/FTC pill showed 67% and 75% reduction in HIV-1 acquisition, respectively, in the discordant heterosexual couples [253]. In addition to protection studies, multiple human studies have been conducted to analyze the mucosal tissue distribution of TFV, and FTC to a lesser extent, showing a favorable exposure and retention, as the two main PK criteria for a promising PrEP agent [254-256].

Based on the high level of protection offered by TFV/FTC pill in most studies described above and favorable PK data, this combinatorial treatment was the first oral PrEP approach to be approved by the FDA in July 2012. Compared to microbicide approach, oral PrEP has been

shown as effective in more clinical trials and also there is more PK data for future approach design. The success of the first HIV-1 PrEP strategy to be implemented for the mother to child HIV-1 transmission, which dramatically reduced the infection rates, points to the fact that, if designed properly, very efficient HIV-1 prevention is possible and achievable [87]. In addition, the outlined studies in the discordant couples emphasize the effect of oral PrEP on the reduction of the viral load and probability of transmission, and this principle is easily transferable to the transmission in the general population [252, 253].

Several protective studies have been conducted for systemic PrEP in humanized mice in the BLT and the RAG-hu models [177, 191]. In addition to prevention studies, the first PK analysis of mucosal drug levels following oral application of the established oral PrEP agent TFV and new oral PrEP candidates MVC and RAL is presented here, with the aim of informing future NHP and clinical trial design [198]. As for topical PrEP, multiple additional questions could be analyzed in the humanized mice for the oral PrEP, as the resistance mutant generation and combinatorial oral PrEP approaches. The studies to identify markers of adherence for the oral PrEP could also be designed in the humanized mice and could potentially contribute to the ultimate human marker characterization. To this end, initial proof of concept studies have been conducted in the RAG-hu mice and are encouraging for their further application as a preclinical oral PrEP model for HIV-1.

1.8.3. BnAb microbicide approach

The alternative approach to the application of antiretrovirals for PrEP is passive administration of bnAb, which mimics an efficient humoral response against HIV-1 at the site of transmission and could be applied as a topical form of PrEP. An array of new bnAb has been discovered recently [115, 116, 257]. Their high potency against wide array of circulating isolates at very low concentrations, with IC_{50} in nanomolar and low micromolar ranges, indicates that

these biological molecules could be good PrEP candidates. Moreover, their topical application in a gel form would allow for already made bnAb to be applied as PrEP, as opposed to their generation by vaccination, for which no successful strategy has been found in three decades of research. Multiple NHP studies have confirmed the efficiency of this approach against mucosal HIV-1 transmission [93, 94, 166]. In addition, several studies in humanized mice have looked at the potential of bnAb PrEP and therapeutic approach against HIV-1 [125, 195, 196, 258]. One of the studies conducted in humanized mice and the first one to test PrEP potential of the bnAb VRC01 microbicide against vaginal HIV-1 challenge is presented here [124].

Even though bnAb microbicide approach has shown promising results thus far, there are several issues with implementing these strategies *in vivo* in humans. The first major obstacle for further development of bnAb as microbicides was thought to be their stability *in vivo*. A recent humanized mouse model study by Klein et al. showed that following subcutaneous application the half-life of bnAb ranges from 0.7 to 6.3 days, which is comparable to and even longer than half-life of antiretrovirals [195]. The long half-lives are favorable for future bnAb applications, and would allow less frequent dosing to increase patient adherence. The second major obstacle to bnAb PrEP application is the high production cost by traditional methods, and this has in part been overcome by new, low cost and high yield plant-based expression systems [127]. Further development of production and purification techniques will allow for massive production of bnAb of high purity, needed for therapeutic and PrEP applications [259].

Future directions for bnAb application include combinatorial approach to achieve full protection, and this could mean application of multiple bnAb which target different epitopes or application of bnAb with antiretrovirals [126, 195, 196]. All bnAb discovered so far have been IgG class antibodies isolated from the plasma of rare elite controllers who are able to successfully generate them against HIV-1. Another approach which is being developed is engineering of IgA class bnAb, which would offer protection in the mucosa, at the site of transmission [260, 261]. The adeno-associated vectors which continuously express bnAb are an

alternative to gel applications, and have shown considerable promise in several animal studies thus far [119, 120]. Importantly, as a preclinical development model humanized mice have an advantage over NHP, especially for long term studies. This is because NHP can develop anti-human Ab to applied bnAb, while no such rejection reaction and reduction in bioavailability exists in the humanized mice.

The first clinical trial of the safety, tolerability, dose-response and pharmacokinetics of a bnAb is underway and it will involve subcutaneous and intravenous application of bnAb VRC01 in HIV-1 positive patients [262]. With the lack of an effective HIV-1 vaccine, bnAb approaches to prevention represent the closest mimic of effective natural immunity and could revolutionize the HIV-1 prevention field in the years to come. The proof of concept study of the VRC01 bnAb microbicide shown here and multiple recently published humanized mouse model studies from other groups show the potential of hu-mice for further exploration and modeling of these preventative approaches.

CHAPTER 2

Topical gel formulation of broadly neutralizing anti-HIV-1 monoclonal antibody VRC01 confers protection against HIV-1 vaginal challenge in a humanized mouse model ¹

2.1 Summary

The new generation broadly neutralizing antibody VRC01 against HIV-1 shows great potential as a topically administered microbicide to prevent sexual transmission. We evaluated its efficacy in a RAG-hu humanized mouse model of vaginal HIV-1 transmission. Mice were challenged vaginally with R5 tropic HIV-1 BaL an hour after intravaginal application of the VRC01 (1 mg/ml concentration) gel. A combination of four first generation bNAbs, namely b12, 2F5, 4E10 and 2G12, was used as a positive efficacy control whereas a non-specific dengue MAb 4G2 was used as negative control. Our results showed that seven out of nine VRC01 antibody administered mice and all of the mice receiving the four bNAb antibody combination were protected against HIV-1 challenge. These findings demonstrate the efficacy of the new bNAb VRC01 as a topical microbicide to protect against HIV-1 vaginal transmission and highlight the use of the RAG-hu mouse model for testing HIV-1 prevention strategies.

2.2 Introduction

HIV-1 incidence continues to be unabated worldwide in the absence of an effective vaccine (UNAIDS 2010 report). Therefore there is an urgency to develop alternative prevention methods to contain this epidemic. In this regard, topically applied microbicide gels to prevent

¹ This chapter contains the results of the study published as cited below.

Milena Veselinovic, C. Preston Neff, Leila R. Mulder, Ramesh Akkina (2012) Topical gel formulation of broadly neutralizing anti-HIV-1 monoclonal antibody VRC01 confers protection against HIV-1 vaginal challenge in a humanized mouse model. *Virology*. 2012 Oct 25;432(2):505-10.doi: 10.1016/j.virol.2012.06.025.

sexual transmission show considerable promise [263-265]. While a number of early clinical trials using non-specific compounds such as nonoxynol-9 failed to show protection, the recent success achieved with the RT inhibitor tenofovir gel in the CAPRISA004 trial provided renewed optimism in the microbicide field [234, 266]. Based on this success, the current strategies are focused on using HIV-1 specific compounds that act against different stages of the viral life cycle. These include entry inhibitors such as maraviroc, nucleoside and non-nucleoside reverse transcriptase inhibitors like tenofovir and TMC 120 and integrase inhibitors exemplified by raltegravir, to name a few [265]. Successful development and deployment of an effective anti-HIV-1 microbicide will empower women to protect themselves against contracting the infection and thus is estimated to prevent millions of new cases of HIV-1.

In practice, prevention of HIV-1 infection by the use of microbicides involves repeated applications of the gels that contain high concentrations of anti-HIV-1 compounds of various chemistries [267-269]. It is possible that such a repeated exposure of sensitive cervico-vaginal mucosal tissues to these drugs during long-term use may result in toxicities causing epithelial damage eventually undermining the protective effects. In this regard, use of biomolecules such as neutralizing antibodies may circumvent potential damage to the mucous membranes thus affording long-term safety. Additionally, combining these with that of other anti-HIV-1 compounds may permit reducing the concentrations of the respective compounds to lower and much safer levels. Given the extensive antigenic variability of HIV-1, any candidate antibody has to be broadly neutralizing to qualify as an effective microbicide to prevent infection. Until recently, studies in this area have been limited due to sparse numbers of available potent broadly neutralizing antibodies (bNAbs) [121, 123, 270, 271]. This limitation is now overcome by the discovery of several new more potent bNAbs [262, 272, 273]. Early evidence that bNAbs do indeed have microbicide potential was derived by the studies of Veazey et al. (2003) in a monkey model [271]. Animals were administered a bNAb antibody b12 vaginally in either saline or hydroxyethyl cellulose gel. Only three out of 12 treated and SHIV-1 challenged animals

became infected and it was found that the duration of protection lasts for at least two hours after administration. These results form a sound basis that neutralizing antibodies have good microbicide potential.

While the previous generation bNAbs such as b12, 2G12, 2F5, and 4E10 were shown to be capable of neutralizing many primary HIV-1 isolates belonging to multiple genetic subtypes, they are not pan-reactive [262]. In recent studies directed towards finding more bNAbs with an ultimate goal of designing better vaccines, newer high throughput strategies were used (Walker et al., 2009 and Wu et al., 2010)[272, 273]. These studies have led to the discovery of more highly potent neutralizing antibodies. These include bNAbs PG9, PG16, HJ16 and VRC01, among others. While b12 and HJ16 can neutralize about 40% of known viral isolates, PG9 and PG16 bNAbs neutralized 73% and 79% of viral isolates tested respectively [272, 274]. The bNAb VRC01 that recognizes the CD4 binding site on HIV-1 gp120 showed even more potency and breadth with a capacity to neutralize 91% of viral strains tested [273]. With these successes as a background, a more intensified search recently yielded even more potent bNAbs such as multiple PGT bNAbs recently discovered by Walker et al. (2011)[275]. The availability of these antibodies opened up many new avenues of HIV-1 therapies and prevention including microbicides.

With regard to testing microbicide strategies *in vivo* using bNAbs as well as other anti-HIV-1 compounds, macaque models have been the leading standard (Veazey et al., 2012)[165]. They were instrumental for the basic groundwork and provided the preliminary efficacy data for subsequent human clinical trials. However, they are expensive, and cannot use HIV-1 itself for testing and therefore rely on SIV and/or varieties of hybrid SHIV-1 viruses for viral challenge [169, 267, 276, 277]. Furthermore, it is not possible to test against drug resistant and genetically diverse HIV-1 strains that exist in the field. Some of these deficiencies can now be overcome by the newer generation humanized mouse models that have emerged [173, 276, 278]. These include RAG-hu mice and BLT mice which were found to have HIV-1 susceptible human cell

reconstitution in cervico-vaginal and rectal mucosal compartments [171, 175]. A number of recent studies have demonstrated their susceptibility to HIV-1 infection by both vaginal and rectal routes [170, 171, 177, 190]. Exploiting this property, both oral pre-exposure prophylaxis and microbicide-based prevention of HIV-1 mucosal transmission has recently been shown using these models [179, 190, 191]. More recently we validated the RAG-hu mouse model for vaginal microbicide testing using maraviroc which showed good efficacy [190].

With the advent of new discoveries that continuously yield a stream of newer and more potent bNAbs and other new biological molecules for potential use as microbicides, it is important that promising candidates need to be evaluated quickly and inexpensively for generating preliminary data for later macaque studies and human clinical trials. With these questions as a background, we conducted the following proof-of-concept studies to evaluate bNAb VRC01 as a potential microbicide. Our results show that these antibodies confer significant protection against HIV-1 vaginal challenge and that RAG-hu mice provide a suitable *in vivo* system to test biomolecules as potential anti-HIV-1 microbicides.

2.3 Materials and methods

Generation of humanized Rag1^{-/-} γc^{-/-} and Rag2^{-/-} γc^{-/-} mice (RAG-hu mice)

Humanized BALB/c- Rag1^{-/-} γc^{-/-} and BALB/c- Rag2^{-/-} γc^{-/-} (RAG-hu) mice were generated using human fetal liver-derived CD34⁺ hematopoietic progenitor cells as previously described [170-172, 174]. Mice were maintained at the Colorado State University Painter Animal Center and all studies have been reviewed and specifically approved by the CSU Institutional Animal Care and Use Committee (Protocol 09-085A). Newborn mice were preconditioned by irradiating with a sublethal dose of 350 rad and then injected intrahepatically with 0.5–1×10⁶ human CD34⁺ cells. Mice were screened for human cell engraftment at 10–12 weeks post-reconstitution. Peripheral blood was collected by tail bleed and red blood cells were lysed by the

Whole Blood Erythrocyte Lysing Kit (R&D Systems, Minneapolis, MN). The white blood cell fraction was stained against the human pan-leukocyte marker CD45 using hCD45-R-PE (Invitrogen) and FACS analyzed to determine the levels of human cell engraftment as previously reported [174]. All mice included in the study were monitored for level of engraftment prior to the beginning of the experiment. Female mice prepared with different donor CD34+ cells with over 50% engraftment were randomly distributed into experimental and control groups (Table 2.1).

Vaginal application of bNAb VRC01 gel and HIV-1 challenge by vaginal route

VCR01 antibody was kindly provided by Vaccine Research Center (VRC), NIAID, NIH, and older generation bNAbs 2G12, b12, 4E10 and 2F5 were obtained from NIH AIDS Research & Reference Reagent Program. To prepare a topical gel formulation VRC01 antibody was incorporated into a 2.2% hydroxyethyl cellulose (HEC) universal placebo gel at a concentration of 1 mg/ml. As a positive effective control, a four bNAb antibody mix was employed. This combinatorial bNAb cocktail gel formulation was prepared by using old generation bNAb antibodies of different specificities that consisted of b12, 4E10, 2F5 and 2G12 antibodies, all incorporated into the 2.2% HEC gel at a final concentration of 0.125 mg/ml for each antibody in the mix (0.5 mg/ml total antibody final concentration). We also prepared a gel with a negative control bNAb (1 mg/ml) using an irrelevant non-HIV-1 antibody 4G2 (a mAb against dengue virus). For these experiments we used mice that have human CD45 cell engraftments of 50% or more (Table 2.1). Female RAG-hu mice were topically administered with VRC01 gel (nine mice), bNAb cocktail gel (five mice) or control 4G2 gel (four mice) an hour before the viral challenge. A 25 µl volume of each of the gel formulations was carefully applied into the vaginal vault of RAG-hu mice. During the whole procedure mice were anesthetized by isoflurane inhalation (duration 7–10 min). An hour post-gel application, mice were challenged vaginally with HIV-1 BaL (3000 TCID) in a 25 µl volume. Gel and viral inoculums were applied by using the bulbous end (1.25 mm in diameter) of a gavage needle (VWR, POPPER, NY) to assure no

mucosal abrasions and tearing would occur [190]. Anesthetized mice were held in an inverted position for 4 min post-inoculation to allow the virus to adsorb and to prevent immediate discharge of virus, as described previously [171, 191]. Control non-treated mice received 2.2% HEC placebo gel. Animals were observed daily and blood samples collected on a weekly basis to assess plasma viremia and CD4 T cell counts.

Measurement of viral loads

HIV-1 infection status and viral loads were assessed by qRT-PCR. RNA was extracted from 25 to 50 µl of EDTA-treated plasma using the QIAamp Viral RNA kit (Qiagen, Valencia, CA). qRT-PCR was performed by using a primer set specific for the HIV-1 LTR sequence and a corresponding LTR specific probe as described previously [171, 172]. C1000 Thermal Cycler (CFX96™ Real-Time System, BIO-RAD) and iScript™ One-Step RT-PCR kit with SYBR® Green (BIO-RAD) were used to perform the real-time qPCR analysis and viral load was expressed as the number of HIV-1 RNA copies per milliliter plasma.

Flow cytometry

Mice were monitored biweekly to analyze the levels of CD4 T cells in the peripheral blood. Whole blood was collected and red blood cells lysed as described previously [172, 174]. Peripheral lymphocytes were stained for hCD45-FITC, hCD3-R-PE and hCD4-PE-Cy5 surface markers (Invitrogen, BD Biosciences) and monitored using a Coulter EPICS XL-MCL FACS analyzer (Beckman Coulter, Fullerton, CA). CD4⁺ T cell levels were calculated as a ratio of the entire CD3 population (CD4⁺CD3⁺:CD4[−]CD3⁺). All mice were screened prior to the gel application and HIV-1 challenge to establish baseline CD4 T cell ratios.

Statistics

Data regarding the number of viral RNA copies, CD4 T cell levels, and infection rates was analyzed using GraphPad Prism version 5 (GraphPad Software, USA). To compare the infection rates between the groups, Fisher's Exact test was applied for analysis of the percent infected curves. P values less than 0.05 were considered to be significant.

2.4 Results

Vaginal application of VRC01 gel confers protection against HIV-1 vaginal challenge in RAG-hu mice

We have previously shown that RAG-hu mice are susceptible to vaginal HIV-1 transmission and can be used to test both oral PrEP and topically administered anti-HIV-1 compounds, namely the CCR5 inhibitor maraviroc and integrase inhibitor raltegravir [190, 191]. Here we evaluated a biological molecule VRC01, a broadly neutralizing monoclonal antibody, for its ability to protect humanized mice against HIV-1 vaginal challenge. Mice were monitored by qRT-PCR on a bi-weekly basis for ten weeks to detect virus in the plasma of virally challenged mice. As a positive effective control, a gel containing a cocktail of four bNAbs, b12, 4E10, 2F5 and 2G12 antibodies, was employed. Our results (Fig. 2.1) showed that all the mice (6/6) receiving either a placebo gel (no antibodies) or a gel containing an irrelevant anti-dengue MAb (4/4) became virus positive within five and three weeks post-viral challenge, respectively. In contrast, in the VRC01 treated group only two out of nine mice (2/9) became virus positive, showing significant protection. In mice receiving the bNAb cocktail of four antibody combination, none were infected (0/5) thus showing complete protection. In mice which were not virally challenged, as expected, no virus could be detected (0/2). With regard to viremia, unprotected mice receiving placebo gel, irrelevant non-specific antibody gel as well as non-protected mice in the VRC01 treated group exhibited persistent viremia after becoming virus positive (Fig. 2.2).

Overall, these data indicate that VRC01 bNAb confers significant protection against HIV-1 vaginal challenge when compared to placebo and 4G2 non-specific antibody treated RAG-hu mice, with P values of 0.0013 and 0.002, respectively. Additionally, when comparing VRC01 and bNAb cocktail treated mice, a P value of 0.2774 indicates that there is no significant difference between these two bNAb treated groups. These data taken together suggest that VRC01 bNAb conferred partial but significant protection and the combinatorial antibody gel provided full protection.

The RAG-hu mouse model mimics the immunopathology of HIV-1 infection in humans, as demonstrated by virally mediated depletion of CD4 T cells. While the qRT-PCR data detected no viremia in microbicide gel protected mice, we wanted to further confirm that there is no evidence of HIV-1 induced pathology as can be seen by stable levels of CD4 T cells. Accordingly, peripheral blood from virally challenged mice was evaluated periodically by FACS analysis for 10 weeks (Fig. 2.3). The baseline CD4 T cell levels were established prior to viral challenge in each of the mice. Typical of HIV-1 infection of these mice, there was a trend of gradual CD4 T cell decline in the irrelevant antibody (4G2) gel treated mice in which viremia was seen. On the contrary, no discernible CD4 T cell loss could be seen in either VRC01 protected or bNAb cocktail gel treated mice. Consistent with the presence of viremia in the two VRC01 mice that became virus positive and were thus deemed non-protected, there was significant CD4 T cell loss. Collectively these data showed that the mice which did not become virus positive also did not show any HIV-1 associated CD4 T cell loss.

Table 2.1. Summary of human cell engraftment levels in humanized mice. Peripheral blood was collected from human CD34 cell reconstituted RAG-hu mice (BALB/c-RAG2^{-/-}γc^{-/-} or BALB/c-RAG1^{-/-}γc^{-/-}, the prefix J is indicative of RAG1) at 10–12 weeks post-engraftment. White blood cell fraction was stained with CD45 FITC conjugated antibody and analyzed by FACS to confirm human cell engraftment prior to treatment and vaginal HIV-1 challenges.

VRC01 mouse no.	Engraftment (%)	Placebo mouse no.	Engraftment (%)
J961	58	1000	78.1
J975	59.3	997	70.4
J977	67.3	944	74.7
J978	63.6	945	63.7
J996	54.1	J1008	67.6
J997	50.9	J1009	87
J998	52.1		
J1033	72.9		
J1035	77		
bNAb cocktail mouse no.	Engraftment (%)	4G2 control mouse no.	Engraftment (%)
J909	69.5	J1010	63.2
J911	78.5	J1011	69.2
J981	54.2	J1012	78.4
J985	73.8	J1013	59.8
J989	55.4		
Negative control mouse no.	Engraftment (%)		
932	62.9		
933	68.2		

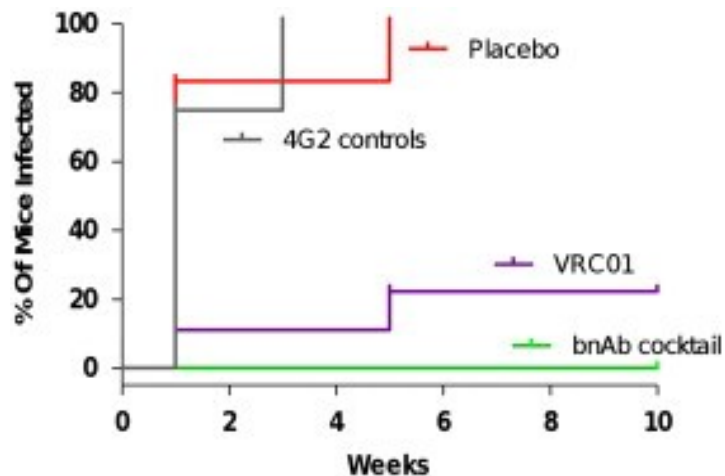


Figure 2.1. Vaginal application of VRC01 gel protects humanized mice against vaginal HIV-1 challenge. RAG-hu mice were challenged by vaginal route 1 h after vaginal administration of VRC01, combinatorial bNAb, irrelevant antibody or placebo gels as described in Materials and methods. Blood was collected bi-

weekly from infected mice and the status of HIV-1 infection was determined by qRT-PCR. Kaplan–Meier plots of time course of appearance of viremia in antibody treated versus non-treated virus challenged mice.

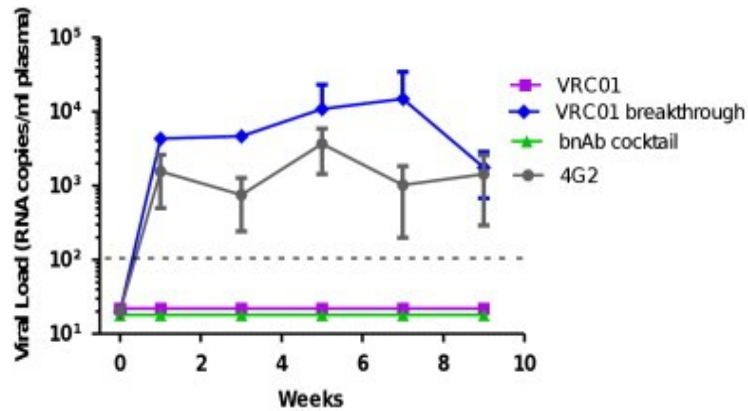


Figure 2.2. Viral RNA loads in mice administered with different bNAbs. RAG-hu mice were challenged by vaginal route an hour after vaginal application of bNAbs gels as described in Materials and methods. Blood was collected bi-weekly. Viral RNA was extracted from plasma and viral RNA loads were determined by qRT-PCR as described in Materials and methods. The dotted line represents limit of PCR detection.

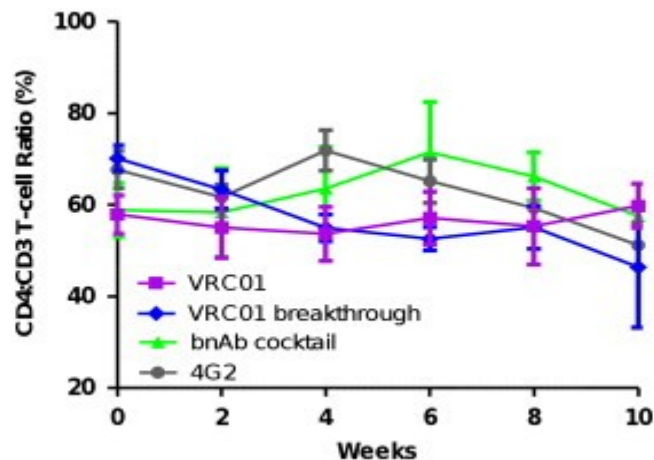


Figure 2.3. CD4 T cell decline in non-protected mice versus protected mice. Levels of CD4 T cells were monitored bi-weekly by FACS to determine their decline in treated versus non-treated mice. Baseline values for each of the mice were established prior to infection as described in Materials and methods and are shown as week 0 values.

2.5 Discussion

A safe and effective microbicide which women can use to protect themselves against contracting HIV-1 infection would greatly help diminish the disease incidence. If found to be effective, use of naturally occurring biological molecules such as antibodies will have considerable edge over other chemical compounds in terms of assuring long term safety and reducing side effects. Here in these preliminary studies we have shown that a new generation potent broadly neutralizing monoclonal antibody VRC01 confers significant protection against HIV-1 vaginal transmission. To our knowledge this is the first report to show the efficacy of the new generation bNAbs as potential HIV-1 microbicides in an *in vivo* system using HIV-1 itself as the challenge virus. While a number of previous macaque studies using previous generation of bNAbs have shown their utility as microbicides [121-123, 271, 279-281], the humanized mouse system we used here permitted efficacy testing in the context of human target cells. Also, unlike in macaque studies, there was no need to condition the animals by progesterone hormonal treatment with Depo-Provera to induce vaginal thinning.

The new generation microbicide compounds are being chosen for their specific inhibitory effects on various stages of the viral life cycle in contrast to previous generation generic non-specific agents [264, 282]. The recently field tested RT inhibitor microbicide compounds such as tenofovir showed promising efficacy but still fall short of the desired full protection level [234]. The inhibitors such as RT and integrase inhibitors would act after viral entry [264, 277]. If the drug levels are insufficient intracellularly in a small sub-population of cells, virus breakthrough is likely. Therefore, if possible it is highly desirable to prevent the first step of viral entry in the viral life cycle. In this regard, compounds such as maraviroc, a CCR5 antagonist, have been shown to prevent vaginal transmission of SHIV-1 virus in macaque studies [283]. We have recently shown its anti-HIV-1 microbicide potential in a humanized mouse system similar to the one we used here [190]. While the inhibitory activity of RTIs is not co-receptor dependent, use of a

single entry inhibitor like CCR5 antagonist maraviroc alone will be ineffective against X4 and dual tropic viruses which are transmitted, albeit at a low level. An advantage of bNAbs in the context of entry inhibitors is that they are effective against all HIV-1 strains irrespective of their co-receptor tropism.

Among bNAbs, VRC01 antibody is the most potent and most broadly neutralizing with activity against 91% of viruses tested [273]. In our present study seven out of nine vaginally challenged mice were protected against HIV-1 infection at 1 mg/ml concentration of the topically applied gel. While the protection conferred is significant, it is not complete. It is possible that a higher concentration of VRC01 may be needed for complete protection. Alternatively an improved formulation that reduces possible biodegradation in the cervico-vaginal environment may improve its efficacy. Moreover, if better retention can be achieved using alternative gel formulations, lower doses of VRC01 might offer the same or higher level of protection, compared to the dose tested here. A recent preliminary report by Pegu et al. (Meeting Abstract Supplement, J. Immunol. April 2011, 186, 155.11) described the protective effect of passively transferred VRC01. In this study, macaques were administered with VRC01 systemically by i.v. route (dose 20 mg/kg). Two days later they were either challenged by vaginal or rectal routes with a SHIV-1 virus. Whereas 3/4 animals got infected in the control groups by both the routes none of the animals (4/4) that received VRC01 were infected by either route thus showing full protection. This data demonstrated that passively administered VRC01 antibody provides sterilizing mucosal immunity.

The microbicide formulation containing a mix of bNAbs 2G12, 4E10, 2F5 and b12 which we used as positive efficacy control in this study provided complete protection in which five out of five animals were protected. This result is consistent with other studies that showed high level of protection by the combination of the bNAbs listed above in different *in vivo* and *in vitro* settings [121-123, 258, 270, 271, 279-281, 284]. This complete protection offered by the combination of four bNAbs with different target epitopes points to the important fact already

recognized in the field—combinatorial approaches are needed for future microbicide formulations and the principle of using multiple antiviral agents must be adopted, keeping in mind the success of HAART therapy for already established HIV-1 infections. In a study pointing to the combinatorial approach by Euler et al. (2011) in which viral isolates from the early stages of HIV-1 epidemic as well as from recent years were tested against VRC01, PG16 and PG8 bNAbs it was found that, even though resistance developed over time for all the bNAbs tested, every strain included in the panel showed high sensitivity to at least one of the bNAbs [285]. A new study by West et al. (2011) showed that a construct which combines VRC01 scFv (single chain Fragment variable) and PG16 IgG yields a potent chimera with greater neutralization breadth thus opening the door for new modes of combining bNAbs with different gp120 epitopes in the future [286]. In addition to combinatorial approaches, efforts to engineer newer bNAbs with increased potency and breadth based on the structure of VRC01 are already under way [287, 288].

Since the present data demonstrated the efficacy of the new generation bNAbs as potential microbicides against HIV-1 infection, a number of other important questions need to be evaluated. Among these are - if combining these with other antibodies and/or with other ARVs will increase the efficacy and breadth of action. For these antibodies to reach wider application in the field as microbicides and to be used by millions of people at risk, it is necessary that they need to be mass produced in the required quantities. This is being currently addressed by newer mass production methods such as utilizing genetically engineered plants [127]. However, possible immunogenicity of plant-expressed molecules and concerns regarding genetically engineered plants in some quarters would need to be resolved for their wider use and acceptance. With regard to large scale testing of a wide variety of biological molecules and chemical ARDs in the future to derive preclinical data, the humanized mouse model we employed here will offer a rapid cost effective *in vivo* model.

CHAPTER 3

HIV-1 Pre-Exposure Prophylaxis: Mucosal Tissue Drug Distribution of RT Inhibitor Tenofovir and Entry Inhibitor Maraviroc in a Humanized Mouse Model 2

3.1 Summary

Pre-exposure prophylaxis (PrEP) strategies utilizing anti-retroviral drugs show considerable promise for HIV-1 prevention. However there is insufficient pharmacokinetic (PK) data on drug concentrations required for protection at the relevant mucosal tissues where the infection is initiated. Here we evaluated the utility of a humanized mouse model to derive PK data on two leading drugs, the RT inhibitor tenofovir (TFV) and CCR5 inhibitor maraviroc (MVC). Following oral dosing, both the drugs and the intracellular active TFV-diphosphate could be detected in vaginal, rectal and intestinal tissues. The drug exposures (AUC_{24h}) were found to be higher in vaginal tissue compared to plasma with even higher levels detected in rectal and intestinal tissues. The overall trends of drug concentrations seen in humanized mice reflect those seen in the human thus establishing the utility of this model complementing the present non-human primate (NHP) models for future pre-clinical evaluations of promising HIV-1 PrEP drug candidates.

3.2 Introduction

² This chapter contains the results of the study published as cited below.

Milena Veselinovic, Kuo-Hsiung Yang, Jonathan LeCureux, Craig Sykes, Leila Remling-Mulder, Angela DM Kashuba, Ramesh Akkina (2014) HIV-1 pre-exposure prophylaxis: Mucosal tissue drug distribution of RT inhibitor Tenofovir and entry inhibitor Maraviroc in a humanized mouse model. *Virology*. 2014 Aug 5;464-465C:253-263. doi: 10.1016/j.virol.2014.07.008.

An estimated 34 million people worldwide were living with HIV-1 in 2011 (UNAIDS global report 2013). The same year 2.5 million new infections were reported, along with 1.7 million HIV-1 related deaths (UNAIDS global report 2013). While the current highly active antiretroviral therapy (HAART) regimens efficiently control the infection and delay progression to AIDS, there is no complete cure due to HIV-1 viral latency [110]. In addition, prolonged therapy can have other problems, including drug resistance and drug toxicity [289]. Preventive vaccines would be ideal to control the spread of HIV-1/AIDS, however no success thus far [205]. Therefore, other approaches are urgently needed to confer protection. In this regard antiretroviral (ARV) drug-based pre-exposure prophylaxis (PrEP) strategies present a practical preventative alternative [230, 231, 290]. Two types of PrEP strategies are currently being pursued with promising results [230, 231, 290]. The first involves oral systemic administration of ARVs, while the second consists of topical vaginal or rectal application of ARV-containing gels with both strategies designed to prevent HIV-1 sexual transmission by mucosal routes. Indeed, field clinical trials had shown promising results with the use of reverse transcriptase inhibitor Tenofovir (TFV) as a microbicide gel in preventing HIV-1 vaginal transmission whereas TFV alone or in combination with Emtricitabine (Truvada) administered orally also provided significant protection [234, 248].

While protection afforded by these above approaches is promising, inconsistencies were found regarding the levels of protection in different clinical trials [234, 245, 248-250, 253]. In the CAPRISA trial, 44% protection was achieved with a TFV microbicide gel, whereas in the VOICE trial, which also used a TFV microbicide gel, the trial was discontinued before conclusion because of a lack of protection [234, 235, 291]. In the IPrEx trial, oral administration of Truvada showed a 44% reduction in HIV-1 infection in men who have sex with men (MSM) [248]. The FEM-PrEP trial, also with oral Truvada, was stopped early due to lack of efficacy in high-risk female population [250]. The TDF2 study also evaluated oral Truvada in men and women and reported a 63% reduction in HIV-1 infection acquisition [249]. The Partners PrEP study, looking at serodiscordant couples, showed a 67% reduction in HIV-1 incidence with oral TFV and a 75%

reduction with Truvada [253]. Additional clinical trials are being conducted in various test populations and results are being awaited [237]. The above conflicting results with regard to variable levels of protection are attributed to differences in ARV dosing regimens, trial designs and target populations, sexual practices and behavioral patterns affecting adherence. A major factor that contributes to protection against HIV-1 is the proper drug concentration to be reached in the target mucosal tissues, such as vaginal and rectal tissue. Tissue drug concentrations are affected by variables such as ARV dose, mode and frequency of administration, tissue permeability and protein binding, as well as drug half-life. Consequently, pharmacokinetic (PK) studies that focus on the mucosal compartments are critical for the future success of PrEP strategies.

Animal models have played crucial role in therapeutic drug development to derive important preclinical data [167, 197]. With regard to assessing HIV-1 PrEP strategies non-human primates (NHP) models have been extremely useful in setting the stage for early human clinical trials [93, 94]. For example, TFV was successfully evaluated as both oral and topical PrEP in rhesus macaque studies [232, 233, 292, 293]. The HIV-1 entry inhibitor maraviroc (MVC) was evaluated similarly in NHP [294, 295]. However, a number of limitations are apparent with NHP models. First of all, they are expensive and use of large numbers of animals often is not feasible. In addition, SIV or SHIV-1, not HIV-1 itself, must be applied as a challenge virus thus precluding NHP use for testing drugs specifically developed against HIV-1. In this context, humanized mice (hu-mice) with a transplanted human immune system have become amenable to test various HIV-1 prevention approaches [167, 169]. Two new generation humanized mouse models are currently available. The hu-HSC humanized mouse model such as RAG-hu employs engraftment of human hematopoietic stem cells (HSC) into newborn immunodeficient ($\text{Rag1}^{-/-}$ or $\text{Rag2}^{-/-}\gamma\text{c}^{-/-}$) mice whereas the BLT mouse model is derived by transplantation of human fetal thymus tissue, liver tissue and HSC [167, 169]. Both models harbor human immune cells in mucosal sites such as vaginal and rectal tissue, and are

susceptible to HIV-1 infection via these routes thus mimicking key aspects of viral mucosal transmission [168, 171, 178]. Both topical and systemic PrEP strategies employing ARVs have been successfully tested using these models [176, 179, 190, 191]. The drugs tested include TFV, MVC, RT inhibitor Emticitabine (FTC) and integrase inhibitor Raltegravir (RAL). Pharmacokinetic and pharmacodynamic (PK-PD) analyses of ARVs in relevant tissue compartments and blood plasma are important to ascertain the relationship between the systemic and local concentrations achieved after drug application and to correlate with antiviral efficacy [230, 246]. Such data can inform the level and duration of effective drug dosing for HIV-1 prevention. While a number of previous studies established the utility of humanized mouse models for efficacy testing of new PrEP strategies and more recently drug distribution in lymphoid tissues [169, 176, 177, 190, 191, 296, 297], no PK studies using these models in the context of mucosal tissues have been described.

Here we evaluated the utility of hu-mice to derive PK data employing two different classes of ARVs - TFV and MVC. Our results show that both ARVs can be readily measured in blood plasma and mucosal compartments encompassing vaginal, rectal and intestinal tissues. The overall drug distribution kinetics in this system were found to be similar to humans thus validating this model for future studies employing more complex combinatorial PrEP dosing regimens.

3.3 Materials and Methods

Generation of humanized mice

Humanized BALB/c Rag1^{-/-} or Rag2^{-/-}γc^{-/-} (Hu-HSC RAG-hu) mice were generated by engrafting human fetal liver-derived CD34⁺ hematopoietic progenitor cells (HPC) as previously described [171, 174]. Mice were maintained at the Colorado State University Painter Animal Center and all studies were approved by the CSU Institutional Animal Care and Use Committee

(Protocol 11-3153A). Newborn mice were preconditioned by irradiating with a sublethal dose of 350 rads and then injected intrahepatically with $0.5\text{--}1 \times 10^6$ human CD34⁺ cells. Mice were screened for human cell engraftment at 10–12 weeks post-reconstitution. Peripheral blood was collected by tail bleed and red blood cells were lysed by the Whole Blood Erythrocyte Lysing Kit (R&D Systems, Minneapolis, MN). The white blood cell fraction was stained against the human pan-leukocyte marker CD45 using hCD45-R-PE (Invitrogen) and FACS analyzed to determine the levels of human cell engraftment as previously reported [171, 174]. Mice with more than 50% human cell engraftment were used for experiments involving hu-mice to assure robust numbers of human cells being present.

Drug administration of antiretrovirals and sample collection

Female mice were administered with either Tenofovir or Maraviroc by oral gavage. Clinical formulations of these drugs in tablet form (Maraviroc (Selzentry) 150 mg, Pfizer Labs; Tenofovir disoproxil fumarate (Viread) 300 mg, Gilead Sciences), were freshly dissolved in sterile PBS prior to oral gavage. Mouse equivalent drug doses were calculated by using an interspecies allometric scaling factor of 12.3 to arrive at 61.5 mg/kg and 62 mg/kg doses for TFV and MVC respectively. Mice (three or five per group) received either TFV (1.23 mg per 20 gram mouse) or MVC (1.24 mg per 20 gram mouse) by oral gavage daily for 5 days. Plasma and tissue samples were collected for each drug following the last gavage at 2h, 8h, and 24h for TFV and at 4h, 12h and 24h for MVC. In addition, 48h plasma samples were collected for one group each of treated animals. All samples except 48h blood plasma were terminal and were collected during mouse necropsies. Tissue samples collected after drug administration consisted of vaginal, rectal and intestinal tissue samples. Tissue and plasma samples were snap frozen in liquid nitrogen within five minutes from the time of tissue collection. For negative controls, plasma and tissue samples were collected from untreated mice and were processed using the protocols described above. Samples were stored at -80°C until drug measurements.

Measurement of drug concentrations in plasma and tissue samples

Quantification of analytes was similar to previously published methods [298]. Briefly, quantification of TFV concentrations in plasma was performed by protein precipitation and LC-MS/MS analysis with an isotopically-labeled internal standard (^{13}C TFV). TFV was eluted from a Waters Atlantis T3 (100 X 2.1mm², 3 μm particle size) analytical column and an API-5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) was used to detect the analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 1-1000 ng/mL using a $1/\text{concentration}^2$ weighted linear regression.

For measuring concentrations in mucosal tissues, TFV and TFV-DP was extracted from tissue homogenate by protein precipitation with isotopically-labeled internal standards (^{13}C TFV and ^{13}C TFV-DP). TFV was eluted from a Waters Atlantis T3 (100 X 2.1mm, 3 μm particle size) analytical column, and TFV-DP was eluted from a Thermo Biobasic AX (50 X 2.1mm, 5 μm particle size) analytical column. An API-5000 triple quadrupole mass spectrometer was used to detect all analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 0.3-300 ng/mL of homogenate for each compound using a $1/\text{concentration}^2$ weighted linear regression. Concentrations were ultimately converted into ng/mg (TFV) or fmol/mg (TFV-DP) tissue for final reporting.

To measure drug concentrations in plasma, MVC was extracted from samples using solid phase extraction with Varian BondElut C-18, 100 mg, 1CC cartridges. Plasma samples were quantified against the internal standard, alprazolam, on an Agilent 1200 series HPLC system using a Zorbax Eclipse XDB (50 x 4.6mm, 1.8 μm particle size) analytical column. An Agilent 1100 MSD was used to detect the analyte and internal standard. The dynamic range of this assay was 1-1000 ng/mL.

For quantification from mucosal tissues, MVC was extracted from tissue homogenates using protein precipitation with the internal standard alprazolam. This resulting extract was

analyzed on an Agilent 1200 series HPLC system using a Zorbax Eclipse XDB (50 x 4.6mm, 1.8 μ m particle size) analytical column. An Agilent 1100 MSD was used to detect the analyte and internal standard. The dynamic range of this assay was 0.007-7 ng/mg.

Statistical analysis

GraphPad Prism version 5 (GraphPad Software, USA) software was used for data analysis, figure generation, calculation of PK parameters and statistical analysis. PK data for TFV, TFV-DP and MVC in hu and non-hu mice were compared using Mann-Whitney two-tailed test. P values <0.05 were considered as significant. Measures of central tendency were expressed as median and inter-quartile range (IQR 25th, 75th percentile).

3.4 Results

TFV and TFV-DP concentrations in plasma and mucosal tissue compartments

To compare the differential drug distribution and accumulation, TFV concentrations were measured in plasma and mucosal tissue compartments namely, vaginal, rectal and intestinal tissues at 2, 8, 24h post-last dose. In addition, plasma concentrations were also determined at 48h. The composite pharmacokinetic data on drug concentrations at various time points in different tissue compartments and plasma of hu-mice are presented in Fig 3.1 A1 whereas the data for individual hu-mice (5 mice per time point) for each of the compartments at different times are presented in Fig 3.1 B1-E1. Similarly, composite pharmacokinetic data and data for individual non-hu mice (3 mice per time point) are presented in Fig 3.1 A2 and Fig 3.1 B2-E2, respectively. Data on TFV C_{max}, T_{max}, half-life ($t_{1/2}$) and area under the curve (AUC) are depicted in Table 3.1 for both hu and non-hu mice.

TFV was easily detected in all compartments at all time points (Fig 3.1 A-E). In hu-mice, highest concentrations in plasma were detected at 2h post dosing (C_{max} 1,990 ng/ml) with a

terminal elimination phase occurring by approximately 24h. The AUC_{48h} for plasma was 11,251 ng*h/ml. The median $t_{1/2}$ in plasma was 17h. In vaginal tissues, C_{max} was at 2h (729 ng/g) with a gradual elimination by 24h. Vaginal tissue AUC_{24h} was 14,946 ng*h/g and TFV concentration in this tissue compartment was higher than in plasma at 8h and 24h. Half-life of TFV in vaginal tissue was 9h. Rectal tissue C_{max} was 56,732 ng/g at 2h with an AUC_{24h} of 1×10^6 ng*h/g and $t_{1/2}$ of 13.5h. In intestinal tissue, C_{max} was 74,677 ng/g at 2h. The AUC was 1.43×10^6 ng*h/g and tissue $t_{1/2}$ was 13.9h. The overall TFV exposure indicated by AUC_{24h} was the highest in intestinal tissue followed by rectal tissue, vaginal tissue and plasma. The AUC_{24h} tissue:plasma ratios for TFV were 1.5 for vaginal, 99 for rectal and 141.7 for intestinal tissue (Fig 3.4). TFV exhibited higher exposures in rectal and intestinal tissue as a group, with concentrations one to two logs higher compared to vaginal tissue and plasma at all time points analyzed (Fig 3.1, Table 3.1). Half-life values of 13.5h and 13.9h in rectal and intestinal tissue respectively suggest similar elimination kinetics from these two tissues with slower elimination rate compared to vaginal tissue ($t_{1/2}$ 9h).

In comparative experiments using non-hu mice, highest TFV concentrations in plasma were also detected at 2h post dosing (median C_{max} 1,550 ng/ml) with a terminal elimination phase by 24h. The AUC_{48h} for plasma was 17,278 ng*h/ml. The median $t_{1/2}$ in plasma was 15h. In vaginal tissues, C_{max} was at 8h (785 ng/g) with a gradual elimination by 24h. Vaginal tissue AUC_{24h} was 9,671 ng*h/g and TFV concentration in this tissue compartment was lower than in plasma at 2h and similar to plasma at 8h and 24h. Half-life of TFV in vaginal tissue was 6.3h. Rectal tissue C_{max} was 76,520 ng/g at 8h with an AUC_{24h} of 1.3×10^6 ng*h/g and $t_{1/2}$ of 53h. In intestinal tissue, C_{max} was 50,334 ng/g at 8h. The AUC was 1×10^6 ng*h/g and $t_{1/2}$ was 61.6h. The overall TFV exposure in non-hu mice, indicated by AUC_{24h} was the highest in rectal tissue followed by intestinal tissue, plasma and vaginal tissue. The AUC_{24h} tissue:plasma ratios for TFV in non-hu mice were 0.7 for vaginal, 95 for rectal and 77.5 for intestinal tissue (Fig 3.4). As in hu-mice, non-hu mice TFV showed higher exposures in rectal and intestinal tissue as a

group, with concentrations one to two logs higher compared to vaginal tissue and plasma (Fig 3.1, Table 3.1). Higher half-life values of 53h and 61.6h in rectal and intestinal tissue respectively were seen in non-hu-mice whereas the $t_{1/2}$ 6.3h seen in vaginal tissue was lower than that in hu-mice. The plasma TFV PK values between the hu and non-hu mice at 24h and 48h time points were found to differ significantly ($p < 0.05$), while in the tissues there were no statistically significant differences.

The prodrug TFV is metabolized into its active form TFV-DP intracellularly in HIV-1 target tissues and cell populations. Accordingly, the levels of TFV-DP were measured in different mucosal tissues (Fig 3.2 and Table 3.1). In hu-mice, the TFV-DP C_{max} was at 2h in rectal tissues (604ng/g) and its AUC_{24h} was 70,194ng*h/g. The $t_{1/2}$ of TFV-DP in rectal compartment was 22.2h. In intestinal tissue TFV-DP C_{max} was 8,841 ng/g at 8h whereas the AUC_{24h} was 172,167 ng*h/g. The intestinal TFV-DP $t_{1/2}$ was 3.5h. The TFV-DP concentrations could not be measured in vaginal tissues consistently as it was detected only once each at 2h and 24h. This is in line with difficulties noted in detection of low TFV-DP concentrations in previous human studies [255, 256]. Overall, the intestinal TFV-DP exposure was found to be higher than in the rectal compartment indicated by the AUC_{24h} intestinal:rectal tissue ratio of 2.45. TFV-DP concentrations were also assessed in non-hu-mice for comparison. The TFV-DP C_{max} was at 24h in rectal tissue (198 ng/g) and its AUC_{24h} was 3,188 ng*h/g. The $t_{1/2}$ of TFV-DP in rectal compartment could not be calculated due to no elimination seen at 24h. In intestinal tissue, C_{max} was 119 ng/g at 8h, the AUC_{24h} was 2,414 ng*h/g and the $t_{1/2}$ was 45.3h. Overall, in non-hu mice the rectal TFV-DP exposure was found to be higher than in the intestinal compartment indicated by the AUC_{24h} . Similar to that in hu-mice above, TFV-DP could not be detected in vaginal compartment. TFV-DP PK differences in hu vs. non-hu mice were statistically significant ($p < 0.05$) at all time points in both rectal and intestinal compartments with the exception of 2h in the intestinal tissue.

Table 3.1. PK parameters for Tenofovir and Tenofovir Diphosphate in humanized and non-humanized mice.

TFV (hu mice)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}[*]	1990	729	56732	74677
Median (IQR)	(898 – 3310)	(581 – 6739)	(21746 – 164561)	(55008 – 288570)
T_{max}	2h	2h	2h	2h
AUC[†]	11251	14946	1 x 10 ⁶	1.43 x 10 ⁶
t_{1/2}[‡]	17	9	13.5	13.9
TFV-DP(hu mice)				
C_{max}[*]	NA	BLQ [*]	604	8,841
Median (IQR)		/	(336 – 6466)	(322 – 28175)
T_{max}	NA	/	2h	8h
AUC[†]	NA	/	70194	172167
t_{1/2}[‡]	NA	/	22.2	3.5
TFV (non-hu mice)				
C_{max}[*]	1,550	785	76,520	50,334
Median (IQR)	(1200-1990)	(224 - 819)	(50493 - 78415)	(38112 - 72930)
T_{max}	2h	8h	8h	8h
AUC[†]	17278	9671	1.3 x 10 ⁶	1 x 10 ⁶
t_{1/2}[‡]	15	6.3	53	61.6
TFV-DP(non-hu mice)				
C_{max}[*]	NA	BLQ	198	119
Median (IQR)				
T_{max}	NA	/	24h	8h
AUC[†]	NA	/	3188	2414
t_{1/2}[‡]	NA	/	/ ^T	45.3

Median (interquartile range, IQR), ng/ml or ng/g; [†]Area under the curve (AUC)_{48h} plasma (ng * h/ml); AUC_{24h} tissues (ng * h/g); ^{*}Half-life (t_{1/2} h); ^{*} TFV-DP detected in 2 samples in humanized mice – once at 2h and once at 24h; ^T no elimination phase; BLQ – below the limit of quantification; NA - not applicable

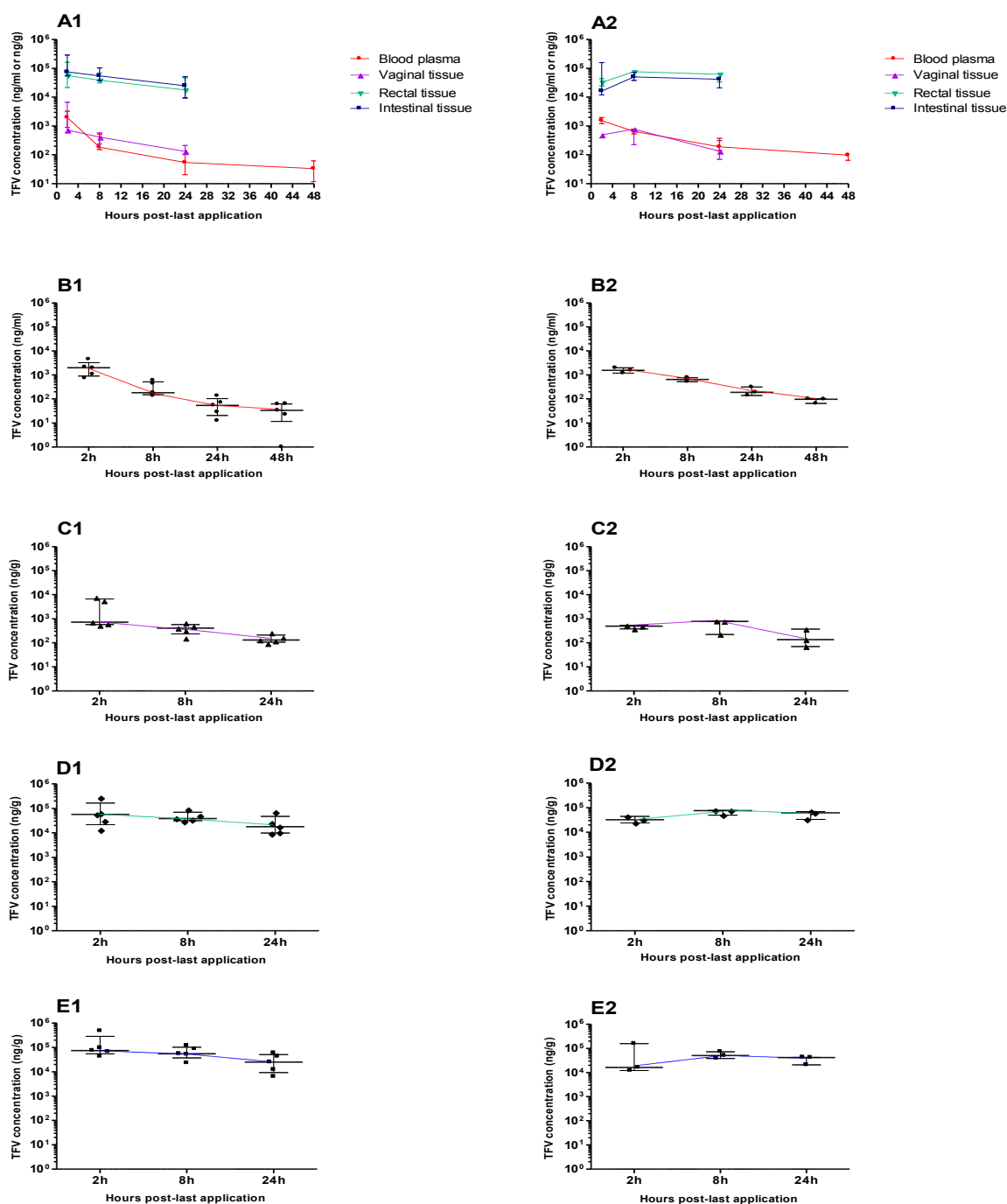


Figure 3.1. PK analysis of orally administered TFV in hu- and non-hu mice. Mice were administered TFV by oral gavage (human equivalent dose 61.5 mg/kg) for 5 days. Post-last dose, plasma and tissue samples were collected at different time points and drug concentrations were determined (ng/ml or ng/g). A1 (hu-mice), A2 (non-hu mice). Composite medians and interquartile ranges (IQR) for plasma vaginal, rectal and intestinal tissue (colon). B1-E1 (hu-mice), B2-E2 (non-hu mice). Individual data points (n=5 hu-

mice, n=3 non-hu mice) for plasma (B), vaginal (C), rectal (D) and intestinal tissue (colon) (E) with composite medians (IQR) shown.

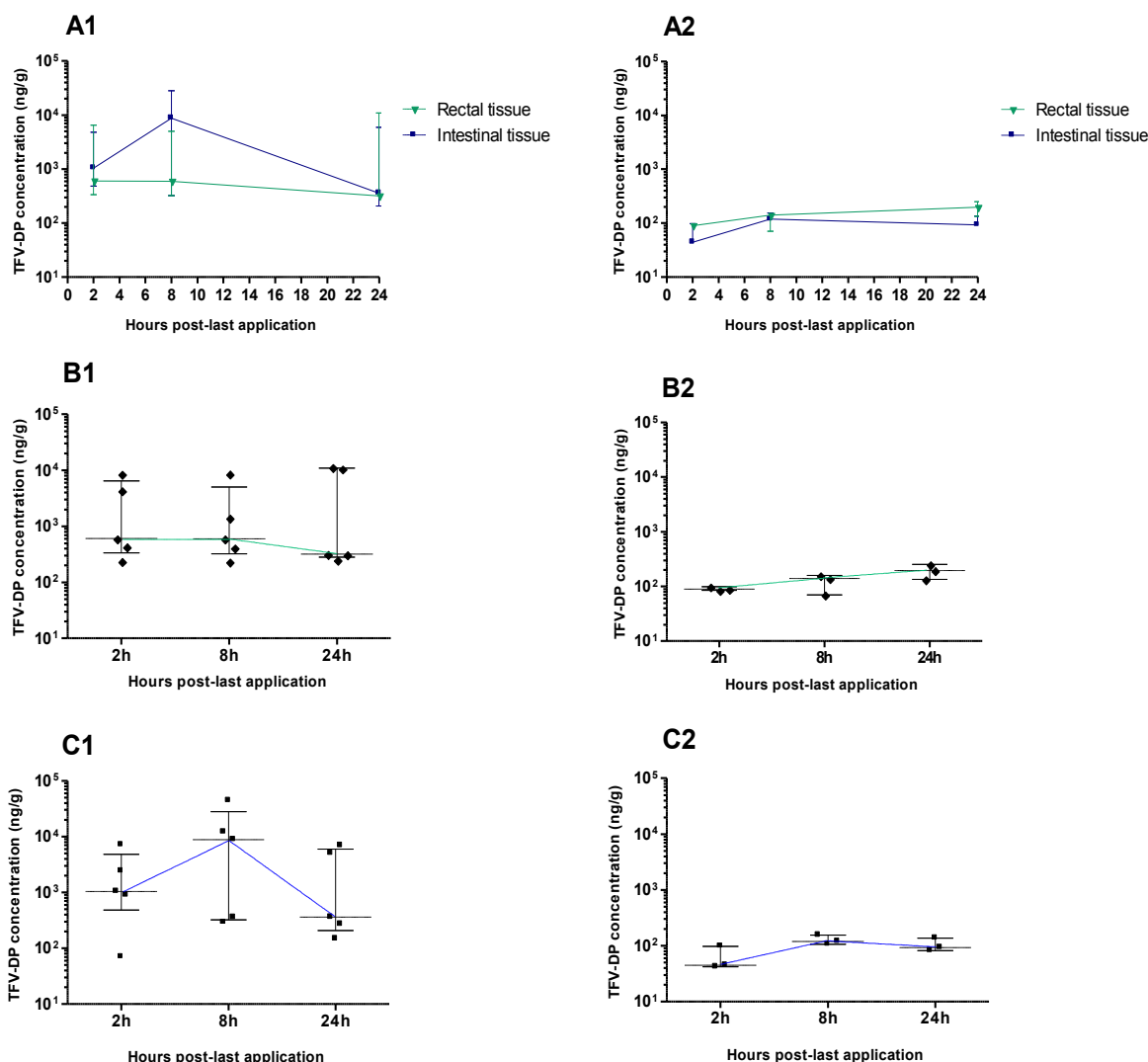


Figure 3.2. PK analysis of TFV DP in hu- and non-hu mice. TFV was gavaged as in Fig 1 above. Intracellular, diphosphorylated metabolite of TFV (TFV-DP) was detected and quantified in tissues at different times post-last dose. A1 (hu-mice), A2 (non-hu mice). Composite median, IQR for TFV DP in rectal and intestinal tissue (colon). B1,C1 (hu mice), B2,C2 (non-hu mice). Individual data points shown (n=5 hu mice, n=3 non-hu mice) with composite median, IQR in rectal (B) and intestinal tissue (C).

MVC concentrations in plasma and mucosal tissue compartments

The HIV-1 entry inhibitor MVC acts extracellularly by interfering with viral binding to the CCR5 co-receptor. In the first set of experiments, non-hu mice were used (5 per each time

point) whereas in the second set of experiments hu-mice (3 per time point) were used to determine if any differences exists between the PK values. The drug concentrations were measured in plasma and vaginal, rectal and intestinal mucosal tissue compartments at 4, 12, 24h post-last dose. In addition, plasma concentrations were also determined at 48h. The composite PK data on MVC drug concentrations at various time points in different tissue compartments and plasma are presented in Fig 3.3 A1 (hu-mice) and 3.3 A2 (non-hu-mice), whereas the data for individual mice for each of the compartments are presented in Fig 3.3 B1-E1 (hu-mice) and Fig 3.3 B2-E2 (non-hu-mice). PK parameters C_{max} , T_{max} , $t_{1/2}$ and AUC are shown in Table 2.

MVC was detected in all compartments at all time points analyzed. For non-hu mice, highest concentrations in plasma post-last dose were detected at 4h (C_{max} 113 ng/ml). The AUC_{48h} for plasma was 3,280 ng*h/ml. The composite $t_{1/2}$ in plasma was 20.1 hrs. In vaginal tissue C_{max} was at 4h (1,764 ng/g) with a gradual elimination up to 12h. Vaginal tissue AUC_{24h} was 11,239 ng*h/g and MVC was detected at higher concentrations than in plasma at 4 h, 12h and 24h. The $t_{1/2}$ of MVC in vaginal tissue was 3.7h. Rectal tissue MVC kinetics was characterized by C_{max} of 3,785 ng/g at 4h, AUC_{24h} of 69,301 ng*h/g and $t_{1/2}$ of 2.9h. In intestinal tissue MVC C_{max} was 18,194 ng/g at 4h, AUC_{24h} was 134,883 ng*h/g and $t_{1/2}$ was 2.7h. The overall MVC exposure, indicated by AUC_{24h} was the highest in intestinal tissue followed by rectal tissue, vaginal tissue and plasma. The AUC_{24h} tissue:plasma ratios for MVC were 3.65 for vaginal, 22.5 for rectal and 43.8 for intestinal tissue (Fig 3.5).

With regard to PK of MVC in hu-mice versus non-hu mice, the overall tissue to plasma distribution trends were similar, with intestinal tissue showing the highest exposure (AUC_{24h} 140,159 ng*h/g), followed by rectal tissue (AUC_{24h} 32,690 ng*h/g), vaginal tissue (AUC_{24h} 2,461 ng*h/g) and plasma (AUC_{24h} 488.6 ng*h/ml, AUC_{48h} 755 ng*h/ml). Concentration of MVC at 4h (C_{max}) in hu-mice was similar to that in non-hu mice in plasma. The MVC concentrations in rectal and intestinal tissues were higher in hu vs. non-hu mice. Significant difference was seen in

plasma wherein the $t_{1/2}$ was 20 times lower in hu vs. non-hu mice (1h vs. 20h), while the $t_{1/2}$ in tissue compartments were slightly lower. Also, lower drug exposure (AUC_{24h}) was seen in hu-mice, with the exception of intestinal tissue. However, the observed differences in drug concentrations between hu-mice and non-hu mice were not found to be statistically significant (Mann-Whitney two-tailed test) except at 4h for intestinal tissue ($p < 0.05$) and 12h for vaginal tissue ($p < 0.05$).

Table 3.2. PK parameters for Maraviroc in humanized and non-humanized mice.

MVC (hu-mice)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
Cmax [*]	102	459	7577	33462
Median (IQR)	(46 – 109)	(228 – 659)	(5492.6 – 9769)	(31958 – 36819)
Tmax	4h	4h	4h	4h
AUC [†]	755	2461	32690	140159
$t_{1/2}$ [‡]	1	2.6	1.5	1.2
MVC (non-hu mice)				
Cmax [*]	113	1764	3785	18194
Median (IQR)	(43 - 797)	(403 – 1982)	(2329 – 8512)	(9893 – 22980)
Tmax	4h	4h	4h	4h
AUC [†]	3280	11239	69301	134883
$t_{1/2}$ [‡]	20.1	3.7	2.9	2.7

^{*}Median (interquartile range, IQR), ng/ml or ng/g; [†]Area under the curve (AUC)_{48h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g); [‡]Half-life ($t_{1/2}$, h)

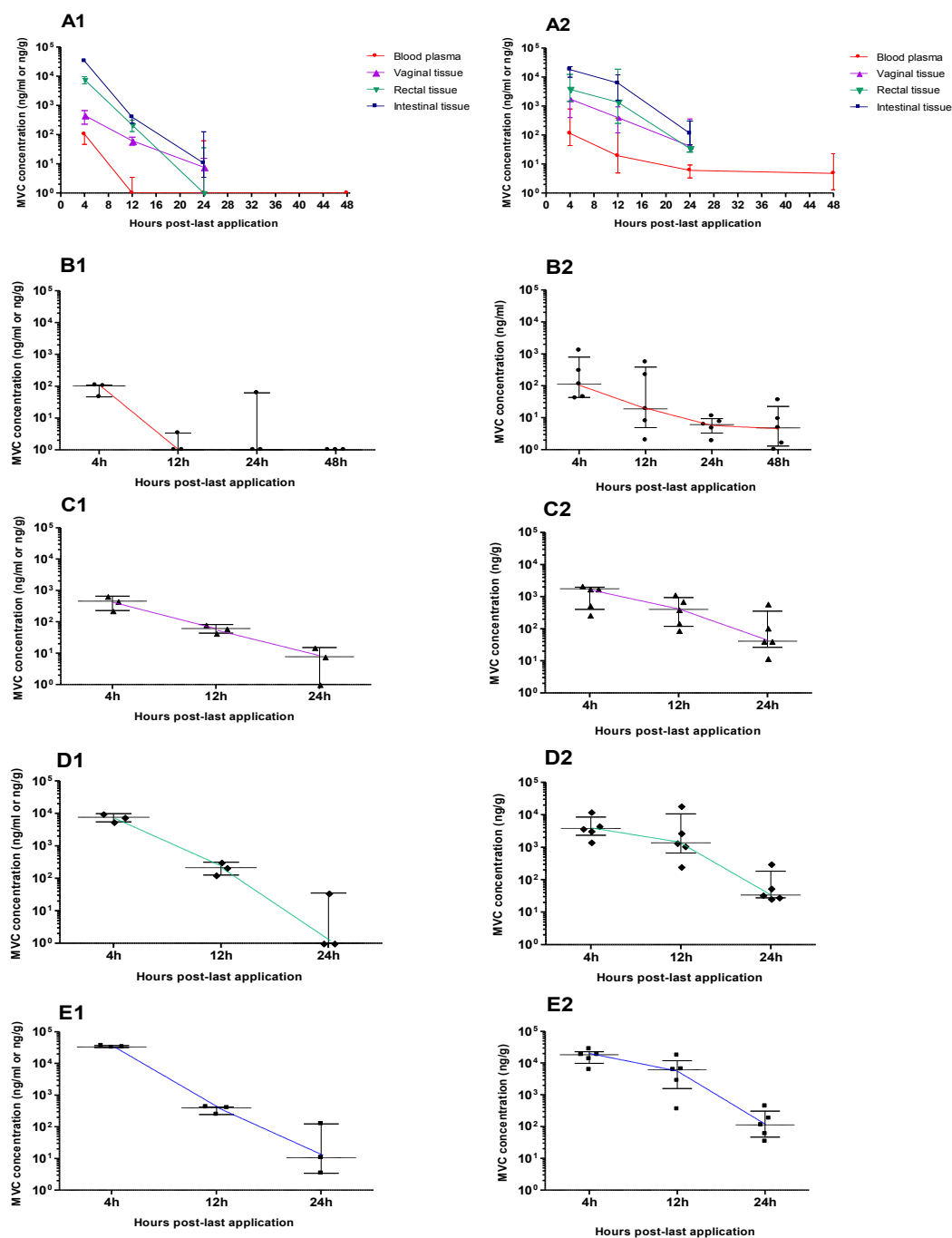


Figure 3.3. PK analysis of orally administered MVC in hu- and non-hu mice. Mice were administered MVC by oral gavage (human equivalent dose 62 mg/kg) for 5 days. Post-last dose, plasma and tissue samples were collected at different time points and drug concentrations were determined (ng/ml or ng/g). A1 (hu mice), A2 (non-hu mice). Composite medians and interquartile ranges (IQR) for plasma, vaginal, rectal and intestinal tissue (colon). B1-E1 (hu mice), B2-E2 (non-hu mice). Individual data points (n=3 hu-

mice, n=5 non-hu mice) for plasma (B), vaginal (C), rectal (D) and intestinal tissue (colon) (E) with composite medians (IQR) shown.

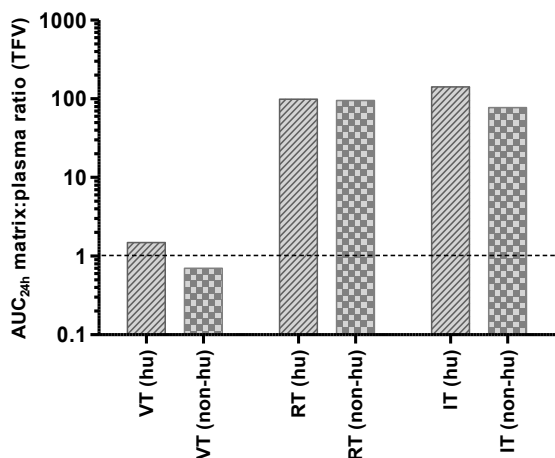


Figure 3.4. Tissue to blood plasma AUC_{24h} ratios of TFV. Tissue to plasma ratios were calculated for AUC_{24h}. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. Values above the line of unity indicate higher drug exposure at mucosal sites compared to plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

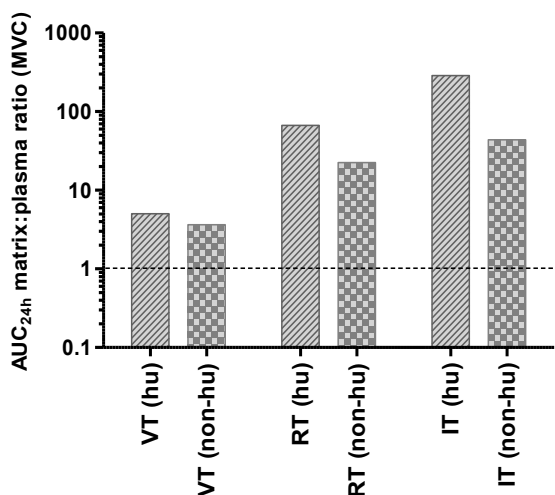


Figure 3.5. Tissue to blood plasma AUC_{24h} ratios of MVC. Tissue to plasma ratios were calculated for AUC_{24h}. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. Values above the line of unity indicate higher drug exposure at mucosal sites compared to plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon)

3.5 Discussion

Nearly all present PrEP strategies employ currently approved ARTs for HIV-1 prevention [246]. However, the doses being used for treating the patients do not accurately represent preventive doses necessary for full protection against HIV-1. Therefore, protective drug concentrations at the mucosal transmission target sites such as vaginal and rectal tissue must be defined to inform optimal dosing. Derivation of this much needed data by experimental studies will represent a significant advancement in the PrEP arena.

Here we utilized a humanized mouse model (RAG-hu) susceptible to HIV-1 mucosal transmission to assess the drug concentrations following oral dosing with two leading PrEP candidates with different modes of action, RT inhibitor TFV and entry inhibitor MVC. Since concurrent measurements after single dosing may under or overestimate true tissue exposures due to different distribution characteristics in tissues compared with plasma, we measured drug concentrations after multiple dosing at steady state kinetics [246]. Our results showed that both TFV and MVC can be readily measured in blood plasma, vaginal, rectal and intestinal tissues. While there were number of previous studies in the human, this is the first experimental study that simultaneously measured the respective drug concentrations in multiple mucosal tissues to correlate their values with those in plasma [230, 246].

Previous studies on TFV in hu-mice showed protection against HIV-1 vaginal challenge following systemic and topical drug application [176, 177, 179]. Here with oral dosing, peak concentrations of TFV were detected at 2h after last dose in each of the compartments tested with a terminal elimination phase at 24-48h. The TFV half-life in blood plasma in hu-mice was comparable to that seen in an earlier human trial [254]. The overall drug exposure determined by AUC_{24hr} for TFV in vaginal tissue was found to be 1.5 higher than in blood plasma and two logs higher than in blood plasma in rectal and intestinal tissue. This is further illustrated by the tissue: plasma AUC_{24h} ratios (T:P ratio) (Fig 3.4), which were 1.5, 99.0 and 141.7 for vaginal,

rectal and intestinal tissues respectively, indicating higher drug exposure in vaginal and rectal tissues relative to plasma. This observed differential accumulation in various tissues is in agreement with the data from multiple human studies and could be due to multiple factors that include distinct mucosal expression and localization of drug transporters [230, 246, 299].

Post-uptake, the prodrug TFV is converted intracellularly to its pharmacologically active form TFV diphosphate (TFV-DP) inhibiting HIV-1-RT. As seen with its prodrug, the TFV-DP exposure was also higher in intestinal tissue than in rectal tissue as depicted by higher AUC_{24h} (172,167 ng*h/g vs. 70,194 ng*h/g) and T:P ratio (141.7 vs. 99). In contrast, the half-life of TFV-DP was higher in rectal tissue (22.2h) compared to the intestinal tissue (3.5h). Differences in TFV-DP concentrations in rectal and intestinal compartments could be due to distinct expression and activity levels of native kinases needed for TFV to TFV-DP conversion, and/or differences in tissue expression of drug transporters, in addition to other factors influencing the PK of the prodrug TFV [230, 246, 299].

Consistent with human studies, higher concentrations of TFV were seen in hu-mouse mucosal tissues compared to blood plasma [256, 300, 301]. The overall TFV tissue exposures were also similar to that in the human as depicted by AUC_{24h} tissue:plasma ratios in vaginal, rectal and intestinal tissues [255]. Sustained TFV and TFV-DP concentrations were seen in the rectal compartment similar to a previous human study [255]. Long lasting intracellular TFV-DP levels seen in hu-mice are also akin to those seen in another previous human trial [302]. The extended half-life seen in the rectal tissue is advantageous for protection in MSM and this was documented in iPrEX study wherein it was found that individuals that did not fully comply with daily recommended dosing were still protected from HIV-1 infection [248]. These findings taken together suggest that overall, the relative PK trends of TFV and its active metabolite TFV-DP in hu-mice are similar to that seen in human studies and clinical trials. Moreover, the hu-mice data further confirm the favorable PK profile of TFV for its use as a PrEP agent.

With regard to the PK profiles of TFV and TFV-DP in hu- versus non-hu mice, while the overall AUC_{24h} trends were similar with drug concentrations being higher in intestinal, rectal tissues than in plasma, differences were also noted in tissue exposure levels, T_{max} and $t_{1/2}$. For example, the TFV AUC_{24h} tissue:plasma ratios in vaginal and intestinal tissue of hu-mice were 2 fold higher compared to non-hu mice whereas the $t_{1/2}$ was longer in rectal and intestinal tissue of non-hu mice (53h and 61.6h, respectively) relative to that in hu-mice (13.5h and 13.9h). With TFV-DP, one to two log higher AUC_{24h} was seen in rectal and in intestinal tissues of hu-mice whereas its $t_{1/2}$ was longer in non-hu mice in both intestinal (45.3h versus 3.5h) and rectal (no elimination phase was detected by 24h, compared to 22.2h $t_{1/2}$ in hu-mice) compartments compared to hu-mice (Table 3.1). While not entirely clear, the observed PK differences between hu- versus non-hu mice can be partly attributed to the absence of circulating lymphoid cell population, and thus drug clearance in the blood compartment as well as the mucosal tissues of non-humanized mice.

With respect to the entry inhibitor MVC, we have previously shown that its oral administration at allometric dosing levels is fully protective against HIV-1 vaginal challenge in hu-mice [191]. MVC exerts its antiviral action extracellularly in contrast to the RT inhibitor TFV which gets converted to its active form intracellularly. Therefore, in our initial experiments we analyzed MVC concentrations in non-hu mice. Maximum MVC concentrations were reached at 4h in plasma and in all the mucosal tissues. The highest exposure to MVC was seen in intestinal tissue (AUC_{24h} T:BP ratio of 43.8), followed by rectal and vaginal tissue (AUC_{24h} ratios of 22.5 and 3.6) The PK trends seen here were comparable to those in human studies [298, 303]. As indicated by AUC_{24h} tissue:plasma ratios, there were higher exposures in vaginal and rectal tissue compartment to that in blood plasma, similar to that seen in previous human clinical trials [298, 303]. In addition, blood plasma T_{max} , elimination rate and AUC_{12h} were similar to those found in human studies [303, 304]. Sustained high rectal drug concentrations up to 12h correlated well with those found in an earlier human study [298].

We next determined the MVC pharmacokinetics in hu-mice to see if any differences exist when compared with non-hu mice. Similar to the results seen in non-hu mice, maximum drug concentrations were reached at 4h in all compartments analyzed. Higher drug exposure (AUC_{24h}) was also detected in rectal and intestinal tissues compared to vaginal tissue. However, a significant difference was the much shorter half-life seen in blood plasma of hu-mice versus non-hu mice (1h vs. 20h). This could be attributable to MVC binding to the CCR5 co-receptor on human cells in the blood and tissue compartments of hu-mice. AUC_{24h} in plasma, vaginal and rectal tissues in non-hu-mice were found to be higher than in hu-mice most likely due to the absence of human cells that would have sequestered most of the drug in the blood cellular compartment. Higher tissue:plasma AUC_{24h} ratios were seen in hu-mice compared to non-hu mice for all tissues (Fig 3.5). This is not unexpected due to lower plasma MVC half-life seen in hu-mice. Relative to that seen in human studies, the AUC_{24h} tissue:plasma ratios were found to be higher in hu-mice. For vaginal tissue, MVC AUC_{24h} VT:BP ratio was 5 in hu-mice compared to 1.9 seen in the human whereas in rectal tissue, the RT:BP AUC ratio was 66.9 relative to the 8-26 range seen in the human. Nevertheless, the overall MVC exposure trends of higher drug concentrations seen in mucosal tissues versus blood plasma are similar between hu-mice and the human. The vaginal concentration of MVC necessary for full HIV-1 prevention in human clinical studies is not currently known. Therefore the concentrations measured in hu-mice wherein MVC was shown to be fully protective against HIV-1 vaginal challenge should inform future dosing strategies in the human [190]. The overall excellent oral bioavailability and high genital tract exposure of MVC relative to plasma as demonstrated in these studies lends further support for it being an ideal candidate for oral PrEP. However in comparison with TFV and its active metabolite TFV-DP, MVC half-life in tissue compartments is much shorter and thus will require consistent dosing and more patient compliance in terms of adherence.

For both the ARVs analyzed, drug concentrations varied widely between individual mice at some time points although the composite PK trends were similar. This is not unlike that

observed in previous NHP and human studies wherein a wide degree of inter and intra-subject variability was seen [93, 94, 255, 256, 298, 301, 303]. While we used hu-mice with robust human cell reconstitution, their levels in individual mice could have partly played a role. However, this appears unlikely since wide variations in drug concentrations were seen in non-hu-mice as well.

With regard to the PK studies like these in the PrEP context, the importance of using hu-mice versus non-hu-mice is apparent. This is illustrated by differences such as MVC plasma half-life being lower in hu-mice compared to non-hu mice. Additionally for compounds such as TFFV which function intracellularly after phosphorylation, use of hu-mice harboring human cells is also more appropriate. Based on the overall data from above, hu-mice susceptible to HIV-1 mucosal transmission can be exploited to assess ARV PK parameters. In addition to complementing results from NHP models, data derived in hu-mice on novel drugs would help inform more detailed subsequent NHP studies where appropriate. Drug concentrations determined to confer protection against HIV-1 itself in hu-mice can be extrapolated to the human to inform HIV-1 prevention doses of novel ARVs for prophylactic purposes.

Since the use of a single drug may not be adequate to confer full protection in a global setting, future oral HIV-1 PrEP strategies will most likely involve a combination of ARVs with different mechanism of action for complete efficacy. However, whether the combinatorial ARVs would retain similar tissue distribution and overall kinetics compared to when applied singly needs to be determined. Such combinatorial studies can be conducted in hu-mice to define PK-PD parameters of various multi-drug regimen approaches to assess potential additive, synergistic and antagonistic effects between different classes of ARVs. In addition, adherence issues of PrEP use and efficacy can also be simulated in hu-mice to derive useful preclinical data [305]. It is also clear from the present data and previous reports that drug distribution and kinetics in different tissues vary [230, 246]. This could be due to multiple factors which include target cell drug influx and efflux properties, differential mucosal expression and distribution of

specific drug transporters and distinct expression of cytochrome P450 (CYP) and other drug metabolizing enzymes in mucosal compartments [299, 306]. Such parameters can be experimentally evaluated in the humanized mouse model in the context of the human immune cell populations targeted by HIV-1. The preclinical knowledge gained from such *in vivo* studies addressing these questions will be very useful for more informed clinical trials of new PrEP strategies.

CHAPTER 4

Mucosal Tissue Drug Distribution of HIV-1 Integrase Inhibitor Raltegravir in a Humanized Mouse Model

4.1 Summary

Raltegravir (RAL), an HIV-1 integrase strand transfer inhibitor, is a potent antiretroviral (ARV) used for the treatment of HIV-1 infection. While PK data are available for therapeutic use, no such data are available for pre-exposure prophylaxis (PrEP) applications. Its irreversible integrase inhibition, low IC_{95} (95% inhibitory concentration) against CCR5 and CXCR4 wild-type and resistant isolates, rapid suppression of viremia, mild side effects and limited drug-drug interaction profile, make it a good candidate for PrEP. In previous clinical studies high inter and intra-patient pharmacokinetic (PK) variability was seen, with no dose-proportional relationship to pharmacodynamic (PD) activity (antiviral efficacy). A comprehensive analysis of RAL PK-PD in mucosal tissues targeted by HIV-1, such as vaginal and rectal tissues, is lacking. To this end, we evaluated the utility of a humanized mouse model to derive PK data on RAL during steady state conditions. Following oral application of human therapeutic dose equivalents, RAL PK was analyzed in plasma and vaginal, rectal and intestinal tissue compartments. RAL exhibited high PK variability in humanized mice, as seen in previous human studies. The general trends of blood plasma versus mucosal tissue distribution mimicked those in humans, as RAL exhibited higher exposure in mucosal tissues at all time points analyzed compared to plasma and median concentrations were one log (vaginal and rectal tissue) to two logs (intestinal tissue) higher than in plasma. The AUC_{24h} tissue:plasma ratios in humanized mice were 6 for vaginal, 8.5 for rectal and 29 for intestinal tissue. Investigating the PK-PD relationship of RAL in this model for PrEP, could inform future dosing strategies using RAL as a PrEP agent.

4.2 Introduction

In the absence of a vaccine, other preventative approaches are needed for the ongoing HIV-1 pandemic. Pre-exposure prophylaxis (PrEP) implies the use of antiretrovirals (ARVs) aimed at blocking HIV-1 mucosal transmission, which represents the dominant route of HIV-1 spread globally. Only one anti-HIV-1 agent, namely the combination of the RT inhibitors Tenofovir and Emtricitabine (Truvada), has been approved for PrEP applications thus far. New drug candidates are needed in order to increase the level of protection offered by PrEP against various globally circulating HIV-1 strains in different vulnerable populations. Raltegravir (RAL) is the first generation HIV-1 integrase strand transfer inhibitor, approved for clinical use in treatment-experienced and antiretroviral therapy (ART)-naïve adult patients, as well as in children and adolescents (2-18 years of age) [307, 308]. It has been shown to be effective against multiple wild-type and multidrug resistant HIV-1 clinical isolates and against both CCR5 and CXCR4 tropic HIV-1 [309]. Its mode of action makes RAL an optimal complement to a reverse transcriptase (RT) inhibitors. RAL has a protein-corrected *in vitro* 95% inhibitory concentration (IC_{95}) of 33nM (14.6 ng/ml) [310]. In treatment-naïve patients, a RAL-containing regimen suppresses HIV-1 RNA replication more rapidly than an Efavirenz (EFV)-based regimen [311]. Other favorable aspects of RAL include mild side effects and minimal drug-drug interactions from its uridine glucuronosyl transferase (UGT)-dependent metabolism [307, 312, 313]. No human homolog of the HIV-1 integrase exists; therefore there is no possibility of unspecific inhibition of human cellular enzymes [307]. Low molecular weight (482.51 Da), slight lipophilicity and 83% protein binding indicate good potential for penetration into tissues following oral application [314]. Moreover, the binding of RAL to the preintegration complex (PIC) is considered irreversible, since the dissociation rate of the drug from the complex is longer than the half-life of the complex itself [315]. These characteristics make RAL a promising candidate for PrEP against HIV-1 mucosal transmission.

Further comprehensive studies are needed to enable RAL pharmacokinetic-pharmacodynamic (PK-PD) modeling for PrEP in vulnerable mucosal tissues, including vaginal, cervical and rectal tissue. Humanized mouse models harbor human immune cells in these mucosal sites which are susceptible to HIV-1 infection via these routes thus mimicking key aspects of viral mucosal transmission [171, 178, 316]. Both topical and systemic PrEP strategies employing ARVs have been successfully tested using these models [176, 179, 190, 191]. Developing a PK-PD model in humanized mice would enable preclinical testing of new PrEP candidates. The data obtained in this small animal model would inform further studies of RAL as a PrEP agent in non-human primate (NHP) models and in clinical trials. Therefore, we have examined RAL PK in a humanized mouse model (RAG-hu mice) under steady-state conditions of oral human therapeutic equivalent doses.

4.3 Materials and methods

Generation of humanized mice

Humanized BALB/c- Rag1^{-/-} γ c^{-/-} and BALB/c- Rag2^{-/-} γ c^{-/-} (RAG-hu) mice were generated using human fetal liver-derived CD34⁺ hematopoietic progenitor cells as previously described [170-172, 174]. Mice were maintained at the Colorado State University Painter Animal Center and all studies have been reviewed and approved by the CSU Institutional Animal Care and Use Committee (Protocol 11-3153A). Newborn mice were injected intrahepatically with 0.5–1×10⁶ human CD34⁺ cells after being preconditioned by irradiating with a sublethal dose of 350 rads. Human cell engraftment was analyzed at 10–12 weeks post-reconstitution. Peripheral blood was collected by tail bleed and Whole Blood Erythrocyte Lysing Kit (R&D Systems, Minneapolis, MN) was used for red blood cell lysis. The white blood cell fraction was stained against the human pan-leukocyte marker CD45 (hCD45-R-PE, Invitrogen) and FACS analyzed to determine

the level of human cell engraftment [174]. Mice with more than 50% human cell engraftment were included in the study, to ensure good level of humanization in mucosal tissues.

Drug administration of antiretrovirals and sample collection

Female mice were administered raltegravir by oral gavage. The clinical formulation of the tablet (Isentress®: 400 mg, Merck & Co) was freshly dissolved in sterile PBS prior to oral gavage. The mouse equivalent dose was calculated by using an interspecies allometric scaling factor of 12.3. Mice received RAL (3.28 mg per 20 gram mouse) by oral gavage daily for 5 days. Initial experiment included 5 mice per time point. Additional mice (2-3) were tested for the time points and compartments for which RAL concentration was below limit of quantification or showed high degree of variability in the initial study. Plasma and vaginal, rectal and intestinal tissue samples were collected following the last gavage at 2h, 8h, and 24h. In addition, 48h plasma samples were collected from one group of treated animals. All samples except 48h blood plasma were terminal and were collected during mouse necropsies. Tissue and plasma samples were snap frozen in liquid nitrogen within five minutes from the time of collection. For negative controls, plasma and tissue samples were collected from untreated mice and were processed using the protocols described above. All samples were stored at -80° C prior to drug concentration analysis.

Measurement of drug concentrations in plasma and tissue samples

Quantification of analytes was similar to previously published methods [317]. RAL was extracted from the matrices by protein precipitation and quantified by LC-MS/MS analysis. Plasma samples were mixed with methanol containing the isotopically-labeled internal standards (²H RAL). Following vortex and centrifugation, the supernatant was removed and evaporated to dryness. The extracts were reconstituted with 1mM ammonium phosphate prior to LC-MS/MS analysis. RAL was eluted from a Phenomenex Synergi Polar-RP (50 X 4.6mm, 4µm

particle size) analytical column. An API- 5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) was used to detect the analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 1-1000 ng/mL for each compound using a $1/\text{concentration}^2$ weighted linear regression.

Tissues were homogenized in 70:30 acetonitrile: 1mM ammonium phosphate and RAL in the resulting homogenate was extracted by protein precipitation with acetonitrile containing isotopically-labeled internal standard (^2H RAL). Following vortex and centrifugation, the supernatant was removed and evaporated to dryness. Samples were analyzed in a similar fashion to plasma. The dynamic range of this assay was 0.3-300ng/mL of homogenate.

Pharmacokinetic and statistical analysis

Pharmacokinetic analysis was performed with Phoenix WinNonlin Pro 5.2 (Certara, L.P., St. Louis MO) and GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA). Median maximal concentration (C_{max}), time to maximal concentration (T_{max}), geometric mean concentration at 24h (C_{trough}), as well as geometric mean concentration and coefficients of variation (CV%) for all time points and compartments were determined in GraphPad Prism. Measures of central tendency were expressed as composite median and inter-quartile range (IQR 25th, 75th percentile). The log-linear trapezoidal method was used to calculate area under the 48h (plasma) or 24h (tissues) time/concentration curve ($\text{AUC}_{48\text{h}}$ or $\text{AUC}_{24\text{h}}$). The AUC tissue to plasma ratios were calculated using $\text{AUC}_{24\text{h}}$ for plasma and tissue compartments. The half-lives ($t_{1/2}$) were determined with Phoenix WinNonlin using the median composite PK profile. Only data points determined to occur in the beta-elimination phase through visual inspection were included in the half-life calculation. PK data in hu and non-hu mice were compared using Mann-Whitney two-tailed test. P values <0.05 were considered as significant.

4.4 Results

RAL concentrations in plasma and mucosal tissue compartments of humanized mice

The composite pharmacokinetic data in plasma and tissue compartments are presented in Fig 4.1 A1. The data for individual mice (5 to 8 mice per time point) for each of the compartments and time points are presented in Fig 4.1 B1-E1. Pharmacokinetic parameters are shown in Table 4.1.

The detection of RAL in hu mice was successful in all compartments and at all time points in 85.1% of analyzed samples. RAL concentrations were below the limit of detection in 14.9% of samples analyzed, at 24h for tissues and at 24h and 48h for plasma (Fig 4.1 B1-E1). Complete data on geometric means and CV% are shown in Table 4.2.

Noncompartmental analysis from the median composite PK profile showed that T_{max} occurred at 2h for all matrices in hu mice. Exposure to RAL, measured as AUC, median C_{max} and C_{trough} , was the highest in the intestinal tissue (Table 4.1: AUC_{24h} : 135,610 ng/g x h, median C_{max} 11692 ng/g, C_{trough} 16.6 ng/g) and was one log higher than in vaginal tissue (Table 4.1: AUC_{24h} : 28172 ng/g x h, median C_{max} 3440 ng/g, C_{trough} 3.2 ng/g). Exposure in the intestinal tissue was also one log higher than in rectal tissue (Table 4.1: AUC_{24h} : 39742 ng/g x h, median C_{max} 1213 ng/g, C_{trough} 5.5 ng/g). Half-life of RAL was 3 times longer in plasma (9.5h) compared to all tissues (3.3h, 3.5h and 2.8h in vaginal, rectal and intestinal tissues). The significantly higher exposure in the intestinal tissue was further confirmed by the AUC_{24h} tissue:plasma ratios. The AUC_{24h} tissue:plasma ratio in the intestines was 29, 5 times higher than in vaginal tissue (AUC_{24h} t:p ratio 6) and 3.5 times higher than in rectal tissue (AUC_{24h} t:p ratio 8.5) (Fig 4.2).

RAL concentrations in plasma and mucosal tissue compartments of non-humanized mice

In Fig 4.1 A2 plasma and tissue composite pharmacokinetic data are shown. In Fig 4.1 B2-E2 the data for individual non-hu mice (3 mice per time point) are presented with median and interquartile range. Pharmacokinetic RAL parameters are shown in Table 4.1 and Table 4.2.

In non-hu mice, RAL was detected in all compartments at all time points analyzed and the variability in drug concentrations in plasma and tissues was less pronounced compared to hu-mice (Fig 4.1 B2-E2). Similar to hu mice, RAL exposure was the highest in intestinal tissue (Table 4.1: AUC_{24h} : 292000 ng/g x h, median C_{max} 18356 ng/g, C_{trough} 312 ng/g), followed by one log lower exposure in rectal compartment (Table 4.1: AUC_{24h} : 63143 ng/g x h, median C_{max} 6781 ng/g, C_{trough} 130 ng/g). The exposure in vaginal tissue was the lowest among tissues (Table 4.1: AUC_{24h} : 6080 ng/g x h, median C_{max} 389 ng/g, C_{trough} 61.3 ng/g) and resembled exposure in blood plasma (Table 4.1: AUC_{48h} : 4118 ng/g x h, median C_{max} 249 ng/g, C_{trough} 36 ng/g). The highest exposure in the intestines and similar exposure in vaginal tissue and plasma was confirmed by AUC_{24h} tissue:plasma ratios. In the intestinal tissue the AUC_{24h} tissue:plasma ratio was 85, about 4.5 times higher than in rectum (AUC_{24h} t:p ratio 18.4) (Fig 4.2). The AUC_{24h} tissue:plasma ratio in vaginal tissue was 1.7, confirming similar exposure to plasma (Fig 4.2). Similarly to hu mice, the half life in plasma (10.4h) was 3.5 times longer than in intestinal and rectal compartment (2.8h in both tissues), while vaginal half-life was longer than in hu mice (6.2h vs. 3.3h). As in hu mice, RAL T_{max} in plasma was at 2h, while T_{max} in all tissues shifted from 2h in hu mice to 8h in non-hu mice (Table 4.1).

Comparison of RAL kinetics in humanized and non-humanized mice

Overall tissue exposure patterns were similar in hu and non-hu mice. There was pronounced RAL accumulation in rectal and intestinal compartments, while vaginal tissue

showed exposure higher than in plasma but lower than in other tissues. RAL tissue exposure, indicated by AUC_{24h} , was within the 10^4 log range in hu and non-hu mice in rectal tissue and within 10^5 log range in the intestines (colon). In vaginal tissue, one log higher RAL exposure (10^4 log range) was seen in hu mice compared to non-hu mice (10^3 log range). In plasma, similar AUC_{24h} , T_{max} and half-life were observed in hu and non-hu mice.

In hu mice T_{max} was reached at an earlier time point (2h) in all tissues, whereas in non-hu mice T_{max} was observed later, at 8h in all tissue compartments. The difference was also observed in vaginal tissue half-life, which was almost twice as long in non-hu mice (6.2h) compared to hu mice (3.3h). In other tissues elimination kinetics and RAL half-life were similar between hu and non-hu mice.

To compare RAL PK data from humanized and non-humanized mice the Mann-Whitney two tailed test analysis was performed based on the composite median profiles. The only difference which was statistically significant ($p < 0.05$) between hu and non-hu composite median profiles was in the intestinal tissue at 8h.

Table 4.1. PK parameters for Raltegravir in humanized and non-humanized mice.

RAL (hu-mice)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}[*]	283 (122.7 – 321)	3440 (731.5 – 9349)	1213 (677.5 – 2981)	11692 (878 – 32332)
C_{trough}^Δ	3.6	3.2	5.5	16.6
T_{max}	2h	2h	2h	2h
AUC[‡]	5510	28172	39742	135610
t_½[†]	9.5	3.3	3.5	2.8
AUC_{24h} T:P Ratio^{**}	/	6	8.5	29
RAL (non-hu)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}[*]	249 (105 – 400)	389 (248.6 – 617.5)	6780.9 (592.7 – 8988.5)	18356 (13811 – 44976.6)
C_{trough}^Δ	36	61.3	130.1	312
T_{max}	2h	8h	8h	8h
AUC[‡]	4118	6080	63143	292009
t_½[†]	10.4	6.2	2.8	2.8
AUC_{24h} T:P Ratio^{**}	/	1.7	18.4	85

^{*} Median (inter-quartile range, IQR) - ng/ml or ng/g; ^Δ Geometric mean of all values at 24h post-last dose – ng/ml or ng/g; [‡] Area under the curve - AUC_{48h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g); [†] Half-life – h; ^{**} AUC_{24h} tissue to plasma ratio

Table 4.2. RAL concentration geometric means (Coefficient of variation, CV%) in humanized and non-humanized mice.

RAL (hu-mice)	Plasma		Vaginal tissue	
	Mean*	CV% [#]	Mean	CV%
2h	210.0	45.0	2531	95.9
8h	256.8	84.0	939.5	85.4
24h ^{**}	3.6	158.8	3.2	104.1
48h	3.9	154.4	/	/

RAL (hu-mice)	Rectal tissue		Intestinal tissue	
	Mean	CV%	Mean	CV%
2h	1342	70.3	1342	70.3
8h	1290	142.0	1290	142.0
24h ^{**}	5.5	158.3	5.5	158.3
48h	/	/	/	/

RAL (non-hu)	Plasma		Vaginal tissue	
	Mean*	CV% [#]	Mean	CV%
2h	218.7	58.7	281.1	71.3
8h	213.3	9.8	391	44.5
24h ^{**}	36	55.7	61.3	12.5
48h	14.5	42.5	/	/

RAL (non-hu)	Rectal tissue		Intestinal tissue	
	Mean	CV%	Mean	CV%
2h	540.5	64.5	540.5	64.5
8h	3306	79.8	3306	79.8
24h ^{**}	130.1	64.7	130.1	64.7
48h	/	/	/	/

* Geometric mean – ng/ml (plasma), ng/g (tissues)

[#] Coefficient of variation - %

^{**} C_{trough}

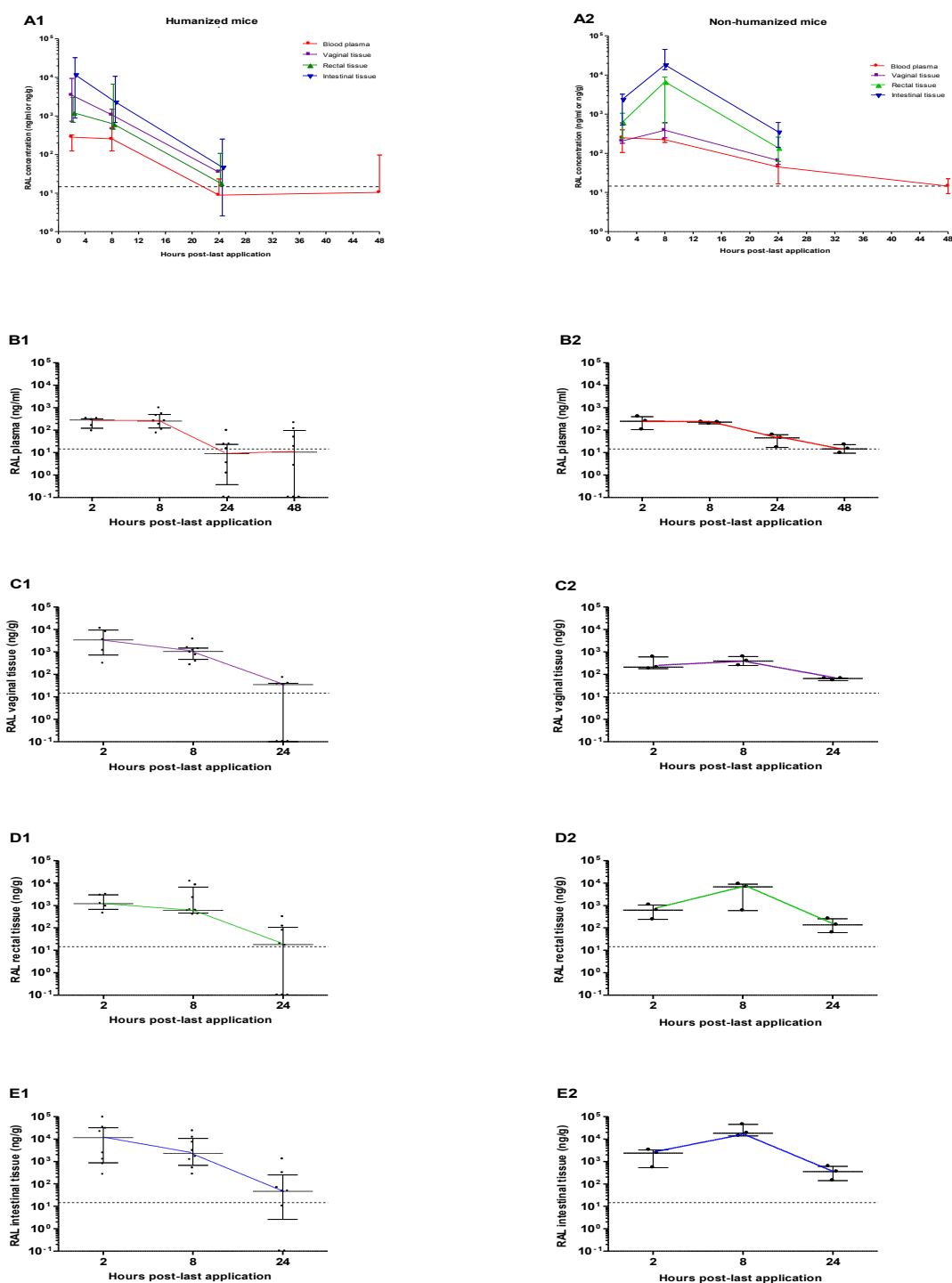


Figure 4.1. PK analysis of orally administered RAL in humanized (hu) and non-humanized (non-hu) mice. Mice were administered RAL by oral gavage (human equivalent dose 164 mg/kg) for 5 days. Post-last dose, plasma and tissue samples were collected at different time points and drug concentrations were determined (ng/ml or ng/g). **A1 (hu mice), A2 (non-hu mice).** Composite medians and interquartile

ranges (IQR) for plasma, vaginal, rectal and intestinal tissue (colon). **B1-E1 (hu mice), B2-E2 (non-hu mice)**. Individual data points (n=5-8 hu mice, n=3 non-hu mice) for plasma (B), vaginal (C), rectal (D) and intestinal tissue (colon) (E) with composite medians (IQR) shown. Dotted line represents RAL *in vitro* 95% inhibitory concentration (IC₉₅) of 33nM (14.6 ng/ml).

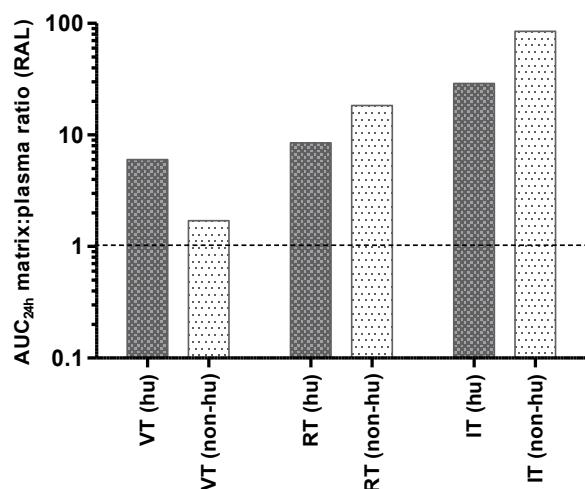


Figure 4.2. Tissue to blood plasma RAL AUC_{24h} ratios in humanized and non-humanized mice. Tissue to plasma ratios were calculated for AUC_{24h}. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. Values above the line of unity indicate higher drug exposure at mucosal sites compared to plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

4.5 Discussion

As a potent and fast acting ARV, RAL is a promising candidate for prophylactic applications; However, PK-PD data are lacking for compartments targeted during viral transmission, such as vaginal and rectal tissue. Humanized mouse models could be useful for analysis of ARV PK-PD to ultimately inform clinical trial design. Here we utilized humanized mice (RAG-hu) to observe and analyze RAL systemic kinetics (blood plasma) and mucosal tissue kinetics (vaginal, rectal and intestinal tissue) following oral application of RAL in human therapeutic equivalent doses and during steady state kinetics. The drug was detected in

majority of tissue and plasma samples (85.1%) and adequate data were obtained to analyze RAL PK under steady state conditions.

Comparison of RAL composite median profiles between humanized and non-humanized mice showed that the only significant difference was observed at 8h in the intestinal tissue ($p < 0.05$). Maximal RAL concentration was reached at a later time point in all tissues of non-hu mice, indicating potential slower drug uptake compared to hu mice. Half-life was similar in all matrices analyzed except in the vaginal tissue, where the elimination rate was slower in non-hu versus hu mice. This could indicate an absence of specific drug metabolism enzymes or specific transporters in the nonhumanized versus humanized vaginal tissue. Plasma kinetics in hu and non-hu mice showed similar AUC_{24h} , T_{max} and half-life, indicating similar systemic drug exposure. The observation of the similar plasma kinetics could indicate that the differences seen between hu and non-hu RAL PK were due to tissue-specific and not systemic factors. Overall, tissue exposure patterns were the same in hu and non-hu mice with rectum and intestines (colon) being the sites of pronounced drug accumulation, and vaginal tissue showing the lowest exposure among tissue compartments. The potential implication is that the PK studies could be done in the non-humanized mice. However, in the context of PK-PD modeling of ARVs for therapeutic or PrEP applications, the drug kinetics observed needs to be correlated to the level of protection offered against HIV-1. This is the crucial element of PK-PD studies and can only be done in the humanized mice, susceptible to HIV-1 infection through systemic and mucosal routes. The data on RAL PK suggests that in future PK-PD modeling, initial PK studies could be done in the non-humanized mice, followed by the HIV-1 challenge (PD) studies with sparse and targeted PK sampling in humanized mice.

As in previous human studies, in hu mice RAL plasma half-life of 9.5 h and T_{max} of 2h were observed [310, 318-321]. In addition, high degree of variability in plasma concentrations and AUC seen in human trials was also observed in hu mice, with CV% of 84% at 8h and 150% at 24h and 48h [310, 318, 319, 321-325]. C_{max} and overall RAL exposure (AUC) in human

clinical studies showed higher values than the ones seen in hu-mice [310, 314, 318-321, 323, 324, 326-330] and the median $C_{\max}$ in hu-mice corresponded better to C_{trough} values seen in clinical trials [318, 324-326]. The biphasic concentration curve described in human studies was not observed in hu-mice, although plasma RAL levels were not analyzed between 8h and 24h, when a secondary peak could have occurred [320, 329]. The overall lower exposure in plasma of RAL-hu mice could be explained by lack of sampling between 8h and 24h, and inability to construct the exposure curve with the secondary peak for AUC calculations. In addition, the differences in enterohepatic recirculation in mice, compared to humans are not known. It has been shown that RAL-glucuronide is hydrolyzed to RAL in the human gastro-intestinal tract, allowing for reabsorption and secondary RAL peak in plasma [313, 331]. The longer half life seen in plasma (9.5h) compared to tissues (around 3h for all) indicates that some level of recirculation occurs in hu mice [320, 329]. The gut microbiome composition, gastrointestinal pH and intraluminal magnesium ion levels, have all been indicated as potential factors for differential reabsorption in humans [332, 333]. The differences in these factors between human and mouse gut need to be further explored, as a better understanding of RAL recirculation mechanisms would allow for more precise modeling of RAL PK and extrapolation of data to human drug metabolism and PK.

In hu mice RAL concentrations were above the established *in vitro* IC_{95} of 33nM (14.6 ng/ml) in 100% of plasma samples at 2h and 8h, and in 50% of samples at 24h and at 48h. Previous clinical studies have not been able to establish plasma dose-proportional relationship to antiviral (PD) activity for RAL. Successful RAL kinetics modeling shown here in hu-mice can be expanded further to more comprehensive PK-PD studies which would offer additional data on RAL plasma kinetics and the relationship to the protection achieved at the sites of HIV-1 transmission, to inform future PrEP approaches.

The main criteria for a promising PrEP drug candidate are the level of drug penetration in targeted sites and tissue drug retention over time. In our study RAL showed high penetration

in vaginal tissue compared to plasma, with the AUC_{24h} tissue:plasma ratio of 6. Previous human studies showed high RAL exposure in CVF, with median reported CVF:plasma AUC ratio of 4 in the study that included infected and uninfected women and CVF:plasma AUC ratio of 2.3 in DIVA study [334, 335]. High RAL exposure throughout gastrointestinal tissue was observed in a recent study by Patterson et al., with the tissue to plasma ratios ranging from 156 to 659, depending on the anatomical biopsy site [336]. The extensive RAL penetration was also seen in our study, where the overall RAL exposure indicated by AUC_{24h} was the highest in intestinal (colonic) tissue followed by rectal tissue, and then vaginal tissue and plasma. Rectal tissue median concentrations were one log higher compared to plasma throughout the 24h study period while intestinal tissue showed two logs higher exposure compared to plasma. The AUC_{24h} tissue:plasma ratios for RAL were 8.5 for rectal and 29 for intestinal tissue, indicating high tissue drug saturation. Differential expression of influx and efflux drug transporters for Tenofovir and Maraviroc was recently reported in human colorectal tissue compared to cervico-vaginal tissue [337]. The corresponding differences in RAL-specific tissue transporter expression between mucosal compartments could explain overall higher saturation seen in the intestinal and rectal compared to vaginal tissue in hu mice. The hydrolysis of RAL-glucuronide in the gut and subsequent RAL reabsorption could also be an additional factor for higher RAL levels in the intestinal compared to vaginal and rectal tissues, seen here and in previous human studies.

In our hu-mouse study RAL levels were maintained above IC_{95} in 100% of vaginal samples at 2h and 8h, and in 57% of samples at 24h. C_{trough} (geometric mean at 24h) for vaginal compartment was 3.2 ng/g, which is lower than the identified plasma level of 21 ng/ml, associated with virological failure in the QDMRK study [326]. The observed threshold RAL plasma level from this therapeutic study might not be directly extrapolated to vaginal tissue and additional PK-PD modeling is needed to define the protective threshold levels in vaginal mucosa for PrEP. This finding might indicate that higher RAL dose would need to be applied for vaginal

PrEP, compared to the established therapeutic dose. Recent study reported cervical tissue levels similar to plasma at 6h and 12h after the last observed dose [338], which could be another indicator for the need to apply higher RAL dose in PrEP applications, compared to the dose used for HIV-1 treatment. In the same study RAL concentrations were found to be decreased with the increase in the biopsy size (weight), and this could point to the fact that RAL distribution is not homogenous in vaginal tissue layers [338]. In hu mice vaginal tissue was collected en bloc, giving results for complete organ exposure. Further studies are needed to define the precise accumulation site(s) of RAL and other ARV in mucosal tissues. Methods such as matrix-assisted laser desorption/ionization (MALDI) enable the identification of ARV accumulation sites through formation of analyte-matrix complexes and represent a powerful tool for future mucosal tissue PK studies [339].

In both rectal and intestinal compartment 100% of samples at 2h and 8h and 62.5% of samples at 24h showed RAL levels higher than IC_{95} , indicating a similar drug retention profile to that seen in vaginal tissue. C_{trough} in rectal and intestinal tissue were 5.5 ng/g and 16.6 ng/g, respectively, showing lower exposure compared to the plasma level of 21 ng/ml, associated with virological failure in the QDMRK study [326]. As outlined for vaginal tissue, such extrapolation of the data from plasma to tissues to define the threshold values in the context of PrEP and protection against mucosal challenge will require additional PK-PD studies. The proof of concept experiments reported here indicate that hu-mice could be applied as a small animal model for future RAL PK-PD studies in mucosal tissues relevant for PrEP design.

Two important findings need to be emphasized for the RAL PK modeling in hu mice. The variability seen in hu-mice mimicked high PK variability seen in humans and the general trends of blood plasma and mucosal distribution mirrored previous findings in humans. High initial RAL saturation achieved at steady state kinetics was maintained up to 8h in all tissues, indicating the potential protection duration against mucosal transmission. At 24h 43% of vaginal samples and 37.5% of rectal and intestinal samples showed RAL concentrations below IC_{95} . The duration of

protection offered by RAL in mucosal compartments could be defined more precisely by additional sampling done between 8h and 24h in the follow up studies.

The data from our study indicate favorable RAL profile for PrEP applications in the heterosexual population and in men who have sex with men (MSM), although the high PK variability seen in this study as in previous human trials makes PK-PD modeling more complex than for most other ARVs. The data also indicate that the dose for PrEP applications will need to be higher than the current therapeutic dose, as the protection in the period lasting 8h in vaginal and rectal tissue would imply multiple daily dosing and could present a significant problem with regard to low adherence rates. Overall, tissue saturation and retention data for RAL in sites of transmission indicates that the PrEP drug concentration would need to be higher than the current HIV-1 treatment dose, to allow for once a day PrEP dosing.

While the data for profiling RAL PK-PD in mucosal sites targeted by the virus are still needed, Phase III study by Merck found that 800 mg once daily RAL is less effective than approved twice a day 400 mg RAL dose, showing that RAL PK does influence the virological response (PD) [326]. This clinical trial emphasized the need for additional RAL PK-PD studies, both for therapeutic and prophylactic applications. In addition, low genetic barrier to resistance, already established for RAL, emphasizes further the need to precisely determine the dose and the time frame for RAL application as a PrEP agent. Recent findings of RAL suboptimal performance compared to protease-inhibitor based therapies in several clinical trials and the finding that RAL plasma concentrations influence the selection of resistant viral mutations over time both point to the importance of further analysis of RAL PK-PD, in the context of therapeutic as well as preventative approaches [340-344].

A comprehensive analysis of RAL PK-PD for PrEP will also include combinatorial regimens, in which antagonistic, agonistic and additive effects of ARVs will be observed, with the ultimate goal of achieving complete protection in the mucosal sites of HIV-1 transmission. In addition to PrEP, the PK-PD analysis in hu mice can be expanded to help define post-exposure

prophylaxis (PEP) approaches. A recent clinical PEP study, which included a cocktail of RAL, TFV and FTC, showed promising results against sexual transmission of HIV-1, indicating further potential RAL applications besides therapy and PrEP [345]. Better characterization of RAL PK-PD in genital tract may also have an impact on preventing mother to child transmission. Finally, defining RAL PK-PD could help in controlling more efficiently replication in viral sanctuary sites in the genital tract and could help reduce the emergence of drug-resistant HIV-1 strains in this compartment. This proof of concept study on RAL PK indicates that hu mice can be applied in future PK-PD modeling. The data gathered here and in future hu mouse experiments will inform study design aimed at defining optimal PK-PD parameters for application of RAL as a PrEP agent.

CHAPTER 5

Combinatorial Pre-Exposure Prophylaxis: Mucosal Tissue Drug Distribution of HIV-1 RT Inhibitor Tenofovir, Integrase Inhibitor Raltegravir and Entry Inhibitor Maraviroc Following Combinatorial Oral Application in a Humanized Mouse Model

5.1 Introduction

With the multidrug approach already in place for the treatment of HIV-1, in the form of combination antiretroviral therapy (cART), similar strategy is feasible for the efficient prevention of the disease. Currently approved prevention drug Truvada combines two RT inhibitors, Tenofovir (TFV) and Emtricitabine (FTC) and has shown a high level of protection in the clinical trials [252, 253]. However, to achieve full protection against HIV-1, an additional agent needs to be included, and Raltegravir (RAL) and Maraviroc (MVC) are among the most suitable drug candidates. Both target different points in the HIV-1 life cycle compared to Truvada, with RAL being the HIV-1 integrase inhibitor and MVC being the CCR5 antagonist and HIV-1 entry inhibitor. Specific early mode of action of MVC and promising preclinical and pharmacokinetic clinical data, especially the favorable drug distribution into the mucosal tissues, make this drug a potential pre-exposure prophylactic (PrEP) agent [298, 303, 304]. As for RAL, its potent antiviral effect due to irreversible inhibition of the HIV-1 integrase, as well as favorable mucosal tissue exposure when applied as a single agent, point to it being a potential PrEP candidate drug [314, 334-336, 338, 346]. However, there still remain multiple questions to be answered before the implementation of oral PrEP strategies. Firstly, the currently applied therapeutic doses might not be adequate for preventative approach [237]. In order to determine the exposure at sites of transmission, such as genital tract and rectal mucosa, detailed pharmacokinetic (PK) analysis is needed following oral application of combinations of ARVs. Secondly, the dose titration for PrEP needs to be done in the context of potential drug to drug

interactions and subsequent antagonistic, agonistic or synergistic effects during combinatorial application. Thirdly, the application of oral PrEP will imply less or more frequent dosing, depending on the formulation, and potential toxicity can be minimized by adequately adjusting the ARV doses by PK studies. Lastly, HIV-1 is a constantly evolving virus which has already generated specific resistant mutants against currently available ARVs. Therefore, PrEP approach can only be justifiable if the advantages of high level of protection outweigh the danger of developing new multidrug-resistant mutants. In that respect, future PK studies which would be combined with protection, pharmacodynamic (PD) studies in preclinical settings would offer initial analysis of PK-PD profiles for new PrEP candidates for further testing in non-human primates (NHP) and clinical trials.

Humanized mouse model has been utilized for testing multiple anti-HIV-1 therapeutics and other approaches thus far [162, 167, 169, 178, 181] and recently it has been applied for single drug PK studies [198]. The aim of the research presented here was to study the pharmacokinetics of currently approved PrEP agent TFV in combination with potential new PrEP candidates RAL and MVC, in a dual drug approach utilizing a humanized mouse model and following oral application of human equivalent therapeutic doses.

5.2 Materials and methods

Antiretroviral drug preparation, administration and sample collection

The mouse-equivalents of human therapeutic doses were calculated using the interspecies scaling factor for a standard 20g RAG-hu mouse, to arrive at 61.5 mg/kg TFV (1.23 mg dose per mouse), 164 mg/kg RAL (3.28 mg dose per mouse) and 62 mg/kg MVC (1.24 mg dose per mouse) [347]. The appropriate amounts of the drugs were pulverized using mortar and pestle, and dissolved in sterile 1xPBS. Female mice were gavaged with the combinations of TFV+MVC, TFV+RAL or RAL+MVC according to the dosing schedule once per day for 5 days.

Plasma and vaginal, rectal and intestinal tissue samples were collected following the last gavage at 2h, 8h, and 24h for TFV+RAL combination. For TFV+MVC and for RAL+MVC combinations the same sets of samples were collected at 5 separate time points -2h, 4h, 8h, 12h and 24h. All samples were collected during mouse necropsies and snap frozen in liquid nitrogen within five minutes from the time of collection. For negative controls, plasma and tissue samples were collected from untreated mice and were processed as described above. All samples were stored at -80° C prior to HPLC analysis.

HPLC analysis of drug concentrations

Quantification of analytes was similar to previously published methods [255, 298, 335]. Briefly, quantification of TFV concentrations in plasma was performed by protein precipitation and LC-MS/MS analysis with an isotopically-labeled internal standard (^{13}C TFV). TFV was eluted from a Waters Atlantis T3 (100 X 2.1mm², 3µm particle size) analytical column and an API-5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) was used to detect the analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 1-1000 ng/mL using a 1/concentration² weighted linear regression. For measuring concentrations in mucosal tissues, TFV and TFV-DP was extracted from tissue homogenate by protein precipitation with isotopically-labeled internal standards (^{13}C TFV and ^{13}C TFV-DP). TFV was eluted from a Waters Atlantis T3 (100 X 2.1mm, 3µm particle size) analytical column, and TFV-DP was eluted from a Thermo Biobasic AX (50 X 2.1mm, 5µm particle size) analytical column. An API-5000 triple quadrupole mass spectrometer was used to detect all analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 0.3-300 ng/mL of homogenate for each compound using a 1/concentration² weighted linear regression. Concentrations were ultimately converted into ng/mg (TFV) or fmol/mg (TFV-DP) tissue for final reporting.

To measure drug concentrations in plasma, MVC was extracted from samples using solid phase extraction with Varian BondElut C-18, 100 mg, 1CC cartridges. Plasma samples were quantified against the internal standard, alprazolam, on an Agilent 1200 series HPLC system using a Zorbax Eclipse XDB (50 x 4.6mm, 1.8 μ m particle size) analytical column. An Agilent 1100 MSD was used to detect the analyte and internal standard. The dynamic range of this assay was 1-1000 ng/mL. For quantification from mucosal tissues, MVC was extracted from tissue homogenates using protein precipitation with the internal standard alprazolam. This resulting extract was analyzed on an Agilent 1200 series HPLC system using a Zorbax Eclipse XDB (50 x 4.6mm, 1.8 μ m particle size) analytical column. An Agilent 1100 MSD was used to detect the analyte and internal standard. The dynamic range of this assay was 0.007-7 ng/mg.

Quantification of RAL plasma concentrations was performed by protein precipitation and LC-MS/MS analysis. Plasma samples were mixed with methanol containing the isotopically-labeled internal standards (2 H RAL). Following vortex and centrifugation, the supernatant was removed and evaporated to dryness. The extracts were reconstituted with 1mM ammonium phosphate prior to LC-MS/MS analysis. RAL was eluted from a Phenomenex Synergi Polar-RP (50 X 4.6mm, 4 μ m particle size) analytical column. An API- 5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) was used to detect the analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 1-1000 ng/mL for each compound using a $1/\text{concentration}^2$ weighted linear regression. The quantitation of RAL in tissues started with the homogenization of each tissue in 70:30 acetonitrile: 1mM ammonium phosphate. A portion of the resulting homogenate was extracted by protein precipitation with acetonitrile containing isotopically-labeled internal standard (2 H RAL). Following vortex and centrifugation, the supernatant was removed and evaporated to dryness. The extracts were reconstituted with 1mM ammonium phosphate prior to LC-MS/MS analysis. RAL was eluted from a Phenomenex Synergi Polar-RP (50 X 4.6mm, 4 μ m particle size) analytical column. An API- 5000 triple quadrupole mass spectrometer was used to

detect the analyte. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 0.3-300ng/mL of homogenate using a $1/\text{concentration}^2$ weighted linear regression.

Statistical analysis

GraphPad Prism version 5 (GraphPad Software, USA) was used for data analysis and statistical analysis. Measures of central tendency were expressed as median and inter-quartile range (IQR 25th, 75th percentile). Median C_{max} values and $\text{AUC}_{24\text{h}}$ values generated in GraphPad Prism were used to calculate comparative combinatorial/single treatment ratios. PK data for single drug application and co-administration were compared using Mann-Whitney two-tailed test. P values <0.05 were considered as significant.

5.3 Results

5.3.1. Pharmacokinetics of combinatorial Raltegravir and Tenofovir oral application

RAL kinetics in plasma and mucosal tissues in co-administration with TFV

In plasma, the highest concentration of RAL was detected at 2h, with median C_{max} of 290 ng/ml. The $\text{AUC}_{24\text{h}}$ was 1831 ng*h/ml and C_{trough} was 6.8 ng/ml. Plasma RAL $t_{1/2}$ was 4.4h. In vaginal tissue median C_{max} was 1076.2 ng/g at 2h, with overall $\text{AUC}_{24\text{h}}$ of 11890 ng*h/g, C_{trough} of 49.1 ng/g, and $t_{1/2}$ of 4.4 h. In rectal tissue, the highest RAL concentration (median C_{max}) of 4170 ng/g was detected at 8h, while the $t_{1/2}$ was 2.2h. Rectal tissue $\text{AUC}_{24\text{h}}$ was 61161 ng*h/g and C_{trough} was 5.22 ng/g. In the intestines, C_{max} was at 2h (median 17206 ng/g), while $t_{1/2}$ was 2.3h. Intestinal $\text{AUC}_{24\text{h}}$ was 254085 ng*h/g and C_{trough} was 188.3 ng/g (Fig 5.1, Table 5.1).

TFV and TFV-DP kinetics in plasma and mucosal tissues in co-administration with RAL

TFV kinetics in co-administration with RAL was characterized by median C_{max} of 500 ng/ml at 2h, AUC_{24h} of 4175 ng*h/ml and $t_{1/2}$ of 9.3h. In vaginal tissue median C_{max} was 1218 ng/g at 2h, with AUC_{24h} of 37450 ng*h/g and $t_{1/2}$ of 8h. Rectal tissue median C_{max} was at 8h (30244 ng/g) and AUC_{24h} was 654458 ng*h/g. It was not possible to determine the $t_{1/2}$ in rectal tissue since there was no elimination phase within 24h period analyzed. In the intestinal tissue, C_{max} was detected at 8h (median 49973 ng/g), while AUC_{24h} was 847754 ng*h/g, and $t_{1/2}$ was 17.1h. In rectal tissue, TFV-DP showed highest concentration (median C_{max}) of 78761 ng/g at 2h and AUC_{24h} was 1.35×10^6 ng*h/g. In the intestines, median C_{max} of TFV-DP was 67068 ng/g at 24h and AUC_{24h} was 1.38×10^6 ng*h/g. The half-life of TFV-DP in rectal and intestinal tissue could not be determined since no elimination phase was detected within the observed 24h period (Fig 5.2, Fig 5.3, Table 5.2).

Single versus co-administration kinetics in plasma and mucosal tissues

The AUC_{24h} tissue to plasma ratios were used to extrapolate relative drug exposure in tissues compared to blood plasma (Fig 5.4, Fig 5.5). Co-administration was shown to increase tissue:plasma AUC_{24h} ratios for both antiretrovirals. Comparing the single to combinatorial application for RAL, tissue:plasma AUC_{24h} ratio was increased from 6.0 to 6.5 in vaginal, from 8.5 to 33.4 in rectal and from 29 to 138.7 in the intestinal compartment. For TFV, a pronounced 6-fold increase in AUC_{24h} ratio, from 1.5 in single application to 9 in RAL co-administration, was seen in vaginal tissue. In rectal and intestinal tissue the AUC_{24h} tissue:plasma TFV ratios were also increased - from 99 and 141.7 in single application to 156.7 and 203 in co-administration with RAL, respectively.

In order to further compare the kinetics in single oral application and co-administration, the median C_{max} combo/single ratios and AUC_{24h} combo/single ratios were calculated for RAL,

TFV and TFV-DP (Table 5.3). In plasma, RAL median combo/single C_{max} ratio was 1.02 and AUC_{24h} ratio was 0.39. In vaginal tissue, RAL C_{max} ratio was 0.31 and AUC_{24h} ratio was 0.42. In rectal and intestinal tissue combo/single C_{max} ratios were 3.44 and 1.47, respectively, and AUC_{24h} ratios were 1.54 and 1.87, respectively. For TFV, the observed combo/single ratios in plasma were 0.25 for C_{max} and 0.37 for AUC_{24h} . In vaginal tissue, TFV C_{max} ratio was 1.67 and AUC_{24h} ratio was 2.51. In rectal and intestinal tissue, TFV C_{max} ratios were 0.53 and 0.67, respectively, while AUC_{24h} ratios were 0.65 and 0.59, respectively. The C_{max} combo/single ratios for TFV-DP were 130.4 and 7.6 for rectal and intestinal tissue. The AUC_{24h} for TFV-DP were 19.23 in rectal and 8 in the intestinal tissue.

Mann-Whitney two tailed t test was used to compare C_{max} medians in single and combinatorial application. For RAL, the only significant difference ($p < 0.05$) was for C_{max} in rectal tissue. For TFV, the only significant alteration was in plasma C_{max} ($p < 0.05$). For TFV-DP, in both rectal and intestinal compartments median C_{max} was significantly altered in combinatorial versus single drug application ($p < 0.05$).

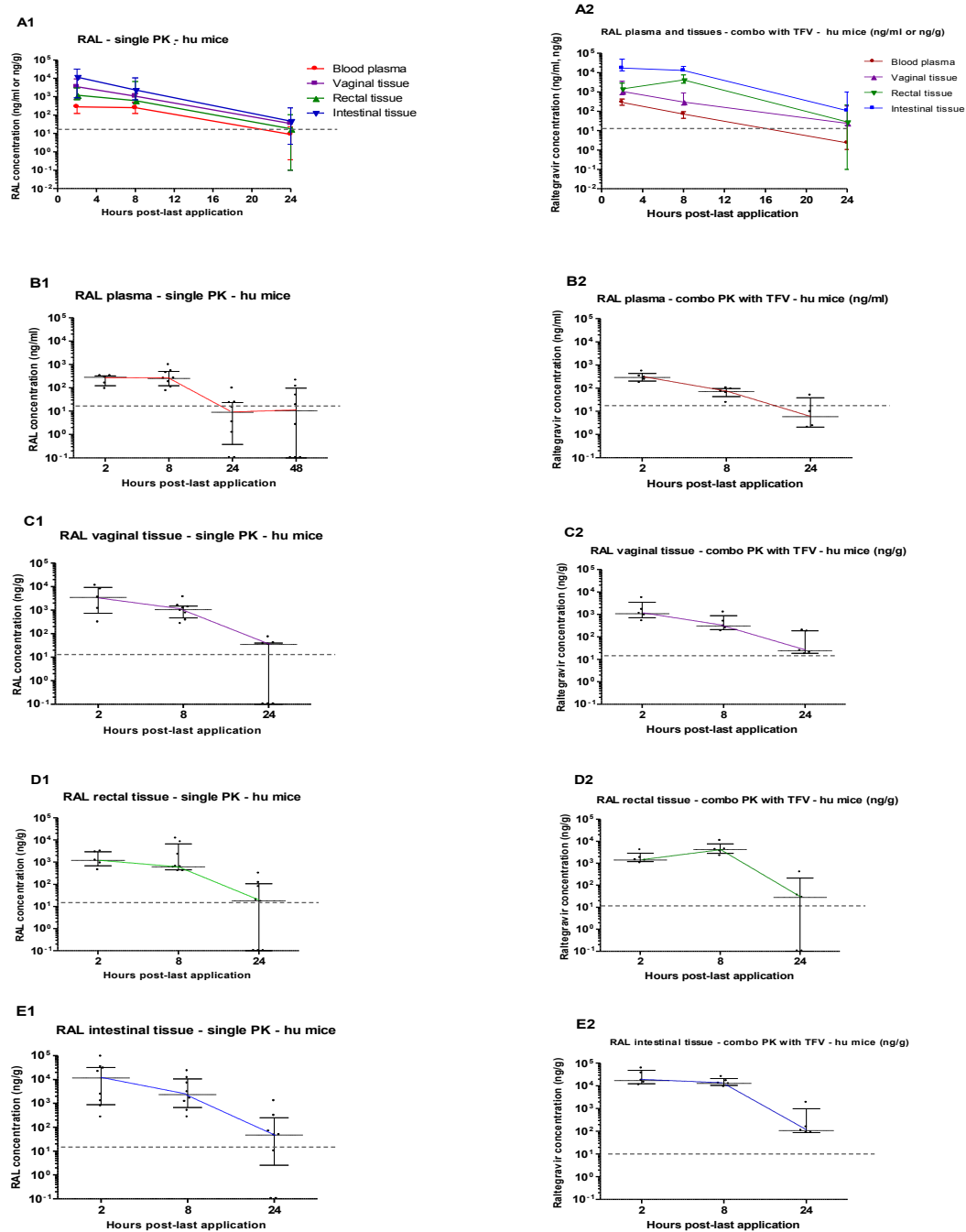


Figure 5.1. Comparison of Raltegravir pharmacokinetics following single (A1-E1) and combinatorial (A2-E2) oral application with Tenofovir. Dotted line represents Raltegravir *in vitro* 95% inhibitory concentration ($IC_{95} = 14.6$ ng/ml).

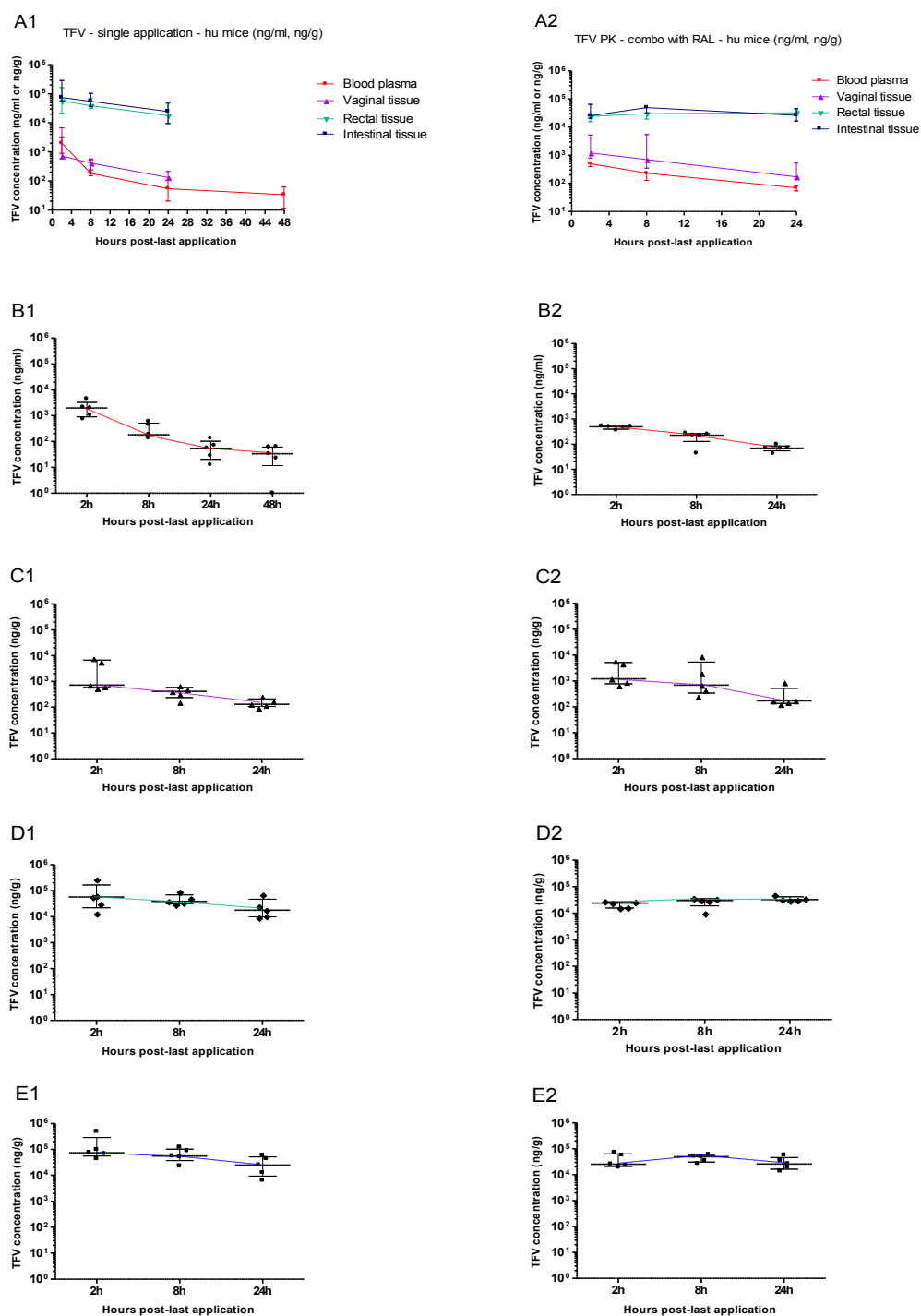


Figure 5.2. Comparison of Tenofovir pharmacokinetics following single (A1-E1) and combinatorial (A2-E2) oral application with Raltegravir.

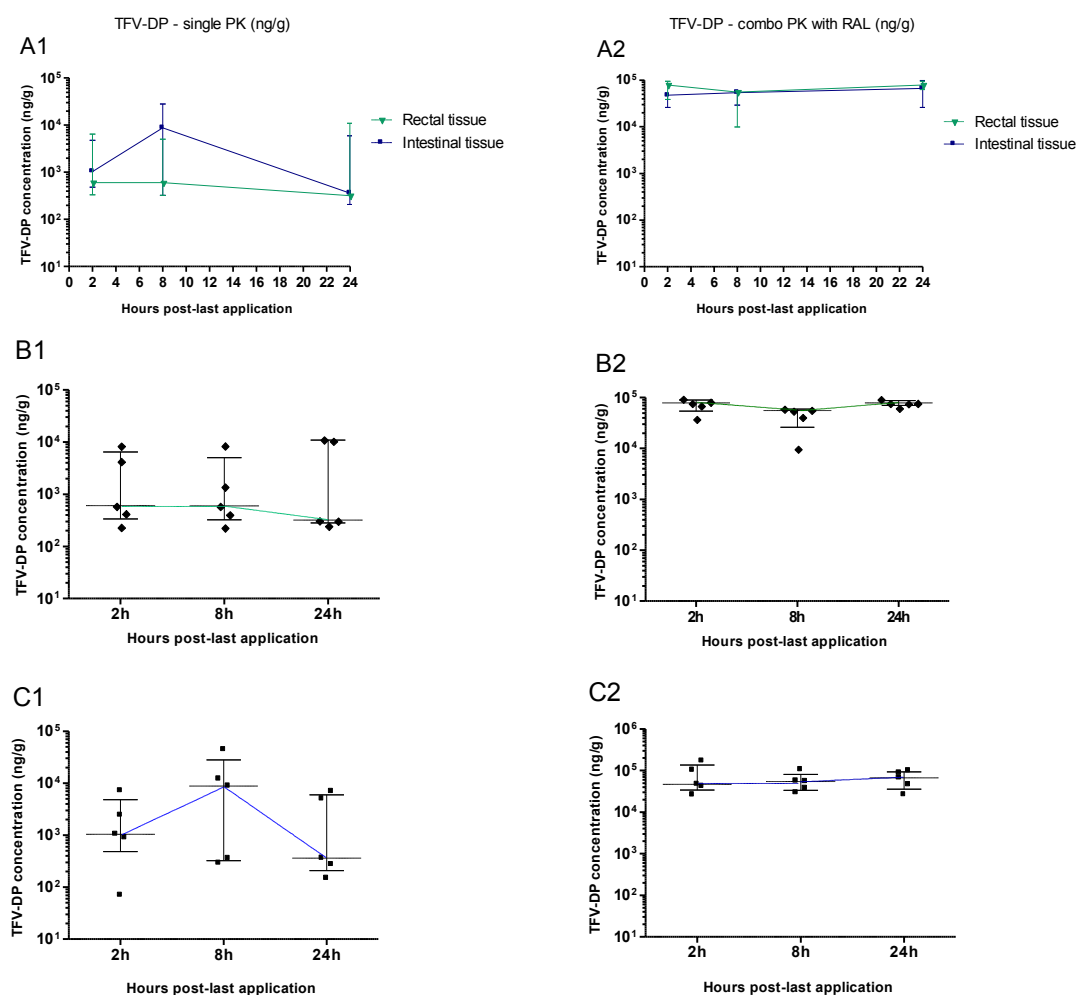


Figure 5.3. Comparison of Tenofovir-diphosphate pharmacokinetics following single (A1-C1) and combinatorial (A2-C2) oral application of Tenofovir with Raltegravir.

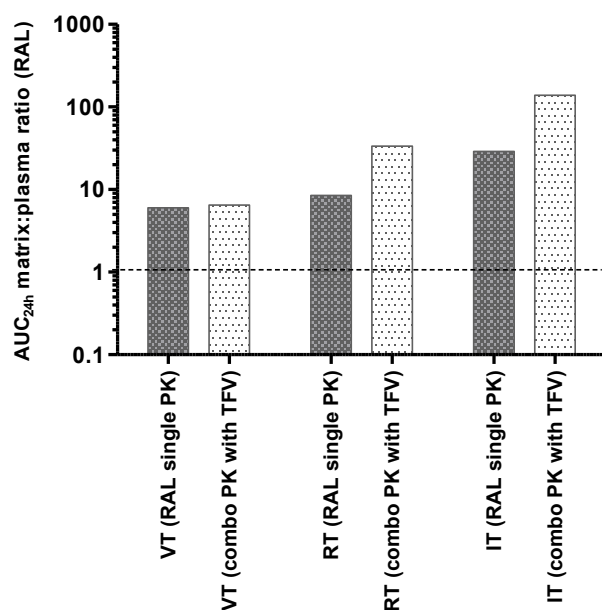


Figure 5.4. AUC_{24h} tissue:plasma ratios for Raltegravir following single and combinatorial oral application with Tenofovir. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

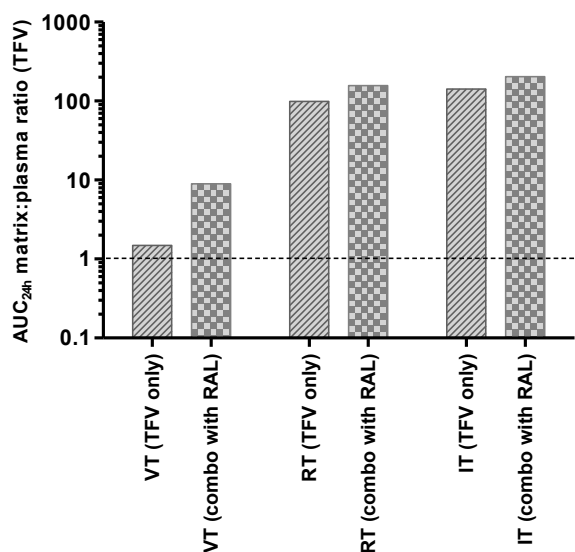


Figure 5.5. AUC_{24h} tissue:plasma ratios for Tenofovir following single and combinatorial oral application with Raltegravir. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

Table 5.1. PK parameters for Raltegravir following single and combinatorial oral application with Tenofovir.

RAL (single PK)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median	283	3440	1213	11692
(IQR)[†]	(122.7 – 321)	(731.5 – 9349)	(677.5 – 2981)	(878 – 32332)
Mean	210	2531	1342	5690
(CV%[∞])	(45%)	(96%)	(70.3%)	(138.7%)
C_{trough}^Δ	3.6	3.2	5.5	16.6
T_{max}	2h	2h	2h	2h
AUC[‡]	4683	28172	39742	135610
t_{1/2}[†]	9	3.1	7.4	3.1
RAL (combo PK w/ TFV)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median	290	1076.2	4170.1	17206.3
(IQR)[†]	(203 – 430.5)	(722.8 – 3508)	(2827-7520)	(12401-48506)
Mean	289.4	1350	4293	22510
(CV%[∞])	(44.2%)	(103.9%)	(66.7%)	(74.4%)
C_{trough}^Δ	6.8	49.1	5.2	188.3
T_{max}	2h	2h	8h	2h
AUC[‡]	1831	11890	61161	254085
t_{1/2}[†]	4.4	4.4	2.2	2.3

Median (inter-quartile range, IQR) - ng/ml or ng/g; [∞] Geometric mean - ng/ml or ng/g (Coefficient of variation - %); [‡] Area under the curve – AUC_{24h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g); [†] Half-life – h; ^Δ Geometric mean of all values at 24h post-last dose – ng/ml or ng/g;

Table 5.2. PK parameters for Tenofovir and Tenofovir-diphosphate following single and combinatorial oral application of Tenofovir with Raltegravir.

TFV (single PK)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median (IQR)	1990 (898 – 3310)	729 (581 – 6739)	56732 (21746 – 164561)	74677 (55008 – 288570)
T_{max}	2h	2h	2h	2h
AUC[†]	11251	14946	1 x 10 ⁶	1.43 x 10 ⁶
t_{1/2}[‡]	17	9	13.5	13.9
TFV-DP (single PK)				
C_{max}				
Median (IQR)	NA	BLQ	604 (336 – 6466)	8841 (322 – 28175)
T_{max}	NA	/	2h	8h
AUC[†]	NA	/	70194	172167
t_{1/2}[‡]	NA	/	22.2	3.5

TFV (combo PK w/ RAL)				
C_{max}				
Median (IQR)	500 (399.5-518)	1218 (784.5-5271)	30244 (19210-35350)	49973 (30672-55364)
T_{max}	2h	2h	8h	8h
AUC[†]	4175	37450	654458	847754
t_{1/2}[‡]	9.3	8	/ [§]	17.1
TFV-DP (combo PK w/ RAL)				
C_{max}				
Median (IQR)	NA	BLQ	78761 (54513-89216)	67068 (36016-93648)
T_{max}	NA	/	2h	24h
AUC[†]	NA	/	1.35 x 10 ⁶	1.38 x 10 ⁶
t_{1/2}[‡]	NA	/	/ [§]	/ [§]

* Median (inter-quartile range, IQR) - ng/ml or ng/g; [†] Area under the curve – AUC_{24h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g); [‡] Half-life – h; [§] no elimination phase; NA- not applicable; BLQ – below limit of quantification

Table 5.3. AUC_{24h} (exposure) and median C_{max} ratios for TFV and RAL oral co-administration.

AUC _{24h} ratio	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
RAL	0.39	0.42	1.54	1.87
TFV	0.37	2.51	0.65	0.59
TFV DP	NA	BLQ	19.23	8.02
C _{max} ratio	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
RAL	1.02	0.31	3.44	1.47
TFV	0.25	1.67	0.53	0.67
TFV DP	NA	BLQ	130.40	7.59

* Median C_{max}; NA- not applicable; BLQ – below the limit of quantification

5.3.2. Pharmacokinetics of combinatorial Maraviroc and Tenofovir oral application

MVC kinetics in plasma and mucosal tissues in co-administration with TFV

In plasma, MVC kinetics was characterized by median C_{max} of 268 ng/ml at 2h, AUC_{24h} of 727.2 ng*h/ml and t_{1/2} of 3.8h. Median C_{max} was 552.8 ng/g in vaginal tissue at 2h, while AUC_{24h} was 3572 ng*h/g and t_{1/2} was 5.1h. In rectal tissue median C_{max} was 12187 ng/g at 8h, while AUC_{24h} was 101053 ng*h/g. Rectal t_{1/2} was 2.7h. Intestinal tissue C_{max} was at 4h, with median of 48270 ng/g. Intestinal AUC_{24h} was 269020 ng*h/g and t_{1/2} was 1.7h (Fig 5.6, Table 5.4).

TFV and TFV-DP kinetics in plasma and mucosal tissues in co-administration with MVC

Median TFV C_{max} in plasma was 495 ng/ml at 2h, while AUC_{24h} was 3791 ng*h/ml and t_{1/2} was 8.8h. In vaginal tissue, median C_{max} was 904.7 ng/ml at 4h. The AUC_{24h} in vaginal tissue was 13768 ng*h/g and t_{1/2} was 35.2h. Rectal tissue C_{max} was 58425 ng/g at 12h, with AUC_{24h} of 935316 ng*h/g and t_{1/2} of 13h. Intestinal TFV kinetics was characterized by median C_{max} of 66171 ng/g at 12h, AUC_{24h} of 1.2 x 10⁶ ng*h/g and t_{1/2} of 11h. For TFV-DP, median C_{max} in rectal

tissue was 141756 ng/g at 12h, AUC_{24h} was 2.89×10^6 g*h/g and $t_{1/2}$ was 62h. In the intestines, TFV-DP reached C_{max} at 24h (median 282034 ng/g), while the AUC_{24h} was 3.61×10^6 ng*h/g. In the intestinal tissue $t_{1/2}$ was not determined as no elimination phase was seen up to 24h (Fig 5.7, Fig 5.8, Table 5.5).

Single versus co-administration kinetics in plasma and mucosal tissues

The AUC_{24h} tissue to plasma ratios were compared for combinatorial and single application of MVC and TFV, in order to delineate the effects on the mucosal tissue exposure (Fig 5.9, Fig 5.10). For MVC, a slight decrease from 5 in single to 4.9 in MVC+TFV application, was seen in AUC_{24h} tissue:plasma ratio for vaginal tissue. In rectal and intestinal tissue, MVC AUC_{24h} tissue:plasma ratios were increased from 67 and 287 in MVC single application to 139 and 370 in combinatorial treatment, respectively. For TFV, an increase in tissue to plasma exposure was seen for all compartments. The AUC_{24h} tissue:plasma ratios were 1.5, 99 and 141.7 in vaginal, rectal and intestinal tissue for TFV only, while they were 3.6, 246 and 316 in the same compartments when TFV was co-applied with MVC.

In order to compare the kinetics in single oral application and co-administration, the median combo/single C_{max} ratios and AUC_{24h} ratios were calculated for MVC, TFV and TFV-DP (Table 5.6). In plasma, MVC median C_{max} ratio was 2.63 and AUC_{24h} ratio was 0.96. In vaginal tissue, MVC C_{max} ratio was 1.2 and AUC_{24h} ratio was 1.45. In rectal and intestinal tissue MVC C_{max} ratios were 1.61 and 1.44, respectively, and AUC_{24h} ratios were 3 and 1.92, respectively. For TFV, the observed ratios in plasma were 0.25 for C_{max} and 0.34 for AUC_{24h} . In vaginal tissue, TFV combo/single C_{max} ratio was 1.24 and AUC_{24h} ratio was 0.92. In rectal and intestinal tissue, TFV C_{max} ratios were 1 and 0.89, respectively, while AUC_{24h} ratios were 0.94 and 0.84, respectively. The C_{max} combo/single ratios for TFV-DP were 234.7 and 31.9 for rectal and intestinal tissue. The AUC_{24h} for TFV-DP were 41.2 in rectal and 21 in the intestinal tissue.

In the two tailed t test the medians for $C_{\max}$ were compared between single and combinatorial application for both drugs, and for TFV-DP. Differences in plasma $C_{\max}$ were found to be significant ($p < 0.05$) for MVC and TFV, while no significant $C_{\max}$ differences were found in tissues. Rectal and intestinal tissue $C_{\max}$ showed significant alterations ($p < 0.05$) for TFV-DP when TFV single administration was compared to combinatorial dosing with MVC.

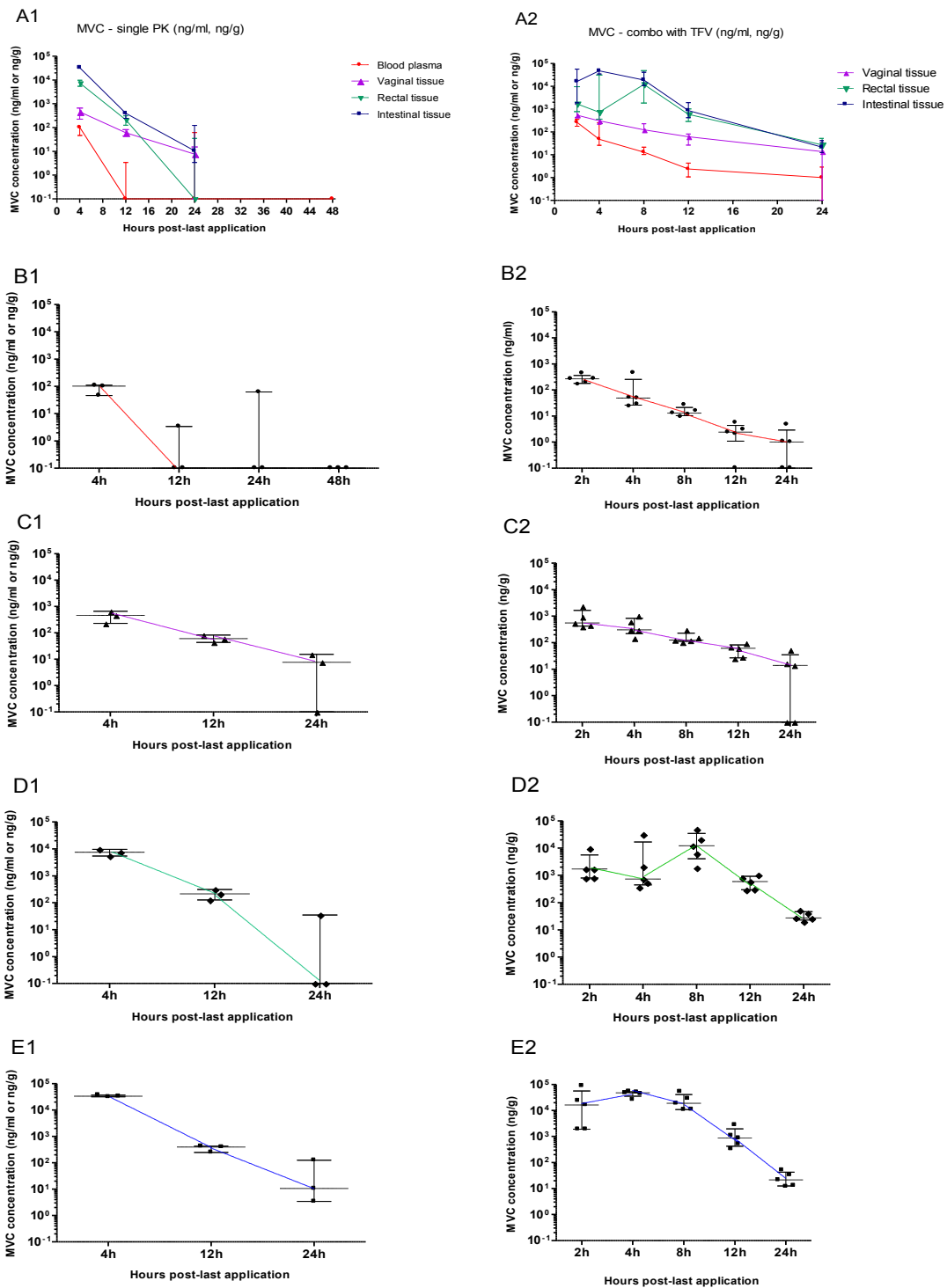


Figure 5.6. Comparison of Maraviroc pharmacokinetics following single (A1-E1) and combinatorial (A2-E2) oral application with Tenofovir.

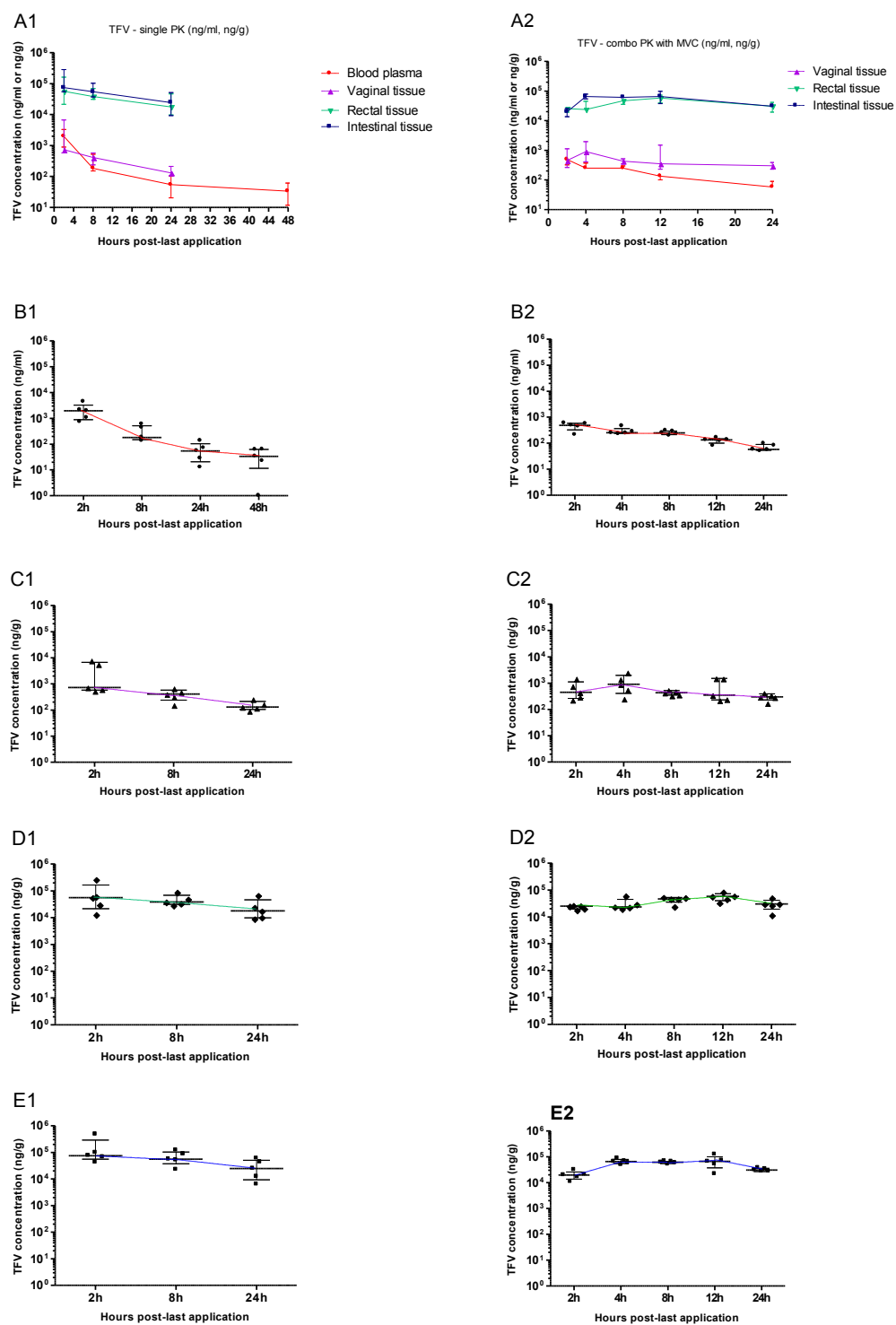


Figure 5.7. Comparison of Tenofovir pharmacokinetics following single (A1-E1) and combinatorial (A2-E2) oral application with Maraviroc.

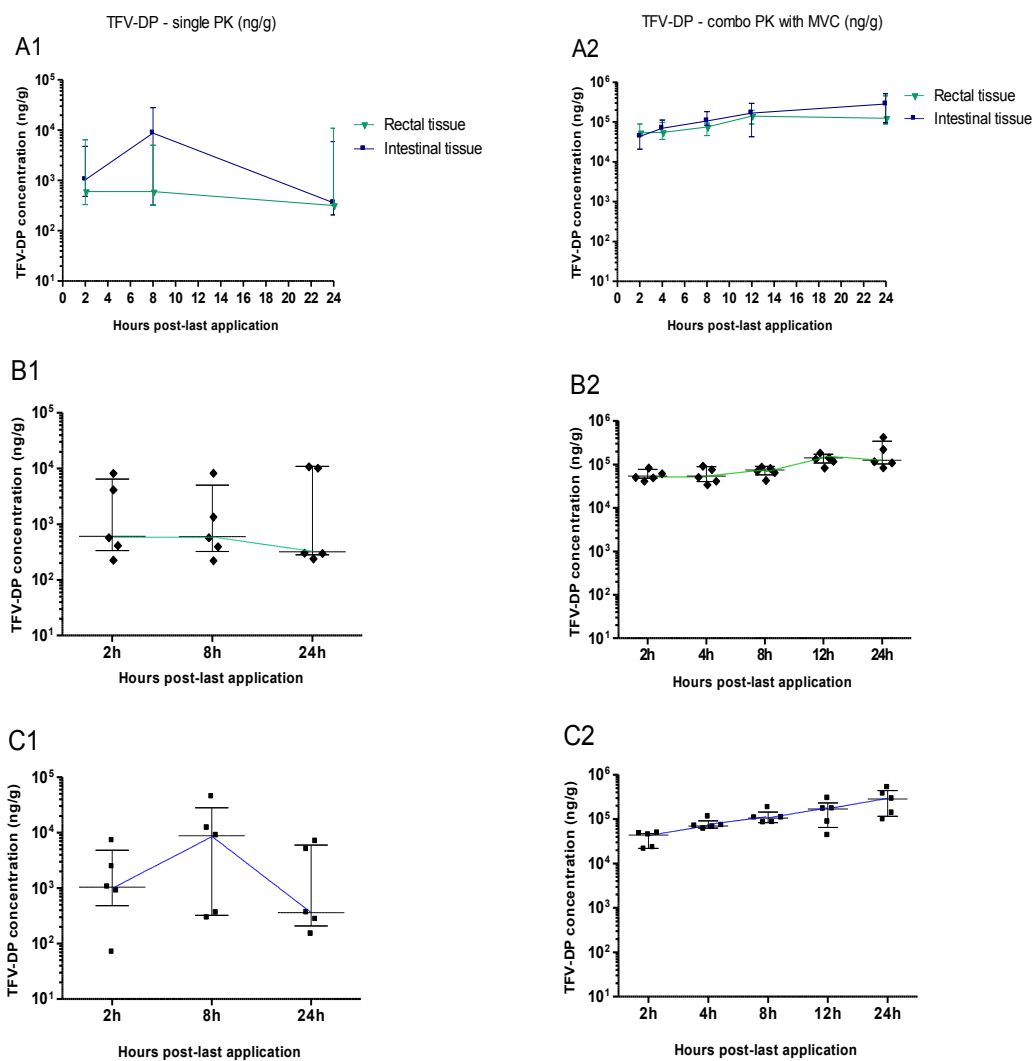


Figure 5.8. Comparison of Tenofovir-diphosphate pharmacokinetics following single (A1-C1) and combinatorial (A2-C2) oral application of Tenofovir with Maraviroc.

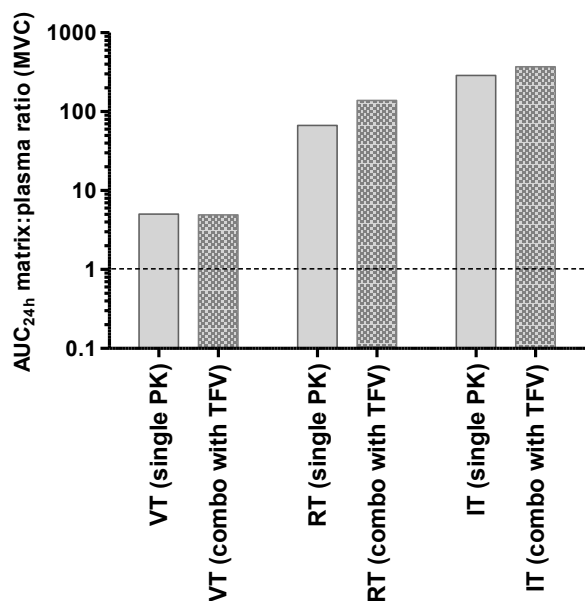


Figure 5.9. AUC_{24h} tissue:plasma ratios for Maraviroc following single and combinatorial oral application with Tenofovir. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

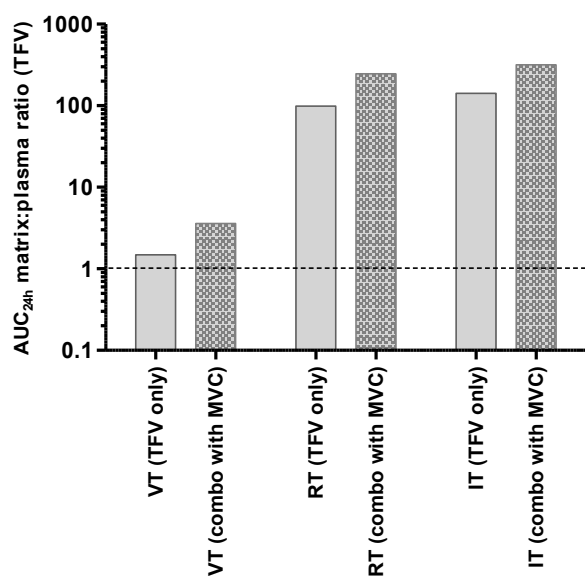


Figure 5.10. AUC_{24h} tissue:plasma ratios for Tenofovir following single and combinatorial oral application with Maraviroc. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

Table 5.4. PK parameters for Maraviroc following single and combinatorial oral application with Tenofovir.

MVC (single PK)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median	102	459	7577	33462
(IQR)	(46 – 109)	(228 – 659)	(5492.6 – 9769)	(31958 – 36819)
T_{max}	4h	4h	4h	4h
AUC[†]	755	2461	32690	140159
t_{1/2}[‡]	1	2.6	1.5	1.2
MVC (combo TFV)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median	268	552.8	12187	48270
(IQR)	(179.5-359.5)	(425.7-1656)	(4034-34812)	(35224-52630)
T_{max}	2h	2h	8h	4h
AUC[†]	727.2	3572	101053	269020
t_{1/2}[‡]	3.8	5.1	2.7	1.7

[†]Median (interquartile range, IQR), ng/ml or ng/g; [†]Area under the curve - AUC_{24h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g);

[‡]Half-life (t_{1/2}, h);

Table 5.5. PK parameters for Tenofovir and Tenofovir-diphosphate following single and combinatorial oral application with Maraviroc.

TFV (single PK)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median (IQR)	1990 (898 – 3310)	729 (581 – 6739)	56732 (21746 – 164561)	74677 (55008 – 288570)
T_{max}	2h	2h	2h	2h
AUC[†]	11251	14946	1 x 10 ⁶	1.43 x 10 ⁶
t_{1/2}[‡]	17	9	13.5	13.9
TFV-DP (single PK)				
C_{max}				
Median (IQR)	NA	BLQ [¥]	604 (336 – 6466)	8841 (322 – 28175)
T_{max}	NA	/	2h	8h
AUC[†]	NA	/	70194	172167
t_{1/2}[‡]	NA	/	22.2	3.5
TFV (combo MVC)				
C_{max}				
Median (IQR)	495 (325-584)	904.7 (410.7-1973)	58425 (40150-72811)	66171 (37557 – 98969)
T_{max}	2h	4h	12h	12h
AUC[†]	3791	13768	935316	1.2 x 10 ⁶
t_{1/2}[‡]	8.8	35.2	13	11
TFV-DP (combo MVC)				
C_{max}				
Median (IQR)	NA	BLQ	141756 (107275-173357)	282034 (115903-442193)
T_{max}	NA	/	12h	24h
AUC[†]	NA	/	2.89 x 10 ⁶	3.61 x 10 ⁶
t_{1/2}[‡]	NA	/	62	/ [¥]

[†]Median (interquartile range, IQR), ng/ml or ng/g; [†]Area under the curve - AUC_{24h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g); [‡]Half-life (t_{1/2}, h); [¥]no elimination phase; NA – not applicable; BLQ – below limit of quantification

Table 5.6. AUC_{24h} (exposure) and median C_{max} ratios for TFV and MVC oral co-administration.

AUC _{24h} ratio	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
MVC	0.96	1.45	3.09	1.92
TFV	0.34	0.92	0.94	0.84
TFV DP	NA	BLQ	41.17	20.97
C _{max} ratio	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
MVC	2.63	1.20	1.61	1.44
TFV	0.25	1.24	1.03	0.89
TFV DP	NA	BLQ	234.70	31.90

* Median C_{max}; NA- not applicable; BLQ – below the limit of quantification

5.3.3. Pharmacokinetics of combinatorial Raltegravir and Maraviroc oral application

RAL kinetics in plasma and mucosal tissues in co-administration with MVC

In plasma, median C_{max} was 468 ng/ml at 2h, while C_{trough} was 5.2 ng/ml. The plasma AUC_{24h} was 3820 ng*h/ml and t_{1/2} was 4h. Vaginal tissue median C_{max} was 797 ng/g at 2h, while C_{trough} was 3.5 ng/g. The AUC_{24h} in vaginal tissue was 5582 ng*h/g and t_{1/2} was 4.6h. In the rectal tissue, highest RAL concentration was detected at 8h (median 4240 ng/g), while C_{trough} was 1.2 ng/g. Rectal AUC_{24h} was 21981 ng*h/g and t_{1/2} was 1h. Intestinal tissue median C_{max} was 13285 ng/g at 8h, while C_{trough} was 14.4 ng/g. The AUC_{24h} in intestinal tissue was 173850 ng*h/g and t_{1/2} was 3.5h (Fig 5.11, Table 5.7).

MVC kinetics in plasma and mucosal tissues in co-administration with RAL

Median C_{max} in plasma was 567 ng/ml at 2h, while AUC_{24h} was 1403 ng*h/ml and t_{1/2} was 5.8h. In vaginal tissue, median C_{max} was 1030 ng/g at 2h, AUC_{24h} was 3549 ng*h/g and t_{1/2} was 6.6h. Rectal tissue median C_{max} was 10181 ng*g/g at 8h, AUC_{24h} was 52760 ng*h/g and t_{1/2} was 1h. Intestinal tissue kinetics was characterized by median C_{max} of 26398 ng/g at 4h, AUC_{24h} of 248605 ng*h/g and t_{1/2} of 2.5h (Fig 5.12, Table 5.8).

Single versus co-administration kinetics in plasma and mucosal tissues

The effect of co-administration on the drug tissue exposure was analyzed by comparing the AUC_{24h} tissue to plasma ratios for RAL and MVC (Fig 5.13, Fig 5.14). For RAL, tissue exposure compared to plasma was reduced in co-administration with MVC in vaginal and rectal tissue, with AUC_{24h} ratios decreasing from 6 to 1.5 in vaginal and from 8.5 to 5.7 in rectal compartment. In the intestinal tissue the drug exposure was enhanced in RAL co-administration with MVC, compared to RAL only, with AUC_{24h} tissue:plasma ratio increase from 29 to 45.5. For MVC, the co-administration with RAL resulted in the exposure decrease in all tissues relative to plasma, compared to single oral MVC application. The AUC_{24h} tissue:plasma ratios were reduced from 5, 67 and 287 for MVC in vaginal, rectal and intestinal tissue to 2.5, 37 and 177 in the same tissues for co-administration with RAL.

In order to compare the kinetics in single oral application and co-administration, the median C_{max} ratios and AUC_{24h} ratios were calculated for RAL and MVC in all compartments analyzed (Table 5.9). In plasma, RAL median C_{max} ratio was 1.65 and AUC_{24h} ratio was 0.82. In vaginal tissue, RAL C_{max} ratio was 0.23 and AUC_{24h} ratio was 0.2. In rectal and intestinal tissue RAL C_{max} ratios were 3.5 and 1.14, respectively, and AUC_{24h} ratios were 0.55 and 1.28, respectively. For MVC, the observed ratios in plasma were 5.56 for C_{max} and 1.86 for AUC_{24h} . In vaginal tissue, MVC C_{max} ratio was 2.24 and AUC_{24h} ratio was 1.44. In rectal and intestinal tissue, MVC C_{max} ratios were 1.34 and 0.79, respectively, while AUC_{24h} ratios were 1.61 and 1.77, respectively.

Mann-Whitney two-tailed analysis was done for median C_{max} in single versus combinatorial application. The only significant difference was for MVC C_{max} ($p < 0.05$) in plasma. For RAL, C_{max} differences in plasma and tissues were found not to be significant ($p > 0.05$).

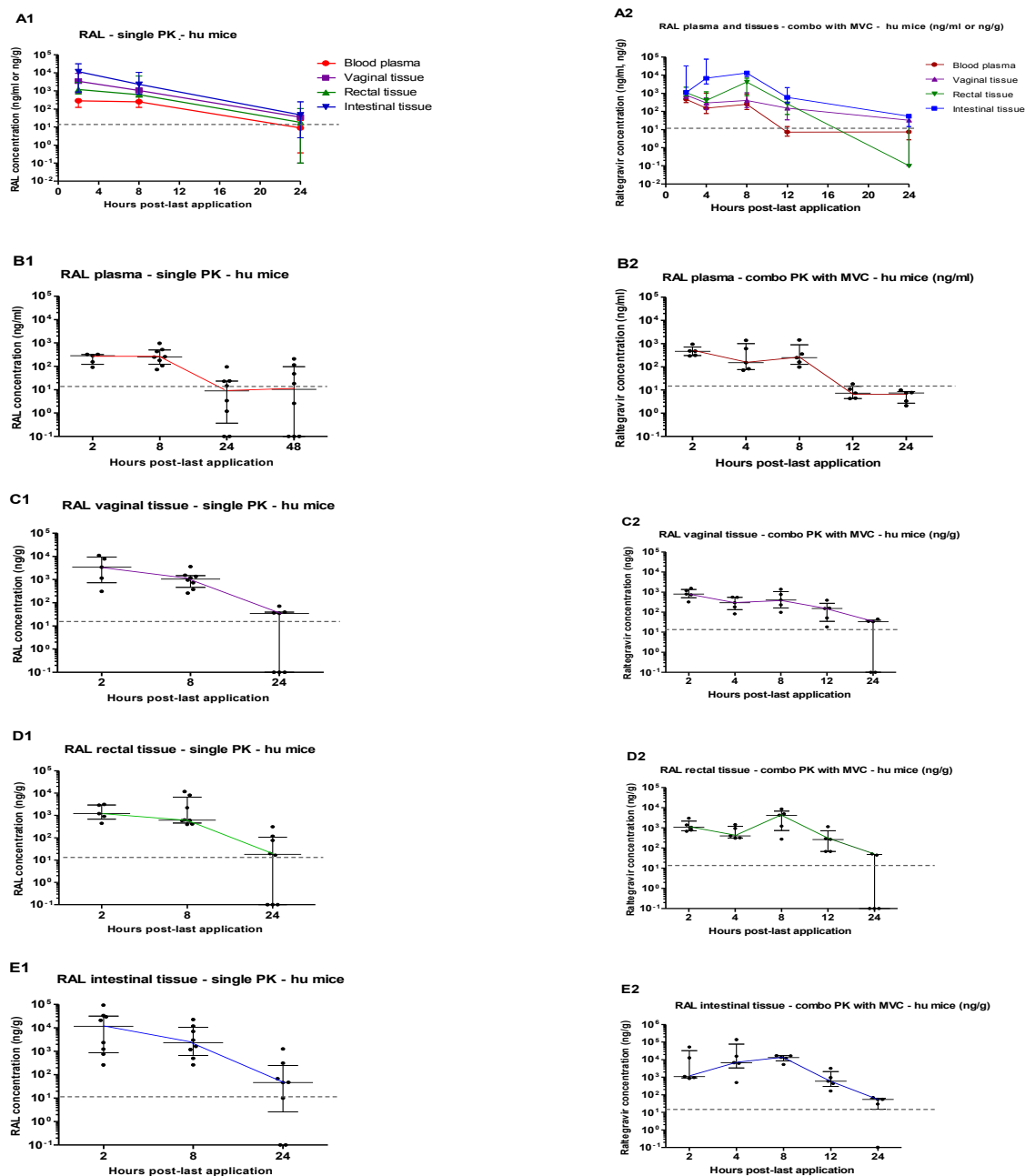


Figure 5.11. Comparison of Raltegravir pharmacokinetics following single (A1-E1) and combinatorial (A2-E2) oral application with Maraviroc. Dotted line represents Raltegravir *in vitro* 95% inhibitory concentration ($IC_{95} = 14.6$ ng/ml).

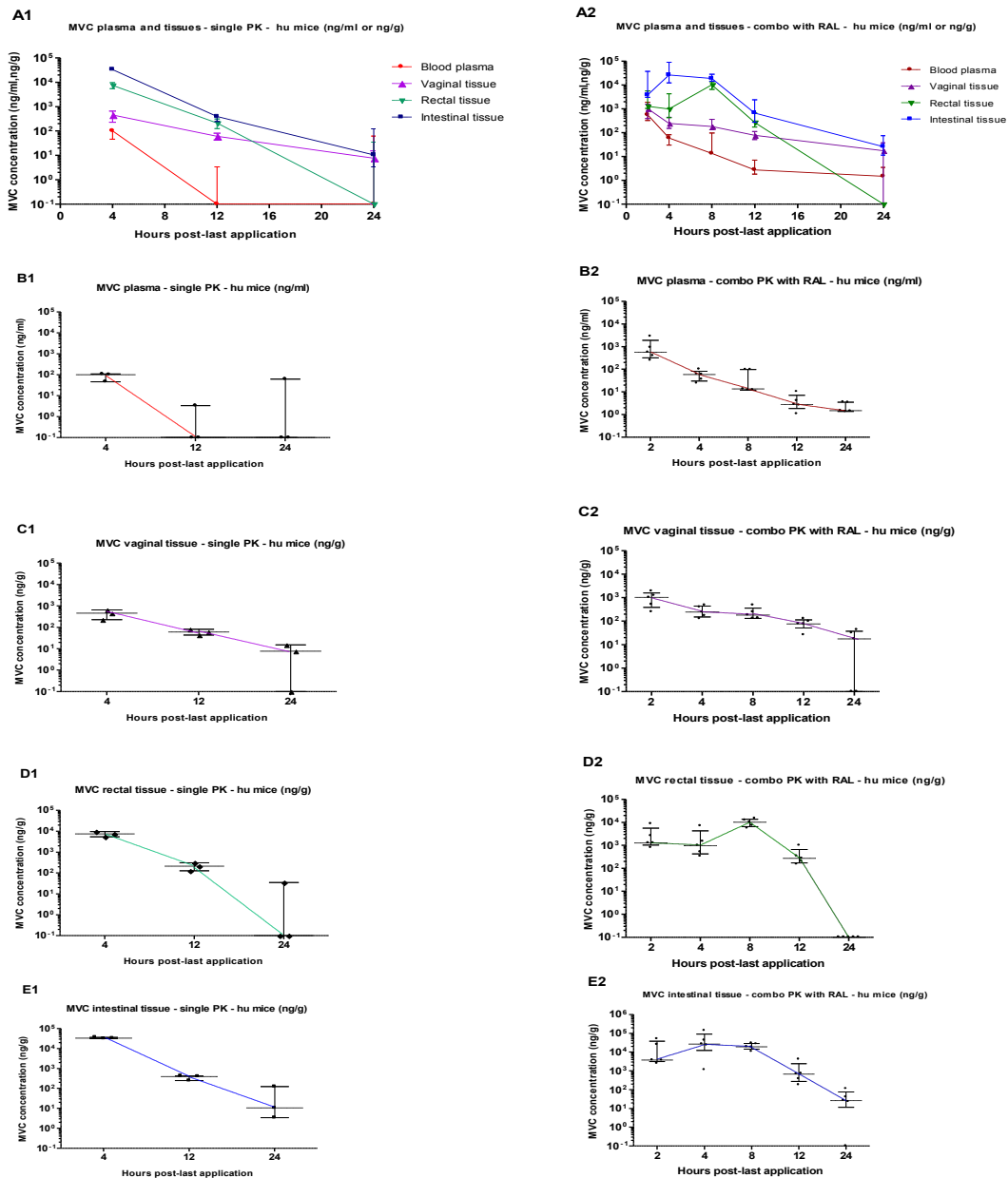


Figure 5.12. Comparison of Maraviroc pharmacokinetics following single (A1-E1) and combinatorial (A2-E2) oral application with Raltegravir

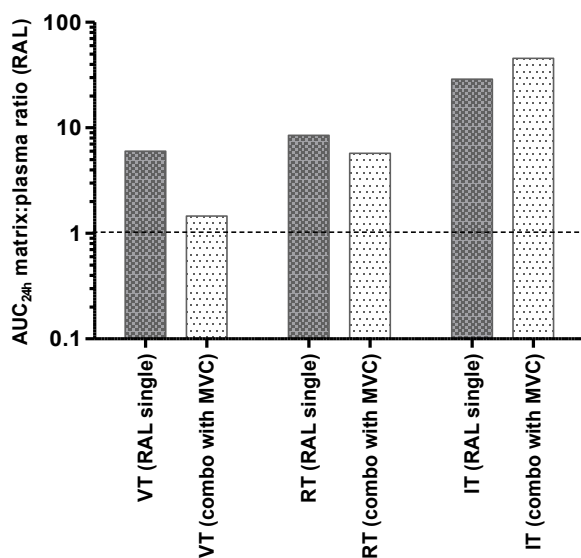


Figure 5.13. AUC_{24h} tissue:plasma ratios for Raltegravir following single and combinatorial oral application with Maraviroc. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

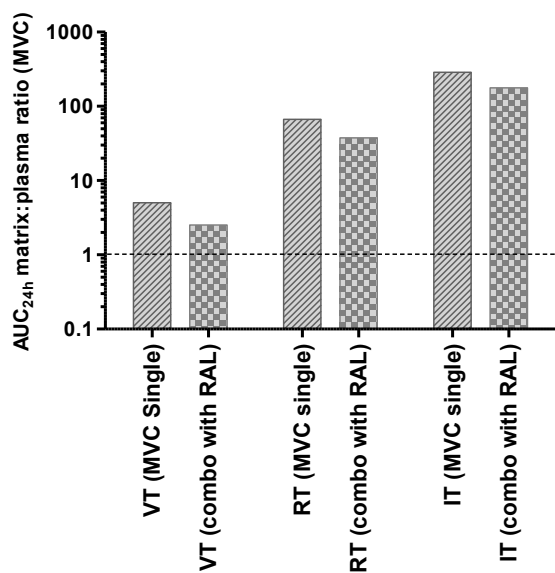


Figure 5.14. AUC_{24h} tissue:plasma ratios for Maraviroc following single and combinatorial oral application with Raltegravir. On the y axis, 1 indicates a line of unity, where mucosal tissue exposure is similar to blood plasma. VT-vaginal tissue, RT-rectal tissue, IT-intestinal tissue (colon).

Table 5.7. PK parameters for Raltegravir following single and combinatorial oral application with Maraviroc.

RAL (single PK)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median (IQR)[*]	283 (122.7 – 321)	3440 (731.5 – 9349)	1213 (677.5 – 2981)	11692 (878 – 32332)
Mean (CV%)[∞]	210 (45%)	2531 (96%)	1342 (70.3%)	5690 (138.7%)
C_{trough}^Δ	3.6	3.2	5.5	16.6
T_{max}	2h	2h	2h	2h
AUC[‡]	4683	28172	39742	135610
t_½[†]	9	3.1	7.4	3.1

RAL (combo PK with MVC)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median (IQR)[*]	468 (306.5-708)	796.9 (526.7-1363)	4240 (748.2-6784)	13285 (8527-16699)
Mean (CV%)[∞]	456.1 (51.7%)	807 (51%)	2278 (85.3%)	11811 (37%)
C_{trough}^Δ	5.2	3.5	1.2	14.4
T_{max}	2h	2h	8h	8h
AUC[‡]	3820	5582	21981	173850
t_½[†]	4	4.6	1	3.5

^{*} Median (inter-quartile range, IQR) - ng/ml or ng/g; [∞] Geometric mean - ng/ml or ng/g (Coefficient of variation - %); [‡] Area under the curve – AUC_{24h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g); [†] Half-life – h; ^Δ Geometric mean of all values at 24h post-last dose – ng/ml or ng/g

Table 5.8. PK parameters for Maraviroc following single and combinatorial oral application with Raltegravir.

MVC (single PK)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median (IQR)	102 (46 – 109)	459 (228 – 659)	7577 (5492.6 – 9769)	33462 (31958 – 36819)
T_{max}	4h	4h	4h	4h
AUC[†]	755	2461	32690	140159
t_½[‡]	1	2.6	1.5	1.2

MVC (combo PK with RAL)	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
C_{max}				
Median (IQR)[*]	567 (322.5-1896)	1030 (384.8-1577)	10181 (6505-13700)	26398 (12352-89693)
T_{max}	2h	2h	8h	4h
AUC[‡]	1403	3549	52760	248605
t_½[†]	5.8	6.6	1	2.5

^{*} Median (interquartile range, IQR), ng/ml or ng/g; [†] Area under the curve - AUC_{24h} for plasma (ng * h/ml); AUC_{24h} for tissues (ng * h/g); [‡] Half-life (t_½, h)

Table 5.9. AUC_{24h} (exposure) and median C_{max} ratios for RAL and MVC oral co-administration.

AUC _{24h} ratio	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
RAL	0.82	0.20	0.55	1.28
MVC	1.86	1.44	1.61	1.77
C _{max} ratio	Plasma	Vaginal tissue	Rectal tissue	Intestinal tissue
RAL	1.65	0.23	3.50	1.14
MVC	5.56	2.24	1.34	0.79

* Median C_{max}

5.3.4. Summary of the comparison between plasma and tissue ARV kinetics following single and dual drug application

Table 5.10. PK parameters relevant to PrEP application. Comparison given for single versus dual drug application for plasma and tissue compartments. A. TFV+RAL combinatorial oral application. B. TFV+MVC combinatorial oral application. C. RAL+MVC combinatorial oral application.**A.**

TFV + RAL combination	Compartment	AUC _{24h}	Median C _{max}	C _{trough}	Tissue: plasma AUC _{24h} ratio	t _{1/2}	Overall kinetics combinatorial vs single
RAL	Plasma	↓	-	↑	NA	↓	Not favorable
	Vaginal tissue	↓	↓	↑↑	-↑	↑	Not favorable
	Rectal tissue	↑	↑↑	-	↑↑	↓	Favorable
	Intestinal tissue	↑	↑	↑↑↑	↑↑	↓	Favorable
TFV	Plasma	↓	↓	NA	NA	↓	Not favorable
	Vaginal tissue	↑	↑	NA	↑	-↓	Favorable
	Rectal tissue	↓	↓	NA	↑	↑	Ambiguous*
	Intestinal tissue	↓	↓	NA	↑	↑	Ambiguous*
TFV-DP	Rectal tissue	↑↑	↑↑↑	NA	NA	↑	Very favorable
	Intestinal tissue	↑↑	↑↑	NA	NA	↑	Very favorable

Table legend: ↑ increase; ↓ decrease; -, -↓ or -↑ little to no change; NA – not applicable; * a decrease in TFV could be due to the observed enhanced conversion to TFV-DP

B.

TFV + MVC combination	Compartment	AUC _{24h}	Median C _{max}	Tissue:plasma AUC _{24h} ratio	t _{1/2}	Overall kinetics combinatorial vs single
MVC	Plasma	-	↑	NA	↑	Favorable
	Vaginal tissue	↑	↑	-	↑	Favorable
	Rectal tissue	↑	↑	↑↑	↑	Favorable
	Intestinal tissue	↑	↑	↑↑	↑	Favorable
TFV	Plasma	↓	↓	NA	↓	Not favorable
	Vaginal tissue	-↓	↑	↑	↑↑	Favorable
	Rectal tissue	-↓*	-	↑↑	-	Favorable
	Intestinal tissue	-↓*	-↓	↑↑	↓	Favorable
TFV-DP	Rectal tissue	↑↑	↑↑↑	NA	↑↑	Very favorable
	Intestinal tissue	↑↑	↑↑	NA	↑↑	Very favorable

Table legend: ↑ increase; ↓ decrease; -, -↓ or -↑ little to no change; NA – not applicable; * a decrease in TFV could be due to the observed enhanced conversion to TFV-DP

C.

RAL +MVC combination	Compartment	AUC _{24h}	Median C _{max}	C _{trough}	Tissue:plasma AUC _{24h} ratio	t _{1/2}	Overall kinetics combinatorial vs single
RAL	Plasma	-↓	↑	↑	NA	↓	Ambiguous
	Vaginal tissue	↓↓	↓↓	-	↓	↑	Not favorable
	Rectal tissue	↓	↑	↓	↓	↓	Not favorable
	Intestinal tissue	↑	↑	-↓	↑	-↑	Favorable
MVC	Plasma	↑	↑	NA	NA	↑	Favorable
	Vaginal tissue	↑	↑	NA	↓	↑	Partially favorable/ Ambiguous
	Rectal tissue	↑	↑	NA	↓	-↓	Partially favorable/ Ambiguous
	Intestinal tissue	↑	-↓	NA	↓	↑	Partially favorable/ Ambiguous

Table legend: ↑ increase; ↓ decrease; -, -↓ or -↑ little to no change; NA – not applicable;

5.4 Discussion

A combinatorial preventative approach which would target HIV-1 on multiple levels and offer high level of protection against sexual transmission is urgently needed. In the present study humanized mice were utilized to model pharmacokinetics of currently approved prophylactic drug Tenofovir (TFV - Truvada formulation with Emtricitabine) in combination with integrase inhibitor Raltegravir (RAL) or entry inhibitor Maraviroc (MVC). In addition, the dual combination of RAL and MVC was also tested. Drug combinations were applied orally in human therapeutic dose equivalents for five consecutive days and PK was analyzed at steady state kinetics. All three drugs were successfully detected in all compartments analyzed, namely vaginal, rectal and intestinal (colonic) tissue and blood plasma.

The Mann-Whitney analysis showed that the only statistically significant C_{max} alterations in the mucosal tissues were for RAL co-administration with TFV in the rectal tissue, and for TVF-DP in rectal and intestinal tissue for both TFV/RAL and TFV/MVC application. In all three cases, the identified significant changes were increases in tissue exposure, both for RAL and for TVF-DP, indicating that all significant C_{max} shifts in co-administration were actually favorable for PrEP applications.

In the TFV and RAL dual application, the resulting PK of RAL showed enhancement in the rectal and intestinal compartments, according to the parameters relevant for PrEP application, namely C_{max} and AUC_{24h} combo/single ratios, C_{trough} , AUC_{24h} tissue:plasma ratios and $t_{1/2}$ (section 5.3.4. Table 5.10 A). The opposite was seen for plasma and vaginal tissue, where combinatorial application with TFV resulted in a decrease of C_{max} and AUC_{24h} , although C_{trough} was increased. Importantly, the vaginal tissue exposure was maintained above the RAL IC_{95} (14.6 ng/g) in all samples at all times analyzed, emphasizing protective kinetics despite the decrease seen in C_{max} and AUC_{24h} . For TFV, in vaginal tissue the combinatorial application with RAL resulted in the more favorable kinetics compared to single TFV dosing. In rectal and

intestinal tissue compartments a striking increase was observed for TFV-DP levels, with one to two log higher values in TFV+RAL treatment compared to TFV only. This represents a highly unexpected finding and the one which would characterize this dual approach as a very potent PrEP candidate, since TFV-DP inhibits the HIV-1 RT intracellularly and represents a direct correlate of the antiviral effect. Previous human TFV/RAL co-administration studies have analyzed the systemic kinetics (blood plasma) and no data were reported regarding mucosal tissues [348]. The synergistic *in vitro* effect against HIV-1 was reported recently for TFV and RAL [349]. Our finding of the enhanced TFV to TFV-DP conversion represents the first potential pharmacokinetic explanation for synergistic protective effect in the *in vivo* settings and warrants further investigation of TFV+RAL combination for PrEP applications.

For TFV/MVC dual application, the kinetics of MVC was enhanced in all tissues, based on multiple PrEP-relevant PK parameters analyzed (Section 5.3.4., Table 5.10 B). The overall AUC_{24h} for TFV was reduced in all tissues, but the tissue:plasma ratios were increased. The most pronounced effect when comparing TFV/MVC combo to single TFV kinetics, was one to two log increase in the TFV-DP levels detected in the rectal and intestinal tissues. This could have contributed to the lower TFV tissue concentration, due to the increase in the intracellular diphosphorylation to generate high levels of TFV-DP seen. Previous TFV/MVC co-administration human studies analyzed blood and urine exposure and no analysis was done for the exposure in mucosal compartments [350]. The increase of TFV conversion to TFV-DP in rectal mucosa and the overall enhancement of MVC kinetics in all tissues are the first reported findings for TFV/MVC mucosal kinetics *in vivo*. For the PrEP applications, this represents a highly promising PK combinatorial profile and further *in vivo* studies should characterize the protective effects against HIV-1 for the complete PK-PD profiling.

The combinatorial RAL/MVC oral application resulted in overall reduction of RAL exposure (AUC_{24h}) and tissue:plasma AUC_{24h} ratios in vaginal and rectal tissue, while the opposite trend was seen in the intestines (Section 5.3.4., Table 5.10 C). Similar decrease in

RAL exposure was seen in the previous MVC co-administration clinical study in blood plasma [351], and our study extends this finding to the mucosal tissues relevant for HIV-1 transmission. Importantly, the RAL tissue levels were maintained above the *in vitro* established IC₉₅ in all tissue compartments up to 24h. For MVC, the effects of RAL/MVC co-administration were the increase in plasma and tissue exposure (AUC_{24h}) and C_{max} levels, while the tissue:plasma AUC_{24h} ratios were reduced. There are no previous clinical studies on MVC PK in mucosal tissues upon co-administration with RAL, and this represents first such report *in vivo*. Since the alterations in MVC PK were ambiguous, with the simultaneous increase in some and decrease in other PK parameters, further investigation is needed to better define mucosal kinetics of MVC in MVC/RAL dual approach. Based on current data it can be concluded that there were no pronounced antagonistic effects in the MVC kinetics when combined with RAL, which also warrants further analysis. Overall, this combination cannot be characterized as promising for PrEP, based on the initial findings. Higher doses than the therapeutic ones applied here might be needed for PrEP and they should be tested to further define combinatorial RAL/MVC kinetics at mucosal sites of HIV-1 transmission.

One caveat of the study was the inability to detect TFV-DP in the vaginal tissue, following TFV/RAL and TFV/MVC dual application, even though TFV itself was successfully quantified in this compartment. This is in line with the previous data obtained in humanized mice following TFV single oral application [198]. In order to overcome this, further modifications might be needed in the vaginal tissue sampling protocol. A recent human study on RAL PK has shown that the increase in biopsy size of vaginal tissue is directly correlated with the decrease in RAL concentration [338]. This finding indicates that the drug distribution might not be equal in all mucosal layers, and that could be extrapolated to TFV, which accumulates intracellularly as RAL. In the studies thus far, vaginal tissue was collected as a whole and a more guided biopsy approach will be tested in the future experiments, in order to obtain only mucosal layers which are potential sites of TFV-DP accumulation. In addition, further insights into drug distribution in

mucosal tissues by detection methods such as MALDI could help guide future sampling protocol modifications [339].

The PK analysis in a small animal model, such as humanized mice, could offer valuable preclinical insights, which could in part guide further NHP studies and subsequent clinical trials. Multiple important questions could be better addressed with such modeling, like the effects of drug interactions on tissue PK following combinatorial dosing, overall toxicity of given combinations and the potential for generation of HIV-1 escape mutants. Importantly, PK-PD studies in a humanized mouse model imply testing drug efficacy against unmodified HIV-1, not modified chimeric SHIV-1 or SIV. In addition, the synergistic, agonistic or antagonistic drug to drug interactions could be characterized on the molecular level within human immune cell subsets in humanized mice. Such characterization is needed in order to offer potential explanations for the robust enhancement of TFV-DP in rectal and intestinal tissue, seen here in the TFV/RAL and TFV/MVC dual approach. The observed TFV-DP increase is also accompanied by favorable kinetics of the second drug in combination, especially in the case of MVC. The findings shown here represent the first reports on mucosal tissue kinetics of TFV, MVC and RAL following oral co-administration, and are encouraging for future PrEP testing of these dual drug combinations in NHP and clinical settings. As the data shown here describes dual PK of selected ARVs, future combinatorial analysis could be expanded to characterize PK of the multidrug (triple or quadruple) approach, as well as PK of the novel nano-formulated or long-lasting combinatorial formulations for PrEP.

CHAPTER 6

Topical PrEP: Pharmacokinetic and Pharmacodynamic (PK-PD) Modeling In Humanized Mice

6.1 Pharmacokinetic study – mucosal tissue exposure of antiretrovirals TFV, MVC and RAL following topical application (microbicide gel formulations) in a humanized mouse model

6.1.1. Introduction

Topical drug application in the form of microbicide gels is one of the two major currently developed pre-exposure prophylaxis strategies, along with oral application of antiretrovirals. The CAPRISA004 study in which Tenofovir (TFV) 1% gel was shown to be protective when applied in a coitally-dependent manner was the first proof of concept human trial for the microbicide approach [234]. However, in the follow up clinical trial VOICE (MTN-003) the same TFV gel formulation was ineffective when applied once daily in a coitally-independent manner [235]. These conflicting results raised several important questions in the field. First question is the individual adherence to the dosing schedule. Any increase in patient non-compliance to the dosing frequency represents a major obstacle to long-term application of PrEP or evaluation of the real protective effects of the approach [236, 238]. The obstacle of low patient adherence can be overcome by the pharmacokinetic analysis of drug retention in mucosal tissues as site of transmission. By obtaining PK data on drug retention at sites of HIV-1 transmission such as vaginal or rectal mucosa, better design of dosing regimens and of the long-lasting time release (depot) formulations would be achievable [95, 229, 243]. The second question is the lack of data on the actual drug concentrations reached in the mucosal tissues following microbicide application [229, 237, 239, 265]. Most PK studies to date have analyzed systemic and mucosal

exposure following oral application of antiretrovirals, and topical application has not been analyzed to the same extent [245, 290, 291].

The outlined questions can be addressed by more comprehensive preclinical testing of candidate PrEP antiretrovirals in a suitable animal model. Humanized mice represent a small animal model in which preclinical analysis can be done in the context of protection against HIV-1, to complement similar studies in NHP, which can address these questions in the context of SHIV-1 or SIV [93, 94, 181, 197]. Having all this in mind, initial topical PK studies have been conducted in RAG-hu mice, one of the new generation humanized mouse models [167]. Humanized mice have already been utilized for testing of multiple protection approaches against HIV-1 in previous studies [171, 176, 177, 179, 190, 296]. However, this is the first analysis of drug concentrations in mucosal site of transmission, namely vaginal mucosa, following intravaginal application of microbicide gel formulations of ARVs. Representative ARVs, belonging to three different classes based on their mode of action were applied in microbicide gel formulations to RAG-hu mice. Namely, this study included the RT inhibitor TFV, the integrase inhibitor Raltegravir (RAL) and the entry inhibitor Maraviroc (MVC). Drug doses applied were based on the previous human trials and data from previous protection studies in humanized mice, and 1% gel formulation of TFV, 5mM formulations of MVC and RAL were applied [190, 234]. To mimic potential exposure-dependent gel application in human trials, drug concentrations were determined one hour post-gel application in vaginal mucosa. All three ARVs analyzed were successfully detected and quantified in vaginal mucosa of RAG-hu mice. This small initial study represents a proof of concept for future more extensive PK analysis in humanized mice, which can be expanded to larger number of animals, the analysis of mucosal PK following combinatorial microbicide gel applications and the analysis of systemic exposure following topical application of ARVs.

6.1.2. Materials and methods

Antiretroviral drug preparation, administration of microbicide gels and sample collection

Hydroxyethylcellulose (HEC) was dissolved in sterile water for the final topical application of HEC to be 2.2%. HEC gel was sterilized by autoclaving and brought to room temperature prior to drug addition. Antiretroviral gels were prepared by dissolving appropriate amounts of TFV, MVC or RAL in HEC gel, to achieve final concentration of 1% TFV, 5 mM concentration of MVC and 5mM concentration of RAL. Five animals for each drug were included in the study. Antiretroviral gels were applied to the anesthetized mice using blunt-end needles, to ensure no trauma or abrasions to vaginal mucosa. Gels were applied in 20 ul volume, in order to achieve complete vaginal mucosa saturation. One hour post-gel application, vaginal tissues were collected during mouse necropsies. Prior to tissue collection en block, vaginal cavity was rinsed multiple times with 1xPBS, in order to eliminate any residual gel present in the vaginal cavity, which could interfere with drug concentration analysis in the mucosa itself. All samples were weighed and snap frozen within 5 minutes of the tissue collection. Samples were stored at -80° C prior to HPLC analysis.

Measurement of drug concentrations in tissue samples

Quantification of analytes was similar to previously published methods [255]. Briefly, for measuring concentrations in vaginal tissue, TFV and TFV-DP was extracted from tissue homogenate by protein precipitation with isotopically-labeled internal standards (¹³C TFV and ¹³C TFV-DP). TFV was eluted from a Waters Atlantis T3 (100 X 2.1mm, 3µm particle size) analytical column, and TFV-DP was eluted from a Thermo Biobasic AX (50 X 2.1mm, 5µm particle size) analytical column. An API-5000 triple quadrupole mass spectrometer was used to detect all analytes. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 0.3-300 ng/mL of homogenate for

each compound using a $1/\text{concentration}^2$ weighted linear regression. Concentrations were ultimately converted into ng/mg (TFV) or fmol/mg (TFV-DP) tissue for final reporting.

For quantification from mucosal vaginal tissue, MVC was extracted from tissue homogenates using protein precipitation with the internal standard alprazolam [298]. This resulting extract was analyzed on an Agilent 1200 series HPLC system using a Zorbax Eclipse XDB (50 x 4.6mm, 1.8 μ m particle size) analytical column. An Agilent 1100 MSD was used to detect the analyte and internal standard. The dynamic range of this assay was 0.007-7 ng/mg.

The quantitation of RAL in vaginal tissue started with the homogenization of each tissue in 70:30 acetonitrile: 1mM ammonium phosphate [352]. A portion of the resulting homogenate was extracted by protein precipitation with acetonitrile containing isotopically-labeled internal standard (^2H RAL). Following vortex and centrifugation, the supernatant was removed and evaporated to dryness. The extracts were reconstituted with 1mM ammonium phosphate prior to LC-MS/MS analysis. RAL was eluted from a Phenomenex Synergi Polar-RP (50 X 4.6mm, 4 μ m particle size) analytical column. An API- 5000 triple quadrupole mass spectrometer was used to detect the analyte. Data were collected using AB Sciex Analyst Chromatography Software (Analyst version 1.6.1). The dynamic range of this assay was 0.3-300ng/mL of homogenate using a $1/\text{concentration}^2$ weighted linear regression.

Statistical analysis

GraphPad Prism version 5 (GraphPad Software, USA) was used for data analysis and statistical analysis. Measures of central tendency were expressed as median and inter-quartile range (IQR 25th, 75th percentile).

6.1.3. Results

Following intravaginal application of 1% TFV, 5mM RAL and 5mM MVC gels, drug concentrations were determined in vaginal tissue at one hour post-gel application in five RAG-

hu mice for each drug. Results are shown in Figure 6.1 A with individual values for each mouse and median with interquartile range (IQR) for each drug. In Figures 6.1B and 1C TFV-DP concentrations are shown for individual mice, as ng/g (1B) or fmol/mg tissue (1C). In Table 6.1 medians with IQR and the coefficients of variation (CV%) are shown for each drug and for intracellular active diphosphorylated TFV metabolite TFV-DP.

Table 6.1. Drug concentrations in vaginal tissue following topical application of TFV, RAL and MVC microbicide gels in humanized mice.

	Median (IQR) [*]	CV% [∞]
TFV	25545 (15505-42031)	55.2%
TFV-DP	550.7 (299.1-2087)	96.9%
MVC	7399 (5555-25103)	75.7%
RAL	30847 (16778-78547)	74.8%

^{*}Median (interquartile range) – ng/g for TFV, MVC and RAL, or fmol/mg for TFV-DP; [∞]Coefficient of variation - %

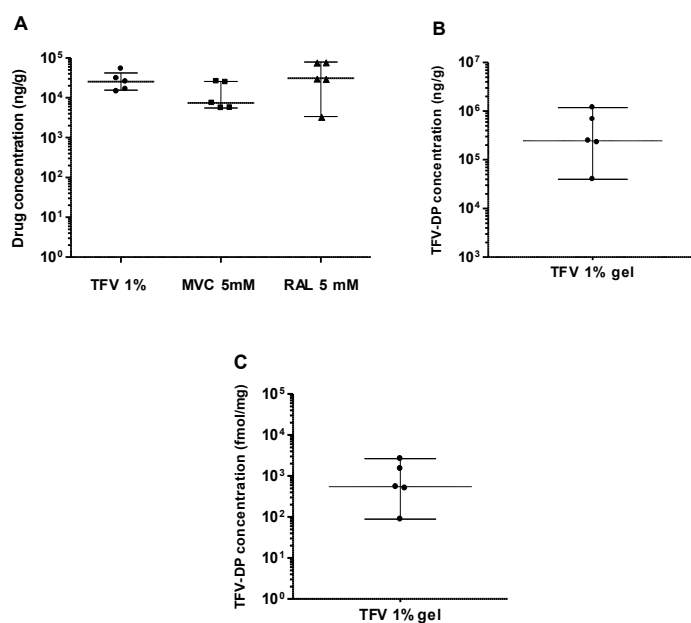


Fig 6.1. PK analysis of vaginally applied TFV, MVC and RAL microbicide gel formulations. A. TFV, MVC and RAL concentrations (ng/g), n=5 for each drug; B. TFV-DP concentration (ng/g). C. TFV-DP concentration (fmol/mg).

6.1.4 Discussion

In this initial study vaginal mucosal tissue drug distribution of three different classes of antiretrovirals - TFV, MVC and RAL, was analyzed after gel formulations were applied intravaginally in RAG-hu mice. Detection in vaginal mucosa of RAG-hu mice was successful for all three drugs and drug levels were quantified using ultrasensitive HPLC methodology. Even though conclusions are limited due to the small number of animals included in the study (n=5 per drug) and analysis at a single time point (1h post-gel application), initial data are encouraging since high drug saturation levels were achieved for all three ARVs. Another initial observation is high degree of variability of drug concentrations, with coefficients of variation (CV%) of 55.2%, 75.7% and 74.8% for TFV, MVC and RAL. Highest level of variability was seen for intracellular active metabolite TFV-DP, which showed CV% of 96.9%. Future planned studies will address high variability following topical application by increasing the number of animals included in the analysis, in order to delineate high variations caused by small study numbers from the potential actual variations in mucosal PK. Importantly, despite high levels of variation, in the majority of mice protective levels defined in available literature were reached for all three ARVs applied, indicating overall favorable PK for protection [245].

Another encouraging finding is that the levels of TFV-DP detected in humanized mice corresponded well to the levels needed in humans for protection. From the previous *ex vivo* studies it is known that the TFV-DP intracellular mucosal concentration needs to be close to or above 1000 fmol/mg tissue for near complete protection [353]. In our study, four out of five mice were near the protection range (>500 fmol/mg) and two out of five had levels higher than 1000 fmol/mg, mimicking very well human exposure and the findings in human CAPRISA004 gel study in which protection was 39% for the same concentration (1%) TFV gel topically applied [234]. In addition, as in human studies where topical application of TFV resulted in much higher tissue saturation than TFV oral application, this was also the trend we found in RAG-hu mice

[245]. Compared to TFV levels reached at steady state following oral application in RAG-hu mice ($10^2 - 10^3$ levels), the concentrations reached here after microbicide gel application were one to two logs higher (10^4 TFV levels), corresponding to previous observations in human trials [198, 245]. The same trend was seen for MVC and RAL, with the concentrations reached after microbicide gel application being one to two logs higher than for oral application at steady state kinetic [198]. For RAL 10^4 levels were reached in vaginal mucosa in topical compared to 10^2 - 10^3 levels in oral application studies [198]. For MVC 10^3 - 10^4 levels in vaginal mucosa were reached in the microbicide study, compared to 10^2 levels at steady state following oral application [198]. This corresponds well to previous comparison of MVC levels reached in NHP studies after oral and topical application [283, 295, 354]. For example, oral MVC application was found to be unprotective against rectal SIV challenge with lower MVC levels reached compared to gel applications in NHP [354]. For RAL, a recent macaque study has shown that 1% RAL gel can protect against high-dose SHIV-1 vaginal challenge (5/6 animals protected) even with gel application three hours post-SHIV-1 challenge [355]. This study expands the potential of microbicide formulations from pre-exposure to post-exposure application, as a form of post-exposure prophylaxis (PeP). Detailed PK studies of drug levels reached over time in mucosal sites of transmission are needed for PeP in addition to PrEP, in order to define the window of opportunity for post-exposure intervention. Humanized mice could be utilized to obtain initial preclinical data for further NHP and human studies of PeP in addition to PrEP.

Based on this initial promising small study in RAG-hu mice, future topical PK analysis could be done as a time course and not at a single time point as here. Parallel analysis of systemic exposure over time, by measuring blood plasma levels in addition to vaginal tissue levels, could offer data on potential systemic exposure and toxicity of microbicide gel formulations. In addition, drug interactions and alterations in mucosal exposure could be analyzed following application of combinatorial ARV gels in RAG-hu mice. All these studies in

humanized mice would offer comprehensive PK data profiles of new topical PrEP and PeP drug candidates and inform further NHP studies and clinical trials.

6.2 Pharmacodynamic (protection) study of antiretrovirals TFV, RAL and UC781 following topical application (gel formulations) in a humanized mouse model

6.2.1. Introduction

The ultimate aim of the pre-exposure prophylaxis modeling in humanized mice is to establish a correlation between drug concentrations reached at sites of transmission (vaginal and rectal mucosa) and the level of protection offered against HIV-1 mucosal challenge. The initial data for mucosal PK was obtained in the small scale study in RAG-hu mice (previous part of the chapter) for 1% TFV, 5 mM MVC and 5 mM RAL vaginal gels. In addition, a previous study has analyzed the protective effect of MVC in a 5mM gel formulation against vaginal HIV-1 challenge in humanize mice [190]. The experiments shown here were aimed at completing this PrEP modeling by conducting protection, pharmacodynamic (PD) studies of 1% TFV gel and 5 mM RAL gel in humanized mice, in the context of intravaginal microbicide application and challenge with CCR5-tropic HIV-1 strain Bal, in accordance with the initial MVC experiment. This type of tropism for the challenge strain was chosen as the vast majority of sexually transmitted HIV-1 in humans are CCR5 co-receptor using subtypes, therefore in order to mimic sexual transmission [87]. In addition to TFV and RAL gels, microbicide formulation of the RT inhibitor UC781 was also tested. Based on *in vitro* and *ex vivo* data from the previous studies, non-nucleoside reverse transcriptase inhibitor UC781 was tested in 0.25% gel formulation [356]. Importantly, this represents the first *in vivo* protection study of UC781 which has shown promising levels of protection against HIV-1 in previous *ex vivo* studies [356-359].

Anticipating that the combinatorial approach is needed for topical PrEP to reach high levels of protection, we have tested TFV and RAL gel, as well as TFV and UC781 gel combinations, in addition to single formulations. The only study in which an antiretroviral applied topically has shown protection is CAPRISA004 trial of the TFV 1% gel [234]. The conducted experiments in humanized mice described here were aimed at increasing the level of protection by combining TFV with another promising RT inhibitor, such as UC781, or by combining TFV with another class of ARV, such as the potent integrase inhibitor RAL. Both combinatorial microbicides did offer higher level of protection compared to individual drugs, while RT inhibitors and RAL alone showed lower and similar protection potential against mucosal HIV-1 Bal challenge. This study further establishes the humanized mouse model for testing preventative approaches to mucosal transmission of HIV-1, as well as offers protection PD data for future comprehensive PK-PD modeling in humanized mice.

6.2.2. Materials and methods

Antiretroviral drug preparation, administration of microbicide gels and HIV-1 Bal vaginal challenge

Hydroxyethylcellulose (HEC) was dissolved in sterile water to a final 2.2% topical application concentration. HEC gel was sterilized by autoclaving and brought to room temperature prior to drug addition. Antiretroviral gels were prepared by dissolving appropriate amounts of TFV, UC781 or RAL in HEC gel, to achieve final concentration of 1% TFV, 0.25% concentration of UC781 and 5mM concentration of RAL. Antiretroviral gels were applied to the anesthetized mice using blunt-end needles, to ensure no trauma or abrasions to vaginal mucosa. Gels were applied in 20 ul volume, in order to achieve complete vaginal mucosa saturation. One hour post-gel application, HIV-1 Bal (3000 TCID) was applied intravaginally in a 25ul volume using a blunt-end needle. Mice were held in a position with elevated pelvis and

lower extremities for 5 min post-inoculation to prevent immediate discharge of virus. Mice in the control group received 2.2% HEC placebo gel (no active antiretroviral component) applied in the same way as ARV gels, followed by the described HIV-1 challenge. Starting at one week post-challenge, blood samples were collected on a weekly basis to assess plasma viremia and CD4 T cell counts.

Measurement of viral loads

HIV-1 infection status and viral loads were assessed by qRT-PCR biweekly. RNA was extracted from 25 to 50 µl of EDTA-treated plasma using the QIAamp Viral RNA kit (Qiagen, Valencia, CA). qRT-PCR was performed by using a primer set specific for the HIV-1 LTR sequence and a corresponding LTR specific probe as described previously [171, 172, 174]. C1000 Thermal Cycler (CFX96™ Real-Time System, BIO-RAD) and iScript™ One-Step RT-PCR kit with SYBR® Green (BIO-RAD) were used to perform the real-time qRT-PCR analysis and viral load was expressed as the number of HIV-1 RNA copies per milliliter plasma.

Flow cytometry

Mice were monitored biweekly to analyze the levels of CD4 T cells in the peripheral blood. Whole blood was collected and red blood cells lysed as described previously [171, 172, 174]. Peripheral lymphocytes were stained for hCD45-FITC, hCD3-R-PE and hCD4-PE-Cy5 surface markers (Invitrogen, BD Biosciences) and monitored using a Coulter EPICS XL-MCL FACS analyzer (Beckman Coulter, Fullerton, CA). CD4⁺ T cell levels were calculated as a ratio of the entire CD3 population (CD4⁺CD3⁺:CD4⁻CD3⁺). All mice were screened prior to the beginning of the study to establish the baseline CD4 T cell ratios.

6.2.3. Results

Study 1. TFV and UC781 topical pre-exposure prophylaxis (PrEP) against HIV-1 Bal intravaginal challenge

Nucleotide reverse transcriptase inhibitor TFV was tested alone and in combination with non-nucleoside reverse transcriptase inhibitor UC-781 in this study. Both drugs were formulated as single drugs or in combination in 2.2% HEC gel and tested against vaginal challenge with CCR5 HIV-1 strain (BaL) in RAG-hu mice. Additional experimental group was comprised of Rag-hu mice which received placebo gel prior to HIV-1 Bal mucosal challenge. As shown in Figure 6.2, combinatorial TFV+UC781 microbicide offered high level of protection, with only one out of five mice becoming infected. In addition, single TFV and UC781 gel formulations were highly protective as well, with 2/7 and 2/5 mice becoming infected, respectively. In the control group one of the mice did not show signs of infection, but the overall infection rate was high (4/5 mice) (Fig 6.2).

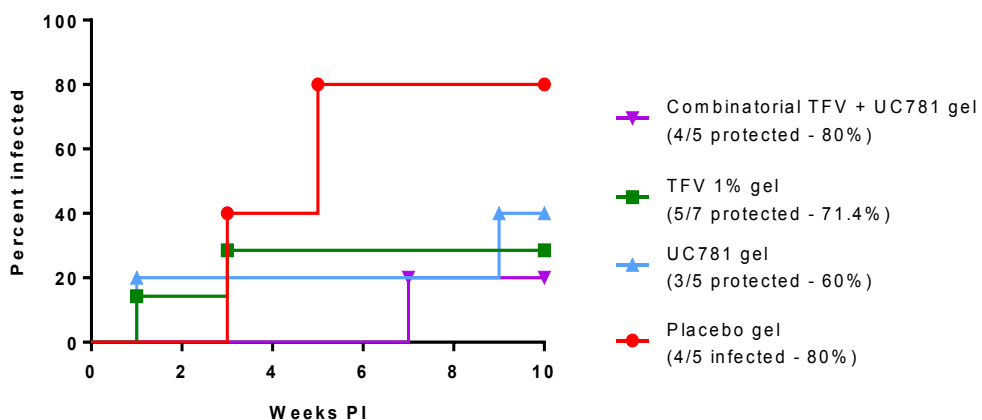
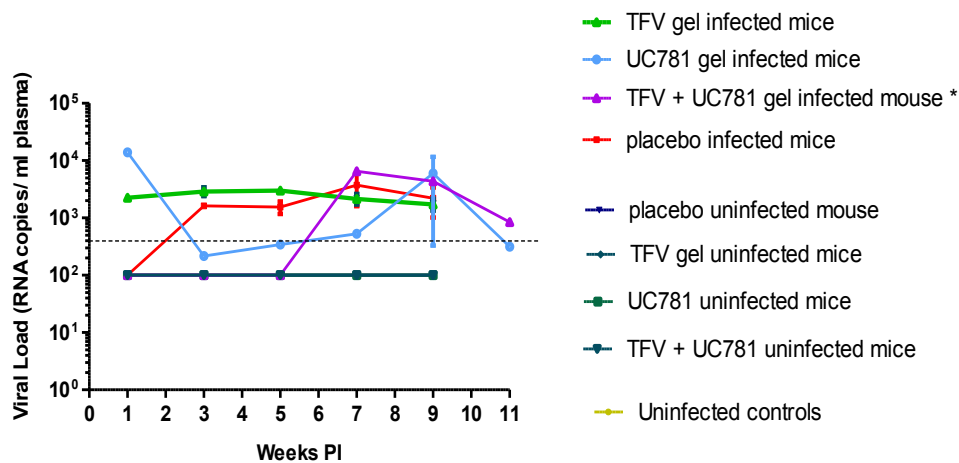


Figure 6.2. Kaplan-Meier plot of percent infected mice over the 10 week course of the experiment.

Infected RAG-hu mice from all groups showed viral load levels between 10^3 and 10^4 viral RNA copies per ml, which is an optimal viral load in hu mice following the HIV-1 Bal challenge

by vaginal mucosal route (Fig 6.3). In TFV and UC781 gel groups, infection was detectable by qRT-PCR early on, from week one post-challenge, and similar trend was seen in the placebo group, where positive viral load could be seen from week three post-challenge. The only mouse that was infected in the TFV+UC781 group showed positive viremia later in the study, at week seven post-challenge, indicating a potential barrier to founder population establishment and initial expansion (possibly at the RT step in the viral cycle), although complete protection from the mucosal challenge was not achieved in this mouse (Fig 6.3).



* Additional time point tested for the only infected mouse in the combinatorial TFV+UC-781 gel group and for second infected mouse for UC781 gel (first positive at week 9), to confirm infection – additional time point shown at week 11 on the graph

Figure 6.3. qRT-PCR viral RNA load analysis. Limit of detection of the qRT-PCR assay shown as a dotted line (400 copies/ml).

In addition to positive viral load by qRT-PCR, infected RAG-hu mice exhibited CD4 T cell depletion, the hallmark of the human HIV-1 infection (Fig 6.4). The CD4 T cell counts were mostly stable up to week four post-challenge, and exhibited the pronounced decrease in infected mice from week four to week six analysis (Fig 6.4). The exception was one of the two infected mice in the UC781 gel group, which showed infection from week one (positive viral load) and the pronounced CD4 depletion in this mouse was seen from week two post-challenge (Fig 6.4). The only infected mouse in the combinatorial TFV+UC781 gel group showed T cell

depletion at week 6, which was followed by detection of HIV-1 viral RNA in this mouse at week 7, as the viral breakthrough was complete (Fig 6.3, Fig 6.4).

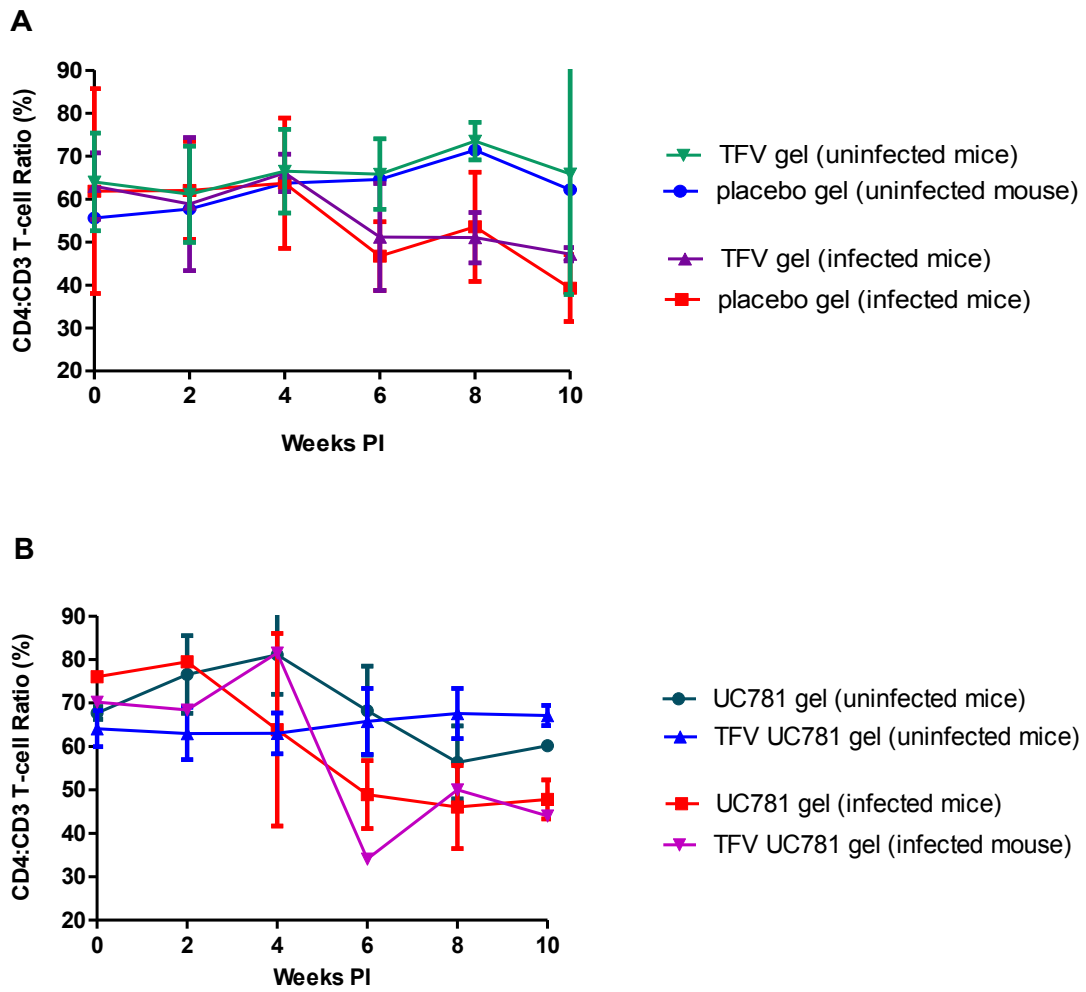


Figure 6.4. CD4 T cell flow cytometry analysis. CD4:CD3 T cell ratio (%) is shown. A. Tenofovir gel treated mice, placebo gel treated mice; B. UC781 gel treated mice, UC781+TFV combinatorial gel treated mice.

Study 2. TFV and RAL topical pre-exposure prophylaxis (PrEP) against HIV-1 Bal intravaginal challenge

In the second study TFV was combined with the integrase inhibitor RAL, and gel application and challenge was done by following the same protocols as in the TFV+UC781 study. Combinatorial TFV+RAL gel showed high level of protection, with only one out of eight animals becoming infected in this group (Fig 6.5). The single TFV and RAL gel formulations showed partial protection, with 3/8 and 3/9 animals becoming infected, respectively (Fig 6.5). All animals in the placebo group (7/7) became infected following intravaginal HIV-1 Bal challenge (Fig 6.5).

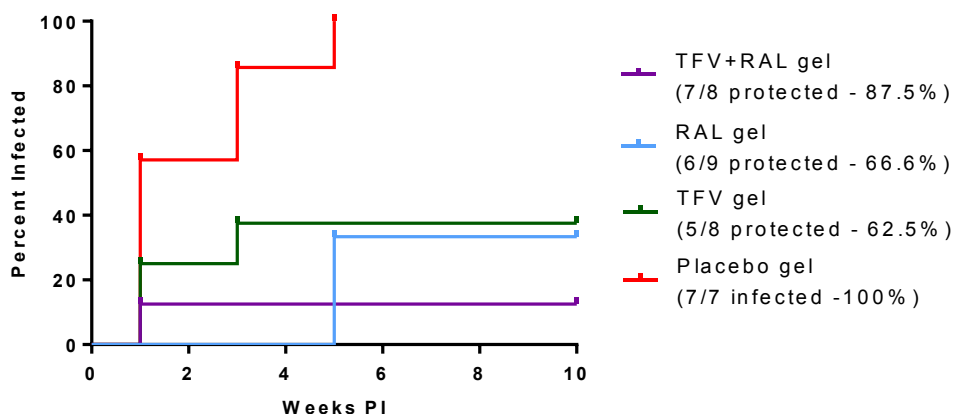


Figure 6.5. Kaplan-Meier plot of percent infected mice over the 10 week course of the experiment.

Infected mice in all groups showed sustained viral loads between 10^3 and 10^4 viral RNA copies per ml of plasma, while placebo mice showed even higher (10^4 - 10^6) levels of viremia in the first three weeks post-challenge (Fig 6.6).

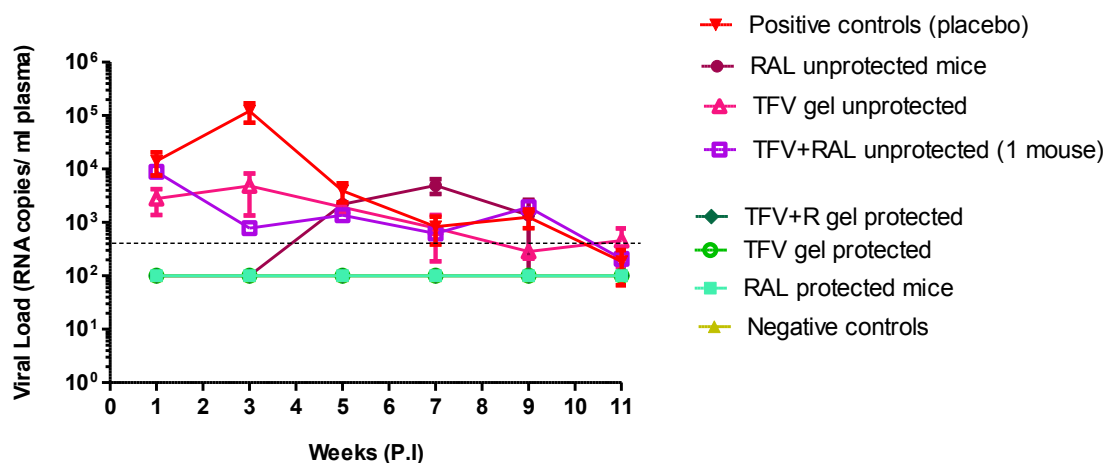


Figure 6.6. qRT-PCR viral RNA load analysis. Limit of detection of the qRT-PCR assay shown as a dotted line (400 copies/ml).

In addition to positive viral load, infected mice exhibited CD4 T cell depletion in the later phase of the study, with the onset of pronounced depletion at week four or week six post-challenge in majority of mice (Fig 6.7). The only infected mouse in the combinatorial TFV+RAL microbicide group showed early pronounced depletion by week four, after which CD4 levels stabilized around 45% level (Fig 6.7).

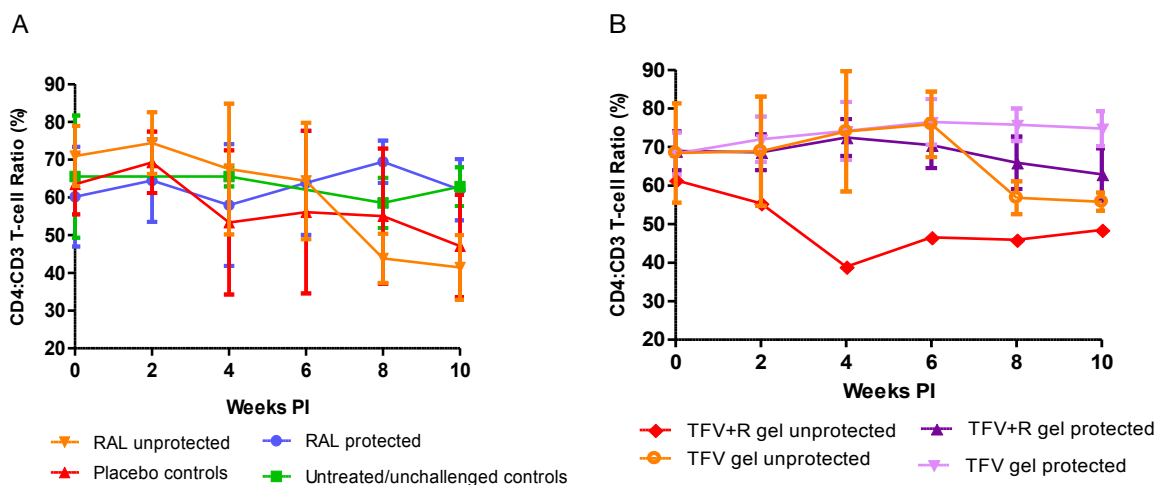


Figure 6.7. CD4 T cell flow cytometry analysis. CD4:CD3 T cell ratio (%) shown. A. Raltegravir gel treated mice, placebo gel treated mice; B. TFV gel treated mice, TFV+RAL combinatorial gel treated mice.

6.2.4. Discussion

Humanized mouse models have been applied in multiple protection studies of different anti-HIV-1 strategies, including oral and topical application of antiretrovirals [167, 173, 178, 197]. In the present study RAG-hu mouse model was utilized to analyze the level of protection offered against vaginal mucosal HIV-1 transmission when microbicide formulations of TFV, UC781 and RAL were applied intravaginally one hour prior to viral challenge. Thus, microbicide gels with 1% TFV, 0.25% UC781 and 5mM RAL were analyzed as potential PrEP agents against CCR5-tropic HIV-1, mimicking mucosal, sexual transmission in humans.

Combinatorial TFV+UC781 and TFV+RAL gels showed high level of protection, while single formulations also showed significant protection against mucosal challenge, confirming in the humanized mouse model the validity of anti-HIV-1 microbicide PrEP approach. The levels of protection offered by TFV 1% gel corresponded well to the previous human trial, with the caveats of this being a single high dose challenge and human exposure being more in the form of series of multiple low-level exposures to HIV-1 [234]. The UC781 RT inhibitor also showed good potential for topical PrEP in this first *in vivo* study ever reported. Moreover, the combination of these two RT inhibitors showed potential agonistic or even synergistic effect in the protection offered against HIV-1. Future PK studies of combinatorial TFV/UC781 microbicide gel would allow for protection levels seen here to be connected to drug concentrations achieved in vaginal mucosa and such initial study for the single drug TFV 1% gel has already been performed in humanized mice (part 1 of this chapter).

The third drug tested here in microbicide form, the integrase inhibitor RAL, has many characteristics of a favorable PrEP agent. Following RAL oral application in humans higher exposure is seen in cervicovaginal fluid (CVF) and vaginal tissue compared to systemic exposure, and this trend was also confirmed in a recent RAG-hu mouse study shown here [334,

338]. The data for topical application of RAL is sparse but a recent NHP study has shown promising results as 1% RAL gel was protective in 5/6 macaques even when applied three hours post-SHIV-1 challenge [355]. In addition, the kinetics of RAL is highly variable compared to other ARVs following oral application, which could impede its PrEP application potential [321, 325]. Microbicide gel RAL formulation, which skips highly variable metabolic steps through which orally applied drug has to go through, would potentially reduce PK variability and show more uniform exposure in vaginal mucosa, an aspect that would be favorable for a future PrEP application of RAL. The synergy in the antiviral effect was also seen for RAL and TFV combination, suggesting that, as in therapy, in the successful preventative approach virus needs to be attacked on multiple levels [237, 360, 361].

Optimizing current PrEP approaches has been a challenge, and even though successful HIV-1 prevention has been demonstrated in recent clinical trials, some of them actually show conflicting results [87]. Major factors contributing to such variations are thought to be low patient compliance (adherence) in some studies and the lack of understanding of mucosal exposure kinetics of ARVs [236-238]. The microbicide approach to HIV-1 PrEP could help resolve both of those obstacles. Increased patient adherence could be achieved with more “user-friendly” long lasting formulations for topical application, such as vaginal rings, as opposed to daily oral PrEP, for which reduced adherence over time has already been shown [229, 236, 243]. The recent proof of concept study in NHP shows favorable kinetics in vaginal tissue for IVR containing TFV, FTC and MVC, implicating the efficiency of the combinatorial PrEP approach used also in our studies [362]. In addition, new generation drug nanoparticle formulations are also being developed for less frequent topical application [243]. For testing such new approaches humanized mice could be applied as an animal model complementary to NHP in preclinical trials and the work shown here is a proof of concept for such studies in the future. The lack of knowledge regarding mucosal levels that are protective can also be addressed in carefully planned multidose and time course studies in humanized mouse model and correlated with

protection against HIV-1, in the studies such as one presented here. As the protective concentrations in human mucosa still need to be determined, in order to inform for optimal PrEP dosing, small animal studies could help accelerate such efforts in the field. Data shown here demonstrates utility of RAG-hu mice for mucosal transmission studies and assessment of ARV microbicide formulations.

Most importantly, this study reports on the high level of protection offered by combinatorial microbicide gel formulations against vaginal HIV-1 challenge, advising for the advancement of these and similar gels in further preclinical trials. Current combinatorial gels potentially being advanced to clinical trials include TFV plus emtricitabine (FTC) and TFV plus dapivirine, and potent protection seen in this study against high dose HIV-1 challenge suggests that combinatorial TFV/RAL and TFV/UC781 gels could be the next candidates in the pipeline for topical PrEP.

CHAPTER 7

Summary and Future Directions

7.1 Summary and future directions

The last decade in the fight against HIV-1 has been marked with the reduction of global incidence of HIV-1, as well as the improvement in patient survival and longer life-span due to the introduction of combination antiretroviral therapy (cART). Nevertheless, with the HIV-1 vaccine still not available, efficient preventative approaches are lacking. In addition, the most vulnerable populations in the global pandemic and the ones with the highest prevalence rate despite the introduction of cART do not have adequate means of protection against sexual transmission of HIV-1, which remains by far the dominant route of viral spread globally. The new approach to combating the pandemic is pre-exposure prophylaxis (PrEP), which implies application of different antiretroviral agents for the prevention of HIV-1 infection. If successful, PrEP would address the currently existing gaps in protection efforts against HIV-1. Two main forms of PrEP are currently being developed. Oral PrEP is the first approach, in which existing therapeutics would be utilized with adjustments needed for their preventative application. The second approach, called topical PrEP, is the application of antiretrovirals or other agents such as broadly neutralizing anti-HIV-1 antibodies (bnAb) at the mucosal sites of HIV-1 transmission. The establishment of correlation between concentrations achieved at sites of transmission (pharmacokinetics, PK) and the level of protection offered against mucosal HIV-1 infection (pharmacodynamics, PD) is crucial for the success of future PrEP strategies. Therefore, as PrEP is being developed, it requires mucosal PK studies to be conducted in parallel with protection PD studies.

In the studies presented here different approaches to HIV-1 PrEP modeling in humanized mice were tested. The topical protection studies included intravaginal gel application

(microbicide approach) of bnAb VRC01 and antiretrovirals (ARVs) Tenofovir (TFV), Raltegravir (RAL) and UC781. The pharmacokinetic studies included analysis of TFV, RAL and Maraviroc (MVC) following oral application and following topical (intravaginal) application. Additional pharmacokinetic analysis was done to analyze ARV kinetics following the combinatorial dual drug TFV+RAL, TFV+MVC and RAL+MVC oral application.

In the PD study shown here second generation bnAb VRC01 offered high level of protection against mucosal challenge with HIV-1, thus demonstrating the potential of this bnAb for further development as a topical agent. Furthermore, the combinatorial gel approach with a cocktail of four first generation bnAb targeting different epitopes on the HIV-1 envelope offered complete protection against HIV-1 vaginal challenge. To expand further this PrEP strategy testing, multiple highly potent new generation antibodies, such as PG or PGT bnAb, directed against conformational epitopes in the gp120 variable loops and Env glycans, could be combined with the CD binding site (CD4bs) directed bnAb, such as VRC01, and gp41 directed bnAb, such as 2F5 and 4E10. The goal would be achieving complete protection against CCR5 tropic HIV-1 mucosal transmission. The protection potential of these bnAb combinatorial gels could be tested further against resistant variants for which transmission rates are increasing in the field. In addition, the escape mutants resistant to currently used antiretrovirals could be tested against bnAb mixes, to see if bnAb can protect against these viruses. This line of study would offer valuable data on PrEP potential but also on therapeutic potential of such bnAb approaches.

In another set of PD studies combinatorial TFV+RAL and TFV+UC781 gels offered higher level of protection compared to the previous single 1% TFV gel studies in humanized mice, NHP and in human clinical trial CAPRISA004. The data obtained are promising for further exploration of the combinatorial topical PrEP approach. Future studies would include testing of other combinations of highly active retrovirals which target early stages in the viral cycle and testing against new resistant variants and drug-resistant mutants emerging in the field. Further

studies could also be aimed at defining the duration of the protection offered and in that respect newer long-lasting, depot or nano-formulated drug gels could be analyzed in the humanized mouse model.

The tissue kinetics of anti-HIV-1 drugs TFV, RAL and MVC following oral application exhibited high degree of analogy to the PK data seen in previous human studies. Importantly, the PrEP potential of these ARVs could be assessed in RAG-hu mice. The assessments were made by using the same set of criteria currently applied for human PrEP candidate evaluation. The ARV $C_{\max}$ and half-lives in mucosal tissues informed on tissue saturation and retention over time and the AUC_{24h} tissue to plasma ratios informed regarding local versus systemic exposure over the studied 24h period. Similar analysis was done for the combinatorial oral PrEP with TFV, RAL and MVC in dual application. These comprehensive reports on mucosal kinetics for co-administration of TFV, RAL and MVC represent the first analysis of tissue PK in the context of PrEP, as all previous co-administration studies followed only blood plasma levels for therapeutic interactions. The data obtained showed the enhancement of TFV-DP in rectal and intestinal tissue when TFV was applied with RAL or MVC. This observation, together with the lack of antagonistic effects in the TFV/MVC and TFV/RAL treatments, is highly encouraging for future PrEP applications. Additionally, in the small scale, initial topical PK study for TFV, RAL and MVC we were able to detect high levels of ARVs in the vaginal mucosa of RAG-hu mice. The PK data was comparable to currently available information on human TFV and TFV-DP levels in vaginal mucosa following gel application. Importantly, as seen in human trials, in the RAG-hu mice topical application resulted in tissue saturations several logs higher compared to oral application, validating the model for future comparison of topical vs. oral PrEP approach for new drug candidates.

Overall, the RAG-hu mouse model was found to be relevant and suitable for future PK-PD evaluation of novel topical and oral PrEP strategies, which could include anti-HIV-1 retrovirals, bnAb against HIV-1 or combinations of the two. Future studies will include the

analysis of tissue drug transporters, drug conversion enzymes and elimination metabolism comparison between human and humanized background, with the aim of developing new generation humanized mouse models. The ultimate goal is the complete humanization of drug metabolism and immune response to HIV-1 for translational PK-PD studies. The completed and ongoing proof of concept studies in our RAG-hu mice hope to contribute to the development of these new models, which will enable HIV-1 PrEP evaluation in the context of human immune system and human liver mimicking to the full extent drug exposure, drug metabolism and drug efficacy against HIV-1 in humans.

REFERENCES

1. Centers for Disease, C., *Pneumocystis pneumonia*--Los Angeles. MMWR Morb Mortal Wkly Rep, 1981. **30**(21): p. 250-2.
2. Ammann, A.J., et al., *Acquired immunodeficiency in an infant: possible transmission by means of blood products*. Lancet, 1983. **1**(8331): p. 956-8.
3. Groopman, J.E., et al., *Virologic studies in a case of transfusion-associated AIDS*. N Engl J Med, 1984. **311**(22): p. 1419-22.
4. Peterman, T.A., D.P. Drotman, and J.W. Curran, *Epidemiology of the acquired immunodeficiency syndrome (AIDS)*. Epidemiol Rev, 1985. **7**: p. 1-21.
5. Curran, J.W., et al., *Epidemiological trends of AIDS in the United States*. Cancer Res, 1985. **45**(9 Suppl): p. 4602s-4604s.
6. Ziegler, J.B., et al., *Postnatal transmission of AIDS-associated retrovirus from mother to infant*. Lancet, 1985. **1**(8434): p. 896-8.
7. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)*. Science, 1983. **220**(4599): p. 868-71.
8. Hahn, B.H., et al., *Molecular cloning and characterization of the HTLV-III virus associated with AIDS*. Nature, 1984. **312**(5990): p. 166-9.
9. Popovic, M., et al., *Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS*. Science, 1984. **224**(4648): p. 497-500.
10. Sarngadharan, M.G., et al., *Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS*. Science, 1984. **224**(4648): p. 506-8.
11. Schupbach, J., et al., *Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS*. Science, 1984. **224**(4648): p. 503-5.
12. Shaw, G.M., et al., *Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome*. Science, 1984. **226**(4679): p. 1165-71.
13. Levy, J.A., et al., *Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS*. Science, 1984. **225**(4664): p. 840-2.
14. Wain-Hobson, S., et al., *Nucleotide sequence of the AIDS virus, LAV*. Cell, 1985. **40**(1): p. 9-17.
15. Allan, J.S., et al., *Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III*. Science, 1985. **228**(4703): p. 1091-4.
16. Dalgleish, A.G., et al., *The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus*. Nature, 1984. **312**(5996): p. 763-7.
17. Klatzmann, D., et al., *T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV*. Nature, 1984. **312**(5996): p. 767-8.
18. Centers for Disease, C., *Classification system for human T-lymphotropic virus type III/lymphadenopathy-associated virus infections*. MMWR Morb Mortal Wkly Rep, 1986. **35**(20): p. 334-9.
19. Yarchoan, R. and S. Broder, *Development of antiretroviral therapy for the acquired immunodeficiency syndrome and related disorders. A progress report*. N Engl J Med, 1987. **316**(9): p. 557-64.
20. Gallo, D., et al., *Comparison of detection of antibody to the acquired immune deficiency syndrome virus by enzyme immunoassay, immunofluorescence, and Western blot methods*. J Clin Microbiol, 1986. **23**(6): p. 1049-51.
21. Thorpe, R., et al., *An improved immunoblotting procedure for the detection of antibodies against HIV*. J Virol Methods, 1987. **16**(1-2): p. 87-96.

22. Landesman, S.H., H.M. Ginzburg, and S.H. Weiss, *The AIDS epidemic*. N Engl J Med, 1985. **312**(8): p. 521-5.
23. Sharp, P.M., et al., *The origins of acquired immune deficiency syndrome viruses: where and when?* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1410): p. 867-76.
24. Gao, F., et al., *Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes*. Nature, 1999. **397**(6718): p. 436-41.
25. Wertheim, J.O. and M. Worobey, *Dating the age of the SIV lineages that gave rise to HIV-1 and HIV-2*. PLoS Comput Biol, 2009. **5**(5): p. e1000377.
26. Zhu, T., et al., *An African HIV-1 sequence from 1959 and implications for the origin of the epidemic*. Nature, 1998. **391**(6667): p. 594-7.
27. Worobey, M., et al., *Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960*. Nature, 2008. **455**(7213): p. 661-4.
28. Hemelaar, J., *The origin and diversity of the HIV-1 pandemic*. Trends Mol Med, 2012. **18**(3): p. 182-92.
29. Tebit, D.M. and E.J. Arts, *Tracking a century of global expansion and evolution of HIV to drive understanding and to combat disease*. Lancet Infect Dis, 2011. **11**(1): p. 45-56.
30. Mir, K.D., et al., *SIV infection in natural hosts: resolution of immune activation during the acute-to-chronic transition phase*. Microbes Infect, 2011. **13**(1): p. 14-24.
31. Etienne, L., et al., *Gene loss and adaptation to hominids underlie the ancient origin of HIV-1*. Cell Host Microbe, 2013. **14**(1): p. 85-92.
32. Sharp, P.M. and B.H. Hahn, *Origins of HIV and the AIDS pandemic*. Cold Spring Harb Perspect Med, 2011. **1**(1): p. a006841.
33. Palm, A.A., et al., *Faster progression to AIDS and AIDS-related death among seroincident individuals infected with recombinant HIV-1 A3/CRF02_AG compared with sub-subtype A3*. J Infect Dis, 2014. **209**(5): p. 721-8.
34. Chiu, I.M., et al., *Nucleotide sequence evidence for relationship of AIDS retrovirus to lentiviruses*. Nature, 1985. **317**(6035): p. 366-8.
35. Wain-Hobson, S., M. Alizon, and L. Montagnier, *Relationship of AIDS to other retroviruses*. Nature, 1985. **313**(6005): p. 743.
36. Baltimore, D., *Expression of animal virus genomes*. Bacteriol Rev, 1971. **35**(3): p. 235-41.
37. Zhao, G., et al., *Mature HIV-1 capsid structure by cryo-electron microscopy and all-atom molecular dynamics*. Nature, 2013. **497**(7451): p. 643-6.
38. Bartesaghi, A., et al., *Prefusion structure of trimeric HIV-1 envelope glycoprotein determined by cryo-electron microscopy*. Nat Struct Mol Biol, 2013. **20**(12): p. 1352-7.
39. Merk, A. and S. Subramaniam, *HIV-1 envelope glycoprotein structure*. Curr Opin Struct Biol, 2013. **23**(2): p. 268-76.
40. Totrov, M., *Estimated secondary structure propensities within V1/V2 region of HIV gp120 are an important global antibody neutralization sensitivity determinant*. PLoS One, 2014. **9**(4): p. e94002.
41. Hwang, S.S., et al., *Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1*. Science, 1991. **253**(5015): p. 71-4.
42. Muesing, M.A., et al., *Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus*. Nature, 1985. **313**(6002): p. 450-8.
43. Gallo, R., et al., *HIV/HTLV gene nomenclature*. Nature, 1988. **333**(6173): p. 504.
44. Starcich, B., et al., *Characterization of long terminal repeat sequences of HTLV-III*. Science, 1985. **227**(4686): p. 538-40.
45. Bryant, M. and L. Ratner, *Myristoylation-dependent replication and assembly of human immunodeficiency virus 1*. Proc Natl Acad Sci U S A, 1990. **87**(2): p. 523-7.

46. Gottlinger, H.G., J.G. Sodroski, and W.A. Haseltine, *Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1*. Proc Natl Acad Sci U S A, 1989. **86**(15): p. 5781-5.
47. Gallay, P., et al., *HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase*. Cell, 1995. **83**(4): p. 569-76.
48. Gallay, P., et al., *HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator*. Cell, 1995. **80**(3): p. 379-88.
49. Harrison, G.P. and A.M. Lever, *The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure*. J Virol, 1992. **66**(7): p. 4144-53.
50. Lapadat-Tapolsky, M., et al., *Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle*. Nucleic Acids Res, 1993. **21**(4): p. 831-9.
51. Paxton, W., R.I. Connor, and N.R. Landau, *Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis*. J Virol, 1993. **67**(12): p. 7229-37.
52. Parkin, N.T., M. Chamorro, and H.E. Varmus, *Human immunodeficiency virus type 1 gag-pol frameshifting is dependent on downstream mRNA secondary structure: demonstration by expression in vivo*. J Virol, 1992. **66**(8): p. 5147-51.
53. Roberts, J.D., K. Bebenek, and T.A. Kunkel, *The accuracy of reverse transcriptase from HIV-1*. Science, 1988. **242**(4882): p. 1171-3.
54. Krishnan, L. and A. Engelman, *Retroviral integrase proteins and HIV-1 DNA integration*. J Biol Chem, 2012. **287**(49): p. 40858-66.
55. Checkley, M.A., B.G. Luttge, and E.O. Freed, *HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation*. J Mol Biol, 2011. **410**(4): p. 582-608.
56. Karn, J. and C.M. Stoltzfus, *Transcriptional and posttranscriptional regulation of HIV-1 gene expression*. Cold Spring Harb Perspect Med, 2012. **2**(2): p. a006916.
57. Feng, S. and E.C. Holland, *HIV-1 tat trans-activation requires the loop sequence within tar*. Nature, 1988. **334**(6178): p. 165-7.
58. Strebel, K., *HIV accessory proteins versus host restriction factors*. Curr Opin Virol, 2013. **3**(6): p. 692-9.
59. Malim, M.H. and P.D. Bieniasz, *HIV Restriction Factors and Mechanisms of Evasion*. Cold Spring Harb Perspect Med, 2012. **2**(5): p. a006940.
60. Gramberg, T., N. Sunseri, and N.R. Landau, *Accessories to the crime: recent advances in HIV accessory protein biology*. Curr HIV/AIDS Rep, 2009. **6**(1): p. 36-42.
61. Basmaciogullari, S. and M. Pizzato, *The activity of Nef on HIV-1 infectivity*. Front Microbiol, 2014. **5**: p. 232.
62. Zaunders, J., W.B. Dyer, and M. Churchill, *The Sydney Blood Bank Cohort: implications for viral fitness as a cause of elite control*. Curr Opin HIV AIDS, 2011. **6**(3): p. 151-6.
63. Guenzel, C.A., C. Herate, and S. Benichou, *HIV-1 Vpr-a still "enigmatic multitasker"*. Front Microbiol, 2014. **5**: p. 127.
64. Strebel, K., *HIV-1 Vpu - an ion channel in search of a job*. Biochim Biophys Acta, 2014. **1838**(4): p. 1074-81.
65. Desimmie, B.A., et al., *Multiple APOBEC3 restriction factors for HIV-1 and one Vif to rule them all*. J Mol Biol, 2014. **426**(6): p. 1220-45.
66. Leblanc, J., J. Weil, and K. Beemon, *Posttranscriptional regulation of retroviral gene expression: primary RNA transcripts play three roles as pre-mRNA, mRNA, and genomic RNA*. Wiley Interdiscip Rev RNA, 2013. **4**(5): p. 567-80.
67. Kuzembayeva, M., et al., *Life of psi: how full-length HIV-1 RNAs become packaged genomes in the viral particles*. Virology, 2014. **454-455**: p. 362-70.

68. Maddon, P.J., et al., *The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain*. Cell, 1986. **47**(3): p. 333-48.
69. McDougal, J.S., et al., *The T4 glycoprotein is a cell-surface receptor for the AIDS virus*. Cold Spring Harb Symp Quant Biol, 1986. **51 Pt 2**: p. 703-11.
70. Borsetti, A., et al., *CD4-independent infection of two CD4(-)/CCR5(-)/CXCR4(+) pre-T-cell lines by human and simian immunodeficiency viruses*. J Virol, 2000. **74**(14): p. 6689-94.
71. Saha, K., et al., *Isolation of primary HIV-1 that target CD8+ T lymphocytes using CD8 as a receptor*. Nat Med, 2001. **7**(1): p. 65-72.
72. Choe, H., et al., *The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates*. Cell, 1996. **85**(7): p. 1135-48.
73. Trkola, A., et al., *CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5*. Nature, 1996. **384**(6605): p. 184-7.
74. Dragic, T., et al., *HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5*. Nature, 1996. **381**(6584): p. 667-73.
75. Tersmette, M., et al., *Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates*. J Virol, 1989. **63**(5): p. 2118-25.
76. Eckert, D.M. and P.S. Kim, *Mechanisms of viral membrane fusion and its inhibition*. Annu Rev Biochem, 2001. **70**: p. 777-810.
77. Schiffner, T., Q.J. Sattentau, and C.J. Duncan, *Cell-to-cell spread of HIV-1 and evasion of neutralizing antibodies*. Vaccine, 2013. **31**(49): p. 5789-97.
78. Fouchier, R.A. and M.H. Malim, *Nuclear import of human immunodeficiency virus type-1 preintegration complexes*. Adv Virus Res, 1999. **52**: p. 275-99.
79. Gaudin, R., et al., *HIV trafficking in host cells: motors wanted!* Trends Cell Biol, 2013. **23**(12): p. 652-62.
80. Christ, F. and Z. Debyser, *The LEDGF/p75 integrase interaction, a novel target for anti-HIV therapy*. Virology, 2013. **435**(1): p. 102-9.
81. Li, M., et al., *Engineered Hyperactive Integrase for Concerted HIV-1 DNA Integration*. PLoS One, 2014. **9**(8): p. e105078.
82. Sloan, R.D. and M.A. Wainberg, *The role of unintegrated DNA in HIV infection*. Retrovirology, 2011. **8**: p. 52.
83. Donahue, D.A. and M.A. Wainberg, *Cellular and molecular mechanisms involved in the establishment of HIV-1 latency*. Retrovirology, 2013. **10**: p. 11.
84. Meng, B. and A.M. Lever, *Wrapping up the bad news: HIV assembly and release*. Retrovirology, 2013. **10**: p. 5.
85. Votteler, J. and W.I. Sundquist, *Virus budding and the ESCRT pathway*. Cell Host Microbe, 2013. **14**(3): p. 232-41.
86. Roy, N., et al., *Mechanisms underlying HIV-1 Vpu-mediated viral egress*. Front Microbiol, 2014. **5**: p. 177.
87. Maartens, G., C. Celum, and S.R. Lewin, *HIV infection: epidemiology, pathogenesis, treatment, and prevention*. Lancet, 2014. **384**(9939): p. 258-71.
88. Pope, M. and A.T. Haase, *Transmission, acute HIV-1 infection and the quest for strategies to prevent infection*. Nat Med, 2003. **9**(7): p. 847-52.
89. Boily, M.C., et al., *Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies*. Lancet Infect Dis, 2009. **9**(2): p. 118-29.
90. Quinn, T.C., et al., *Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group*. N Engl J Med, 2000. **342**(13): p. 921-9.
91. Hladik, F. and M.J. McElrath, *Setting the stage: host invasion by HIV*. Nat Rev Immunol, 2008. **8**(6): p. 447-57.

92. Polis, C.B. and K.M. Curtis, *Use of hormonal contraceptives and HIV acquisition in women: a systematic review of the epidemiological evidence*. Lancet Infect Dis, 2013. **13**(9): p. 797-808.
93. Evans, D.T. and G. Silvestri, *Nonhuman primate models in AIDS research*. Curr Opin HIV AIDS, 2013. **8**(4): p. 255-61.
94. Fennessey, C.M. and B.F. Keele, *Using nonhuman primates to model HIV transmission*. Curr Opin HIV AIDS, 2013. **8**(4): p. 280-7.
95. Mauck, C.K., et al., *Toward early safety alert endpoints: exploring biomarkers suggestive of microbicide failure*. AIDS Res Hum Retroviruses, 2013. **29**(11): p. 1475-86.
96. Dezzutti, C.S., et al., *HIV-1 infection of female genital tract tissue for use in prevention studies*. J Acquir Immune Defic Syndr, 2013. **63**(5): p. 548-54.
97. Dezzutti, C.S. and F. Hladik, *Use of human mucosal tissue to study HIV-1 pathogenesis and evaluate HIV-1 prevention modalities*. Curr HIV/AIDS Rep, 2013. **10**(1): p. 12-20.
98. Zhang, Z., et al., *Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells*. Science, 1999. **286**(5443): p. 1353-7.
99. Hladik, F., et al., *Initial events in establishing vaginal entry and infection by human immunodeficiency virus type-1*. Immunity, 2007. **26**(2): p. 257-70.
100. Arthos, J., et al., *HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells*. Nat Immunol, 2008. **9**(3): p. 301-9.
101. Piguet, V. and R.M. Steinman, *The interaction of HIV with dendritic cells: outcomes and pathways*. Trends Immunol, 2007. **28**(11): p. 503-10.
102. Murooka, T.T., et al., *HIV-infected T cells are migratory vehicles for viral dissemination*. Nature, 2012. **490**(7419): p. 283-7.
103. Shaw, G.M. and E. Hunter, *HIV transmission*. Cold Spring Harb Perspect Med, 2012. **2**(11).
104. Guadalupe, M., et al., *Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy*. J Virol, 2003. **77**(21): p. 11708-17.
105. Brechley, J.M., et al., *CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract*. J Exp Med, 2004. **200**(6): p. 749-59.
106. Mehandru, S., et al., *Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract*. J Exp Med, 2004. **200**(6): p. 761-70.
107. Keele, B.F., et al., *Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection*. Proc Natl Acad Sci U S A, 2008. **105**(21): p. 7552-7.
108. Little, S.J., et al., *Viral dynamics of acute HIV-1 infection*. J Exp Med, 1999. **190**(6): p. 841-50.
109. Mellors, J.W., et al., *Prognosis in HIV-1 infection predicted by the quantity of virus in plasma*. Science, 1996. **272**(5265): p. 1167-70.
110. Ruelas, D.S. and W.C. Greene, *An integrated overview of HIV-1 latency*. Cell, 2013. **155**(3): p. 519-29.
111. Barouch, D.H. and S.G. Deeks, *Immunologic strategies for HIV-1 remission and eradication*. Science, 2014. **345**(6193): p. 169-74.
112. Mosier, D.E., *HIV-1 envelope evolution and vaccine efficacy*. Curr Drug Targets Infect Disord, 2005. **5**(2): p. 171-7.
113. Boutwell, C.L., et al., *Viral evolution and escape during acute HIV-1 infection*. J Infect Dis, 2010. **202** Suppl 2: p. S309-14.

114. Perreau, M., Y. Levy, and G. Pantaleo, *Immune response to HIV*. Curr Opin HIV AIDS, 2013. **8**(4): p. 333-40.
115. Mascola, J.R. and B.F. Haynes, *HIV-1 neutralizing antibodies: understanding nature's pathways*. Immunol Rev, 2013. **254**(1): p. 225-44.
116. Kwong, P.D., J.R. Mascola, and G.J. Nabel, *Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning*. Nat Rev Immunol, 2013. **13**(9): p. 693-701.
117. van Gils, M.J. and R.W. Sanders, *Broadly neutralizing antibodies against HIV-1: templates for a vaccine*. Virology, 2013. **435**(1): p. 46-56.
118. Gao, F., et al., *Cooperation of B Cell Lineages in Induction of HIV-1-Broadly Neutralizing Antibodies*. Cell, 2014. **158**(3): p. 481-91.
119. Balazs, A.B., et al., *Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission*. Nat Med, 2014. **20**(3): p. 296-300.
120. Balazs, A.B., et al., *Antibody-based protection against HIV infection by vectored immunoprophylaxis*. Nature, 2012. **481**(7379): p. 81-4.
121. Hessel, A.J., et al., *Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques*. Nat Med, 2009. **15**(8): p. 951-4.
122. Hessel, A.J., et al., *Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers*. PLoS Pathog, 2009. **5**(5): p. e1000433.
123. Hessel, A.J., et al., *Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L*. J Virol, 2010. **84**(3): p. 1302-13.
124. Veselinovic, M., et al., *Topical gel formulation of broadly neutralizing anti-HIV-1 monoclonal antibody VRC01 confers protection against HIV-1 vaginal challenge in a humanized mouse model*. Virology, 2012. **432**(2): p. 505-10.
125. Stoddart, C.A., et al., *Efficacy of broadly neutralizing monoclonal antibody PG16 in HIV-infected humanized mice*. Virology, 2014. **462-463**: p. 115-25.
126. Halper-Stromberg, A., et al., *Broadly Neutralizing Antibodies and Viral Inducers Decrease Rebound from HIV-1 Latent Reservoirs in Humanized Mice*. Cell, 2014.
127. Fox, J.L., *HIV drugs made in tobacco*. Nat Biotechnol, 2011. **29**(10): p. 852.
128. Munier, C.M., et al., *The role of T cell immunity in HIV-1 infection*. Curr Opin Virol, 2013. **3**(4): p. 438-46.
129. Fukazawa, Y., et al., *Lymph node T cell responses predict the efficacy of live attenuated SIV vaccines*. Nat Med, 2012. **18**(11): p. 1673-81.
130. Hansen, S.G., et al., *Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine*. Nature, 2011. **473**(7348): p. 523-7.
131. Lederman, M.M., et al., *Residual immune dysregulation syndrome in treated HIV infection*. Adv Immunol, 2013. **119**: p. 51-83.
132. Paiardini, M. and M. Muller-Trutwin, *HIV-associated chronic immune activation*. Immunol Rev, 2013. **254**(1): p. 78-101.
133. Coffin, J. and R. Swanstrom, *HIV pathogenesis: dynamics and genetics of viral populations and infected cells*. Cold Spring Harb Perspect Med, 2013. **3**(1): p. a012526.
134. Garg, H., J. Mohl, and A. Joshi, *HIV-1 induced bystander apoptosis*. Viruses, 2012. **4**(11): p. 3020-43.
135. Marchetti, G., C. Tincati, and G. Silvestri, *Microbial translocation in the pathogenesis of HIV infection and AIDS*. Clin Microbiol Rev, 2013. **26**(1): p. 2-18.
136. Hunt, P.W., *Th17, gut, and HIV: therapeutic implications*. Curr Opin HIV AIDS, 2010. **5**(2): p. 189-93.

137. Estes, J.D., *Pathobiology of HIV/SIV-associated changes in secondary lymphoid tissues*. Immunol Rev, 2013. **254**(1): p. 65-77.
138. Okoye, A.A. and L.J. Picker, *CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure*. Immunol Rev, 2013. **254**(1): p. 54-64.
139. Wood, K.L., H.L. Twigg, 3rd, and A.I. Doseff, *Dysregulation of CD8+ lymphocyte apoptosis, chronic disease, and immune regulation*. Front Biosci (Landmark Ed), 2009. **14**: p. 3771-81.
140. Moir, S. and A.S. Fauci, *Insights into B cells and HIV-specific B-cell responses in HIV-infected individuals*. Immunol Rev, 2013. **254**(1): p. 207-24.
141. Rustagi, A. and M. Gale, Jr., *Innate antiviral immune signaling, viral evasion and modulation by HIV-1*. J Mol Biol, 2014. **426**(6): p. 1161-77.
142. Landau, N.R., *The innate immune response to HIV-1: to sense or not to sense*. DNA Cell Biol, 2014. **33**(5): p. 271-4.
143. Cummins, N.W. and A.D. Badley, *Mechanisms of HIV-associated lymphocyte apoptosis: 2010*. Cell Death Dis, 2010. **1**: p. e99.
144. Kirschner, D., G.F. Webb, and M. Cloyd, *Model of HIV-1 disease progression based on virus-induced lymph node homing and homing-induced apoptosis of CD4+ lymphocytes*. J Acquir Immune Defic Syndr, 2000. **24**(4): p. 352-62.
145. Brainard, D.M., et al., *Decreased CXCR3+ CD8 T cells in advanced human immunodeficiency virus infection suggest that a homing defect contributes to cytotoxic T-lymphocyte dysfunction*. J Virol, 2007. **81**(16): p. 8439-50.
146. Porichis, F. and D.E. Kaufmann, *Role of PD-1 in HIV pathogenesis and as target for therapy*. Curr HIV/AIDS Rep, 2012. **9**(1): p. 81-90.
147. Fauci, A.S., *Pathogenesis of HIV disease: opportunities for new prevention interventions*. Clin Infect Dis, 2007. **45 Suppl 4**: p. S206-12.
148. Arts, E.J. and D.J. Hazuda, *HIV-1 antiretroviral drug therapy*. Cold Spring Harb Perspect Med, 2012. **2**(4): p. a007161.
149. Margolis, A.M., et al., *A review of the toxicity of HIV medications*. J Med Toxicol, 2014. **10**(1): p. 26-39.
150. Lake, J.E. and J.S. Currier, *Metabolic disease in HIV infection*. Lancet Infect Dis, 2013. **13**(11): p. 964-75.
151. Panel of experts from the Metabolic Disorders Study Group, N.A.P.A.S.G., *Executive summary of the consensus document on metabolic disorders and cardiovascular risk in patients with HIV infection*. Enferm Infecc Microbiol Clin, 2014.
152. Zapata, H.J. and A.C. Shaw, *Aging of the human innate immune system in HIV infection*. Curr Opin Immunol, 2014. **29C**: p. 127-136.
153. Pathai, S., et al., *Is HIV a model of accelerated or accentuated aging?* J Gerontol A Biol Sci Med Sci, 2014. **69**(7): p. 833-42.
154. Clifford, D.B. and B.M. Ances, *HIV-associated neurocognitive disorder*. Lancet Infect Dis, 2013. **13**(11): p. 976-86.
155. Meintjes, G., J. Scriven, and S. Marais, *Management of the immune reconstitution inflammatory syndrome*. Curr HIV/AIDS Rep, 2012. **9**(3): p. 238-50.
156. Chang, C.C., et al., *HIV and co-infections*. Immunol Rev, 2013. **254**(1): p. 114-42.
157. Archin, N.M. and D.M. Margolis, *Emerging strategies to deplete the HIV reservoir*. Curr Opin Infect Dis, 2014. **27**(1): p. 29-35.
158. Xing, S. and R.F. Siliciano, *Targeting HIV latency: pharmacologic strategies toward eradication*. Drug Discov Today, 2013. **18**(11-12): p. 541-51.
159. Boehm, D., et al., *BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism*. Cell Cycle, 2013. **12**(3): p. 452-62.
160. Sherrill-Mix, S., et al., *HIV latency and integration site placement in five cell-based models*. Retrovirology, 2013. **10**: p. 90.

161. Lusich, M. and M. Giacca, *Regulation of HIV-1 latency by chromatin structure and nuclear architecture*. J Mol Biol, 2014.
162. Garcia-Lerma, J.G. and W. Heneine, *Animal models of antiretroviral prophylaxis for HIV prevention*. Curr Opin HIV AIDS, 2012. **7**(6): p. 505-13.
163. McGary, C.S., G. Silvestri, and M. Paiardini, *Animal models for viral infection and cell exhaustion*. Curr Opin HIV AIDS, 2014. **9**(5): p. 492-9.
164. Barouch, D.H. and B. Korber, *HIV-1 vaccine development after STEP*. Annu Rev Med, 2010. **61**: p. 153-67.
165. Veazey, R.S., et al., *Animal models for microbicide studies*. Curr HIV Res, 2012. **10**(1): p. 79-87.
166. Hatzioannou, T. and D.T. Evans, *Animal models for HIV/AIDS research*. Nat Rev Microbiol, 2012. **10**(12): p. 852-67.
167. Akkina, R., *New generation humanized mice for virus research: comparative aspects and future prospects*. Virology, 2013. **435**(1): p. 14-28.
168. Nischang, M., et al., *Modeling HIV infection and therapies in humanized mice*. Swiss Med Wkly, 2012. **142**: p. w13618.
169. Denton, P.W. and J.V. Garcia, *Humanized mouse models of HIV infection*. AIDS Rev, 2011. **13**(3): p. 135-48.
170. Akkina, R., et al., *Humanized Rag1^{-/-} gammac^{-/-} mice support multilineage hematopoiesis and are susceptible to HIV-1 infection via systemic and vaginal routes*. PLoS One, 2011. **6**(6): p. e20169.
171. Berges, B.K., et al., *Mucosal transmission of R5 and X4 tropic HIV-1 via vaginal and rectal routes in humanized Rag2^{-/-} gammac^{-/-} (RAG-hu) mice*. Virology, 2008. **373**(2): p. 342-51.
172. Berges, B.K., et al., *Humanized Rag2^{-/-}gammac^{-/-} (RAG-hu) mice can sustain long-term chronic HIV-1 infection lasting more than a year*. Virology, 2010. **397**(1): p. 100-3.
173. Berges, B.K. and M.R. Rowan, *The utility of the new generation of humanized mice to study HIV-1 infection: transmission, prevention, pathogenesis, and treatment*. Retrovirology, 2011. **8**: p. 65.
174. Berges, B.K., et al., *HIV-1 infection and CD4 T cell depletion in the humanized Rag2^{-/-} gamma c^{-/-} (RAG-hu) mouse model*. Retrovirology, 2006. **3**: p. 76.
175. Sun, Z., et al., *Intrarectal transmission, systemic infection, and CD4⁺ T cell depletion in humanized mice infected with HIV-1*. J Exp Med, 2007. **204**(4): p. 705-14.
176. Chateau, M.L., et al., *Rectal transmission of transmitted/founder HIV-1 is efficiently prevented by topical 1% tenofovir in BLT humanized mice*. PLoS One, 2013. **8**(3): p. e60024.
177. Denton, P.W., et al., *Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice*. PLoS Med, 2008. **5**(1): p. e16.
178. Denton, P.W. and J.V. Garcia, *Mucosal HIV-1 transmission and prevention strategies in BLT humanized mice*. Trends in microbiology, 2012. **20**(6): p. 268-74.
179. Denton, P.W., et al., *One percent tenofovir applied topically to humanized BLT mice and used according to the CAPRISA 004 experimental design demonstrates partial protection from vaginal HIV infection, validating the BLT model for evaluation of new microbicide candidates*. Journal of virology, 2011. **85**(15): p. 7582-93.
180. Chiba, K., *Perspective of humanized mouse models for assessing PK/PD and toxic profile of drug candidates in preclinical study*. Drug Metab Pharmacokinet, 2014. **29**(1): p. 1-2.
181. Peltz, G., *Can 'humanized' mice improve drug development in the 21st century?* Trends Pharmacol Sci, 2013. **34**(5): p. 255-60.
182. Brehm, M.A., et al., *Generation of improved humanized mouse models for human infectious diseases*. J Immunol Methods, 2014.

183. Gonzalez, L., N. Strbo, and E.R. Podack, *Humanized mice: novel model for studying mechanisms of human immune-based therapies*. Immunol Res, 2013. **57**(1-3): p. 326-34.
184. Hofer, U., et al., *Pre-clinical modeling of CCR5 knockout in human hematopoietic stem cells by zinc finger nucleases using humanized mice*. J Infect Dis, 2013. **208 Suppl 2**: p. S160-4.
185. Murooka, T.T. and T.R. Mempel, *Intravital microscopy in BLT-humanized mice to study cellular dynamics in HIV infection*. J Infect Dis, 2013. **208 Suppl 2**: p. S137-44.
186. Chang, J.J. and M. Altfeld, *Immune activation and the role of TLRs and TLR agonists in the pathogenesis of HIV-1 infection in the humanized mouse model*. J Infect Dis, 2013. **208 Suppl 2**: p. S145-9.
187. Epstein, A.A., et al., *Combinatorial assessments of brain tissue metabolomics and histopathology in rodent models of human immunodeficiency virus infection*. J Neuroimmune Pharmacol, 2013. **8**(5): p. 1224-38.
188. Denton, P.W., et al., *Generation of HIV latency in humanized BLT mice*. J Virol, 2012. **86**(1): p. 630-4.
189. Marsden, M.D., et al., *HIV latency in the humanized BLT mouse*. J Virol, 2012. **86**(1): p. 339-47.
190. Neff, C.P., et al., *A topical microbicide gel formulation of CCR5 antagonist maraviroc prevents HIV-1 vaginal transmission in humanized RAG-hu mice*. PloS one, 2011. **6**(6): p. e20209.
191. Neff, C.P., et al., *Oral pre-exposure prophylaxis by anti-retrovirals raltegravir and maraviroc protects against HIV-1 vaginal transmission in a humanized mouse model*. PloS one, 2010. **5**(12): p. e15257.
192. Dash, P.K., et al., *Long-acting nanoformulated antiretroviral therapy elicits potent antiretroviral and neuroprotective responses in HIV-1-infected humanized mice*. AIDS, 2012. **26**(17): p. 2135-44.
193. Zhou, J., et al., *Systemic administration of combinatorial dsRNAs via nanoparticles efficiently suppresses HIV-1 infection in humanized mice*. Mol Ther, 2011. **19**(12): p. 2228-38.
194. Neff, C.P., et al., *An aptamer-siRNA chimera suppresses HIV-1 viral loads and protects from helper CD4(+) T cell decline in humanized mice*. Sci Transl Med, 2011. **3**(66): p. 66ra6.
195. Klein, F., et al., *HIV therapy by a combination of broadly neutralizing antibodies in humanized mice*. Nature, 2012. **492**(7427): p. 118-22.
196. Horwitz, J.A., et al., *HIV-1 suppression and durable control by combining single broadly neutralizing antibodies and antiretroviral drugs in humanized mice*. Proc Natl Acad Sci U S A, 2013. **110**(41): p. 16538-43.
197. Veazey, R.S., *Animal models for microbicide safety and efficacy testing*. Curr Opin HIV AIDS, 2013. **8**(4): p. 295-303.
198. Veselinovic, M., et al., *HIV pre-exposure prophylaxis: Mucosal tissue drug distribution of RT inhibitor Tenofovir and entry inhibitor Maraviroc in a humanized mouse model*. Virology, 2014. **464-465C**: p. 253-263.
199. Yoshizato, K. and C. Tateno, *A mouse with humanized liver as an animal model for predicting drug effects and for studying hepatic viral infection: where to next?* Expert Opin Drug Metab Toxicol, 2013. **9**(11): p. 1419-35.
200. Rathinam, C., et al., *Efficient differentiation and function of human macrophages in humanized CSF-1 mice*. Blood, 2011. **118**(11): p. 3119-28.
201. Strowig, T., et al., *Transgenic expression of human signal regulatory protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice*. Proc Natl Acad Sci U S A, 2011. **108**(32): p. 13218-23.

202. Pitisuttithum, P., et al., *Safety and reactogenicity of canarypox ALVAC-HIV (vCP1521) and HIV-1 gp120 AIDSVAX B/E vaccination in an efficacy trial in Thailand*. PLoS One, 2011. **6**(12): p. e27837.
203. Haynes, B.F., et al., *Progress in HIV-1 vaccine development*. J Allergy Clin Immunol, 2014. **134**(1): p. 3-10.
204. Greenspan, N.S., *Design Challenges for HIV-1 Vaccines Based on Humoral Immunity*. Front Immunol, 2014. **5**: p. 335.
205. Excler, J.L., M.L. Robb, and J.H. Kim, *HIV-1 vaccines: Challenges and new perspectives*. Hum Vaccin Immunother, 2014. **10**(6).
206. Su, B. and C. Moog, *Which Antibody Functions are Important for an HIV Vaccine?* Front Immunol, 2014. **5**: p. 289.
207. Chanzu, N. and B. Ondondo, *Induction of Potent and Long-Lived Antibody and Cellular Immune Responses in the Genitorectal Mucosa Could be the Critical Determinant of HIV Vaccine Efficacy*. Front Immunol, 2014. **5**: p. 202.
208. Sui, Y., et al., *Vaccine-induced myeloid cell population dampens protective immunity to SIV*. J Clin Invest, 2014. **124**(6): p. 2538-49.
209. Smith, P.L., H. Tanner, and A. Dalgleish, *Developments in HIV-1 immunotherapy and therapeutic vaccination*. F1000Prime Rep, 2014. **6**: p. 43.
210. Stephenson, K.E. and D.H. Barouch, *A global approach to HIV-1 vaccine development*. Immunol Rev, 2013. **254**(1): p. 295-304.
211. Excler, J.L., et al., *Nonneutralizing Functional Antibodies: a New "Old" Paradigm for HIV Vaccines*. Clin Vaccine Immunol, 2014. **21**(8): p. 1023-1036.
212. Parks, W.P., et al., *HIV-1 inhibition by azidothymidine in a concurrently randomized placebo-controlled trial*. J Acquir Immune Defic Syndr, 1988. **1**(2): p. 125-30.
213. Flexner, C. and M. Saag, *The antiretroviral drug pipeline: prospects and implications for future treatment research*. Curr Opin HIV AIDS, 2013. **8**(6): p. 572-8.
214. Wensing, A.M., et al., *2014 update of the drug resistance mutations in HIV-1*. Top Antivir Med, 2014. **22**(3): p. 642-50.
215. Johnson, V.A., et al., *Update of the drug resistance mutations in HIV-1: March 2013*. Top Antivir Med, 2013. **21**(1): p. 6-14.
216. Gandhi, M. and R.T. Gandhi, *Single-pill combination regimens for treatment of HIV-1 infection*. N Engl J Med, 2014. **371**(3): p. 248-59.
217. Organization, W.H., in *Consolidated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing HIV Infection: Recommendations for a Public Health Approach*. 2013: Geneva.
218. Walker, B.D. and M.S. Hirsch, *Antiretroviral therapy in early HIV infection*. N Engl J Med, 2013. **368**(3): p. 279-81.
219. Sabin, C.A., et al., *Rating evidence in treatment guidelines: a case example of when to initiate combination antiretroviral therapy (cART) in HIV-positive asymptomatic persons*. AIDS, 2013. **27**(12): p. 1839-46.
220. Rodger, A.J., et al., *Mortality in well controlled HIV in the continuous antiretroviral therapy arms of the SMART and ESPRIT trials compared with the general population*. AIDS, 2013. **27**(6): p. 973-9.
221. Chung, J., D.L. DiGiusto, and J.J. Rossi, *Combinatorial RNA-based gene therapy for the treatment of HIV/AIDS*. Expert Opin Biol Ther, 2013. **13**(3): p. 437-45.
222. Peterson, C.W., et al., *Combinatorial anti-HIV gene therapy: using a multipronged approach to reach beyond HAART*. Gene Ther, 2013. **20**(7): p. 695-702.
223. Hutter, G., et al., *Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation*. N Engl J Med, 2009. **360**(7): p. 692-8.
224. Wang, C. and Q. Zhou, *Systemic delivery of siRNA: challenging but promising*. Recent Pat Drug Deliv Formul, 2012. **6**(1): p. 19-30.

225. Tebas, P., et al., *Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV*. N Engl J Med, 2014. **370**(10): p. 901-10.
226. Kurth, A.E., et al., *Combination HIV prevention: significance, challenges, and opportunities*. Curr HIV/AIDS Rep, 2011. **8**(1): p. 62-72.
227. Padian, N.S., et al., *HIV prevention transformed: the new prevention research agenda*. Lancet, 2011. **378**(9787): p. 269-78.
228. Ortblad, K.F., R. Lozano, and C.J. Murray, *The burden of HIV: insights from the Global Burden of Disease Study 2010*. AIDS, 2013. **27**(13): p. 2003-17.
229. Adams, J.L. and A.D. Kashuba, *Formulation, pharmacokinetics and pharmacodynamics of topical microbicides*. Best Pract Res Clin Obstet Gynaecol, 2012. **26**(4): p. 451-62.
230. Romano, J., et al., *Pharmacokinetics and pharmacodynamics in HIV prevention; current status and future directions: a summary of the DAIDS and BMGF sponsored think tank on pharmacokinetics (PK)/pharmacodynamics (PD) in HIV prevention*. AIDS Res Hum Retroviruses, 2013. **29**(11): p. 1418-27.
231. Nicol, M.R. and A.D. Kashuba, *Pharmacologic opportunities for HIV prevention*. Clin Pharmacol Ther, 2010. **88**(5): p. 598-609.
232. Garcia-Lerma, J.G., et al., *Prevention of rectal SHIV transmission in macaques by daily or intermittent prophylaxis with emtricitabine and tenofovir*. PLoS Med, 2008. **5**(2): p. e28.
233. Parikh, U.M., et al., *Complete protection from repeated vaginal simian-human immunodeficiency virus exposures in macaques by a topical gel containing tenofovir alone or with emtricitabine*. J Virol, 2009. **83**(20): p. 10358-65.
234. Abdool Karim, Q., et al., *Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women*. Science, 2010. **329**(5996): p. 1168-74.
235. McEnery, R., *Oral tenofovir arm of VOICE trial discontinued early*. IAVI Rep, 2011. **15**(5): p. 21.
236. Gengiah, T.N., et al., *Adherence challenges with drugs for pre-exposure prophylaxis to prevent HIV infection*. Int J Clin Pharm, 2014. **36**(1): p. 70-85.
237. Cohen, M.S., et al., *Antiretroviral treatment of HIV-1 prevents transmission of HIV-1: where do we go from here?* Lancet, 2013. **382**(9903): p. 1515-24.
238. Cohen, M.S., et al., *Antiviral agents and HIV prevention: controversies, conflicts, and consensus*. AIDS, 2012. **26**(13): p. 1585-98.
239. Friend, D.R. and P.F. Kiser, *Assessment of topical microbicides to prevent HIV-1 transmission: concepts, testing, lessons learned*. Antiviral Res, 2013. **99**(3): p. 391-400.
240. Akil, A., et al., *Increased Dapivirine tissue accumulation through vaginal film codelivery of dapivirine and Tenofovir*. Mol Pharm, 2014. **11**(5): p. 1533-41.
241. Nel, A.M., et al., *Pharmacokinetic assessment of dapivirine vaginal microbicide gel in healthy, HIV-negative women*. AIDS Res Hum Retroviruses, 2010. **26**(11): p. 1181-90.
242. Nel, A.M., et al., *Pharmacokinetics of 2 dapivirine vaginal microbicide gels and their safety vs. Hydroxyethyl cellulose-based universal placebo gel*. J Acquir Immune Defic Syndr, 2010. **55**(2): p. 161-9.
243. Whaley, K.J., et al., *Novel approaches to vaginal delivery and safety of microbicides: biopharmaceuticals, nanoparticles, and vaccines*. Antiviral Res, 2010. **88 Suppl 1**: p. S55-66.
244. Friend, D.R. and G.F. Doncel, *Combining prevention of HIV-1, other sexually transmitted infections and unintended pregnancies: Development of dual-protection technologies*. Antiviral Res, 2010. **88 Suppl 1**: p. S47-54.
245. Karim, S.S., et al., *Drug concentrations after topical and oral antiretroviral pre-exposure prophylaxis: implications for HIV prevention in women*. Lancet, 2011. **378**(9787): p. 279-81.

246. Thompson, C.G., M.S. Cohen, and A.D. Kashuba, *Antiretroviral pharmacology in mucosal tissues*. J Acquir Immune Defic Syndr, 2013. **63 Suppl 2**: p. S240-7.
247. Smith, K., et al., *HIV-1 treatment as prevention: the good, the bad, and the challenges*. Curr Opin HIV AIDS, 2011. **6**(4): p. 315-25.
248. Grant, R.M., et al., *Preexposure chemoprophylaxis for HIV prevention in men who have sex with men*. N Engl J Med, 2010. **363**(27): p. 2587-99.
249. Thigpen, M.C., et al., *Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana*. N Engl J Med, 2012. **367**(5): p. 423-34.
250. Van Damme, L., et al., *Preexposure prophylaxis for HIV infection among African women*. N Engl J Med, 2012. **367**(5): p. 411-22.
251. Choopanya, K., et al., *Antiretroviral prophylaxis for HIV infection in injecting drug users in Bangkok, Thailand (the Bangkok Tenofovir Study): a randomised, double-blind, placebo-controlled phase 3 trial*. Lancet, 2013. **381**(9883): p. 2083-90.
252. Cohen, M.S., et al., *Prevention of HIV-1 infection with early antiretroviral therapy*. N Engl J Med, 2011. **365**(6): p. 493-505.
253. Baeten, J.M., et al., *Antiretroviral prophylaxis for HIV prevention in heterosexual men and women*. N Engl J Med, 2012. **367**(5): p. 399-410.
254. Kearney, B.P., J.F. Flaherty, and J. Shah, *Tenofovir disoproxil fumarate: clinical pharmacology and pharmacokinetics*. Clin Pharmacokinet, 2004. **43**(9): p. 595-612.
255. Patterson, K.B., et al., *Penetration of tenofovir and emtricitabine in mucosal tissues: implications for prevention of HIV-1 transmission*. Sci Transl Med, 2011. **3**(112): p. 112re4.
256. Vourvahis, M., et al., *The pharmacokinetics and viral activity of tenofovir in the male genital tract*. J Acquir Immune Defic Syndr, 2008. **47**(3): p. 329-33.
257. Burton, D.R., et al., *Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses*. Science, 2012. **337**(6091): p. 183-6.
258. Luo, X.M., et al., *Dimeric 2G12 as a potent protection against HIV-1*. PLoS Pathog, 2010. **6**(12): p. e1001225.
259. Rosenberg, Y., et al., *Rapid high-level production of functional HIV broadly neutralizing monoclonal antibodies in transient plant expression systems*. PLoS One, 2013. **8**(3): p. e58724.
260. McKinley, S.A., et al., *Modeling neutralization kinetics of HIV by broadly neutralizing monoclonal antibodies in genital secretions coating the cervicovaginal mucosa*. PLoS One, 2014. **9**(6): p. e100598.
261. Abdel-Motal, U.M., et al., *Prolonged expression of an anti-HIV-1 gp120 minibody to the female rhesus macaque lower genital tract by AAV gene transfer*. Gene Ther, 2014.
262. Mascola, J.R. and D.C. Montefiori, *The role of antibodies in HIV vaccines*. Annu Rev Immunol, 2010. **28**: p. 413-44.
263. Lederman, M.M., et al., *Topical application of entry inhibitors as "virstats" to prevent sexual transmission of HIV infection*. Retrovirology, 2008. **5**: p. 116.
264. Omar, R.F. and M.G. Bergeron, *The future of microbicides*. Int J Infect Dis, 2011. **15**(10): p. e656-60.
265. Shattock, R.J. and Z. Rosenberg, *Microbicides: topical prevention against HIV*. Cold Spring Harb Perspect Med, 2012. **2**(2): p. a007385.
266. Abdool Karim, S.S. and C. Baxter, *Overview of microbicides for the prevention of human immunodeficiency virus*. Best Pract Res Clin Obstet Gynaecol, 2012. **26**(4): p. 427-39.
267. Hladik, F. and G.F. Doncel, *Preventing mucosal HIV transmission with topical microbicides: challenges and opportunities*. Antiviral Res, 2010. **88 Suppl 1**: p. S3-9.
268. Kelly, C.G. and R.J. Shattock, *Specific microbicides in the prevention of HIV infection*. J Intern Med, 2011. **270**(6): p. 509-19.

269. Klasse, P.J., R.J. Shattock, and J.P. Moore, *Which topical microbicides for blocking HIV-1 transmission will work in the real world?* PLoS Med, 2006. **3**(9): p. e351.
270. Parren, P.W., et al., *Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro.* J Virol, 2001. **75**(17): p. 8340-7.
271. Veazey, R.S., et al., *Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120.* Nat Med, 2003. **9**(3): p. 343-6.
272. Walker, L.M., et al., *Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target.* Science, 2009. **326**(5950): p. 285-9.
273. Wu, X., et al., *Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1.* Science, 2010. **329**(5993): p. 856-61.
274. Corti, D., et al., *Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals.* PLoS One, 2010. **5**(1): p. e8805.
275. Walker, L.M., et al., *Broad neutralization coverage of HIV by multiple highly potent antibodies.* Nature, 2011. **477**(7365): p. 466-70.
276. Denton, P.W. and J.V. Garcia, *Novel humanized murine models for HIV research.* Curr HIV/AIDS Rep, 2009. **6**(1): p. 13-9.
277. Klasse, P.J., R. Shattock, and J.P. Moore, *Antiretroviral drug-based microbicides to prevent HIV-1 sexual transmission.* Annu Rev Med, 2008. **59**: p. 455-71.
278. Shultz, L.D., et al., *Humanized mice as a preclinical tool for infectious disease and biomedical research.* Ann N Y Acad Sci, 2011. **1245**: p. 50-4.
279. Ferrantelli, F., et al., *Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies.* J Infect Dis, 2004. **189**(12): p. 2167-73.
280. Hofmann-Lehmann, R., et al., *Postnatal pre- and postexposure passive immunization strategies: protection of neonatal macaques against oral simian-human immunodeficiency virus challenge.* J Med Primatol, 2002. **31**(3): p. 109-19.
281. Ruprecht, R.M., et al., *Protection of neonatal macaques against experimental SHIV infection by human neutralizing monoclonal antibodies.* Transfus Clin Biol, 2001. **8**(4): p. 350-8.
282. Ferguson, L.M. and L.C. Rohan, *The importance of the vaginal delivery route for antiretrovirals in HIV prevention.* Ther Deliv, 2011. **2**(12): p. 1535-50.
283. Veazey, R.S., et al., *Protection of rhesus macaques from vaginal infection by vaginally delivered maraviroc, an inhibitor of HIV-1 entry via the CCR5 co-receptor.* J Infect Dis, 2010. **202**(5): p. 739-44.
284. Gauduin, M.C., et al., *Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1.* Nat Med, 1997. **3**(12): p. 1389-93.
285. Euler, Z., et al., *Activity of broadly neutralizing antibodies, including PG9, PG16, and VRC01, against recently transmitted subtype B HIV-1 variants from early and late in the epidemic.* J Virol, 2011. **85**(14): p. 7236-45.
286. West, A.P., Jr., et al., *Single-chain Fv-based anti-HIV proteins: potential and limitations.* J Virol, 2012. **86**(1): p. 195-202.
287. Scheid, J.F., et al., *Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding.* Science, 2011. **333**(6049): p. 1633-7.
288. Wu, X., et al., *Focused evolution of HIV-1 neutralizing antibodies revealed by structures and deep sequencing.* Science, 2011. **333**(6049): p. 1593-602.
289. Pennings, P.S., *HIV Drug Resistance: Problems and Perspectives.* Infect Dis Rep, 2013. **5**(Suppl 1): p. e5.

290. Heneine, W. and A. Kashuba, *HIV prevention by oral preexposure prophylaxis*. Cold Spring Harb Perspect Med, 2012. **2**(3): p. a007419.
291. van der Straten, A., et al., *Unraveling the divergent results of pre-exposure prophylaxis trials for HIV prevention*. AIDS, 2012. **26**(7): p. F13-9.
292. Garcia-Lerma, J.G., et al., *Intermittent prophylaxis with oral Truvada protects macaques from rectal SHIV infection*. Sci Transl Med, 2010. **2**(14): p. 14ra4.
293. Nuttall, J., et al., *Pharmacokinetics of tenofovir following intravaginal and intrarectal administration of tenofovir gel to rhesus macaques*. Antimicrob Agents Chemother, 2012. **56**(1): p. 103-9.
294. Forbes, C.J., et al., *Non-aqueous silicone elastomer gels as a vaginal microbicide delivery system for the HIV-1 entry inhibitor maraviroc*. J Control Release, 2011. **156**(2): p. 161-9.
295. Malcolm, R.K., et al., *Pharmacokinetics and efficacy of a vaginally administered maraviroc gel in rhesus macaques*. J Antimicrob Chemother, 2013. **68**(3): p. 678-83.
296. Choudhary, S.K., et al., *Suppression of human immunodeficiency virus type 1 (HIV-1) viremia with reverse transcriptase and integrase inhibitors, CD4+ T-cell recovery, and viral rebound upon interruption of therapy in a new model for HIV treatment in the humanized Rag2-/-[gamma]c-/- mouse*. J Virol, 2009. **83**(16): p. 8254-8.
297. Denton, P.W., et al., *Targeted cytotoxic therapy kills persisting HIV infected cells during ART*. PLoS Pathog, 2014. **10**(1): p. e1003872.
298. Brown, K.C., et al., *Single and multiple dose pharmacokinetics of maraviroc in saliva, semen, and rectal tissue of healthy HIV-negative men*. The Journal of infectious diseases, 2011. **203**(10): p. 1484-90.
299. Nicol, M.R., et al., *Expression of six drug transporters in vaginal, cervical, and colorectal tissues: Implications for drug disposition in HIV prevention*. J Clin Pharmacol, 2013.
300. Kwara, A., et al., *Antiretroviral drug concentrations and HIV RNA in the genital tract of HIV-infected women receiving long-term highly active antiretroviral therapy*. Clin Infect Dis, 2008. **46**(5): p. 719-25.
301. Dumond, J.B., et al., *Antiretroviral drug exposure in the female genital tract: implications for oral pre- and post-exposure prophylaxis*. AIDS, 2007. **21**(14): p. 1899-907.
302. Hawkins, T., et al., *Intracellular pharmacokinetics of tenofovir diphosphate, carbovir triphosphate, and lamivudine triphosphate in patients receiving triple-nucleoside regimens*. J Acquir Immune Defic Syndr, 2005. **39**(4): p. 406-11.
303. Dumond, J.B., et al., *Maraviroc concentrates in the cervicovaginal fluid and vaginal tissue of HIV-negative women*. J Acquir Immune Defic Syndr, 2009. **51**(5): p. 546-53.
304. Abel, S., et al., *Assessment of the pharmacokinetics, safety and tolerability of maraviroc, a novel CCR5 antagonist, in healthy volunteers*. Br J Clin Pharmacol, 2008. **65 Suppl 1**: p. 5-18.
305. Adams, J.L., et al., *Tenofovir diphosphate and emtricitabine triphosphate concentrations in blood cells compared with isolated peripheral blood mononuclear cells: a new measure of antiretroviral adherence?* J Acquir Immune Defic Syndr, 2013. **62**(3): p. 260-6.
306. To, E.E., C.W. Hendrix, and N.N. Bumpus, *Dissimilarities in the metabolism of antiretroviral drugs used in HIV pre-exposure prophylaxis in colon and vagina tissues*. Biochem Pharmacol, 2013. **86**(7): p. 979-90.
307. Karmon, S.L. and M. Markowitz, *Next-generation integrase inhibitors : where to after raltegravir?* Drugs, 2013. **73**(3): p. 213-28.
308. Liedtke, M.D., et al., *Long-term efficacy and safety of raltegravir in the management of HIV infection*. Infect Drug Resist, 2014. **7**: p. 73-84.

309. Summa, V., et al., *Discovery of raltegravir, a potent, selective orally bioavailable HIV-integrase inhibitor for the treatment of HIV-AIDS infection*. J Med Chem, 2008. **51**(18): p. 5843-55.
310. Markowitz, M., et al., *Antiretroviral activity, pharmacokinetics, and tolerability of MK-0518, a novel inhibitor of HIV-1 integrase, dosed as monotherapy for 10 days in treatment-naïve HIV-1-infected individuals*. J Acquir Immune Defic Syndr, 2006. **43**(5): p. 509-15.
311. Rockstroh, J.K., et al., *Long-term treatment with raltegravir or efavirenz combined with tenofovir/emtricitabine for treatment-naïve human immunodeficiency virus-1-infected patients: 156-week results from STARTMRK*. Clin Infect Dis, 2011. **53**(8): p. 807-16.
312. Nguyen, B.Y., et al., *Raltegravir: the first HIV-1 integrase strand transfer inhibitor in the HIV armamentarium*. Ann N Y Acad Sci, 2011. **1222**: p. 83-9.
313. Kassahun, K., et al., *Metabolism and disposition in humans of raltegravir (MK-0518), an anti-AIDS drug targeting the human immunodeficiency virus 1 integrase enzyme*. Drug Metab Dispos, 2007. **35**(9): p. 1657-63.
314. Iwamoto, M., et al., *Safety, tolerability, and pharmacokinetics of raltegravir after single and multiple doses in healthy subjects*. Clin Pharmacol Ther, 2008. **83**(2): p. 293-9.
315. Grobler JA, M.P., Ly S, et al, *Functionally irreversible inhibition of integration by slowly dissociating strand transfer inhibitors*, in *10th International Workshop on Clinical Pharmacology of HIV Therapy*. 2009: Amsterdam.
316. Nischang, M., et al., *Modeling HIV infection and therapies in humanized mice*. Swiss medical weekly, 2012. **142**: p. w13618.
317. Patterson, K.B., et al., *Penetration of tenofovir and emtricitabine in mucosal tissues: implications for prevention of HIV-1 transmission*. Science translational medicine, 2011. **3**(112): p. 112re4.
318. Cattaneo, D., et al., *Comparison of the in vivo pharmacokinetics and in vitro dissolution of raltegravir in HIV patients receiving the drug by swallowing or by chewing*. Antimicrob Agents Chemother, 2012. **56**(12): p. 6132-6.
319. Ananworanich, J., et al., *Pharmacokinetics of and short-term virologic response to low-dose 400-milligram once-daily raltegravir maintenance therapy*. Antimicrob Agents Chemother, 2012. **56**(4): p. 1892-8.
320. Wohl, D.A., et al., *Raltegravir pharmacokinetics in treatment-naïve patients is not influenced by race: results from the raltegravir early therapy in African-Americans living with HIV (REAL) study*. Antimicrob Agents Chemother, 2013. **57**(2): p. 784-8.
321. Fayet Mello, A., et al., *Cell disposition of raltegravir and newer antiretrovirals in HIV-infected patients: high inter-individual variability in raltegravir cellular penetration*. J Antimicrob Chemother, 2011. **66**(7): p. 1573-81.
322. Cattaneo, D., et al., *Inter- and intra-patient variability of raltegravir pharmacokinetics in HIV-1-infected subjects*. J Antimicrob Chemother, 2012. **67**(2): p. 460-4.
323. Rizk, M.L., et al., *Pharmacokinetics and pharmacodynamics of once-daily versus twice-daily raltegravir in treatment-naïve HIV-infected patients*. Antimicrob Agents Chemother, 2012. **56**(6): p. 3101-6.
324. Sandkovsky, U., et al., *Measurement of plasma and intracellular concentrations of raltegravir in patients with HIV infection*. AIDS, 2012. **26**(17): p. 2257-9.
325. Siccardi, M., et al., *Inpatient and outpatient pharmacokinetic variability of raltegravir in the clinical setting*. Ther Drug Monit, 2012. **34**(2): p. 232-5.
326. Eron, J.J., Jr., et al., *Raltegravir once daily or twice daily in previously untreated patients with HIV-1: a randomised, active-controlled, phase 3 non-inferiority trial*. Lancet Infect Dis, 2011. **11**(12): p. 907-15.

327. Neely, M., et al., *Pharmacokinetics and pharmacogenomics of once-daily raltegravir and atazanavir in healthy volunteers*. Antimicrob Agents Chemother, 2010. **54**(11): p. 4619-25.
328. Jackson, A., et al., *Plasma and intracellular pharmacokinetics of darunavir/ritonavir once daily and raltegravir once and twice daily in HIV-infected individuals*. J Acquir Immune Defic Syndr, 2011. **58**(5): p. 450-7.
329. Molto, J., et al., *Plasma and intracellular (peripheral blood mononuclear cells) pharmacokinetics of once-daily raltegravir (800 milligrams) in HIV-infected patients*. Antimicrob Agents Chemother, 2011. **55**(1): p. 72-5.
330. Wang, L., et al., *Pharmacokinetic modeling of plasma and intracellular concentrations of raltegravir in healthy volunteers*. Antimicrob Agents Chemother, 2011. **55**(9): p. 4090-5.
331. Monteagudo, E., et al., *Studies of metabolism and disposition of potent human immunodeficiency virus (HIV) integrase inhibitors using 19F-NMR spectroscopy*. Xenobiotica, 2007. **37**(9): p. 1000-12.
332. Moss, D.M., et al., *Predicting intestinal absorption of raltegravir using a population-based ADME simulation*. J Antimicrob Chemother, 2013. **68**(7): p. 1627-34.
333. Nwosu, F.C., et al., *Gut Microbiota in HIV Infection: Implication for Disease Progression and Management*. Gastroenterol Res Pract, 2014. **2014**: p. 803185.
334. Clavel, C., et al., *Raltegravir concentrations in the genital tract of HIV-1-infected women treated with a raltegravir-containing regimen (DIVA 01 study)*. Antimicrob Agents Chemother, 2011. **55**(6): p. 3018-21.
335. Patterson KB, P.H., White N, et al, *Pharmacokinetics of raltegravir in the blood plasma and genital tract of HIV-positive and HIV-negative women*, in *18th International AIDS Conference*. 2010.
336. Patterson, K.B., et al., *Differential penetration of raltegravir throughout gastrointestinal tissue: implications for eradication and cure*. AIDS, 2013. **27**(9): p. 1413-9.
337. Nicol, M.R., et al., *Expression of six drug transporters in vaginal, cervical, and colorectal tissues: Implications for drug disposition in HIV prevention*. J Clin Pharmacol, 2013. **54**(5): p. 574-583.
338. Mitchell, C., et al., *Correlation between plasma, intracellular and cervical tissue levels of raltegravir at steady state dosing in healthy women*. Antimicrob Agents Chemother, 2014.
339. Thompson CG, R.E., Sykes C, Fedoriw Y, Luciw P, Muddiman DC, et al, *Characterizing antiretroviral distribution within active viral reservoirs using mass spectrometry imaging*, in *15th International Workshop on Clinical Pharmacology of HIV and Hepatitis Therapy*. 2014: Washington D.C.
340. Kozal, M.J., et al., *A nucleoside- and ritonavir-sparing regimen containing atazanavir plus raltegravir in antiretroviral treatment-naïve HIV-infected patients: SPARTAN study results*. HIV Clin Trials, 2012. **13**(3): p. 119-30.
341. Eron, J.J., et al., *Switch to a raltegravir-based regimen versus continuation of a lopinavir-ritonavir-based regimen in stable HIV-infected patients with suppressed viraemia (SWITCHMRK 1 and 2): two multicentre, double-blind, randomised controlled trials*. Lancet, 2010. **375**(9712): p. 396-407.
342. Taiwo, B., et al., *Efficacy of a nucleoside-sparing regimen of darunavir/ritonavir plus raltegravir in treatment-naïve HIV-1-infected patients (ACTG A5262)*. AIDS, 2011. **25**(17): p. 2113-22.
343. Garrido, C., et al., *Mechanisms involved in CD4 cell gains in HIV-infected patients switched to raltegravir*. AIDS, 2012. **26**(5): p. 551-7.
344. Cahn, P., et al., *Dolutegravir versus raltegravir in antiretroviral-experienced, integrase-inhibitor-naïve adults with HIV: week 48 results from the randomised, double-blind, non-inferiority SAILING study*. Lancet, 2013. **382**(9893): p. 700-8.

345. Mayer, K.H., et al., *Raltegravir, tenofovir DF, and emtricitabine for postexposure prophylaxis to prevent the sexual transmission of HIV: safety, tolerability, and adherence*. J Acquir Immune Defic Syndr, 2012. **59**(4): p. 354-9.
346. Barau, C., et al., *High concentration of raltegravir in semen of HIV-infected men: results from a substudy of the EASIER-ANRS 138 trial*. Antimicrob Agents Chemother, 2010. **54**(2): p. 937-9.
347. Stoddart, C.A., et al., *Validation of the SCID-hu Thy/Liv mouse model with four classes of licensed antiretrovirals*. PLoS One, 2007. **2**(7): p. e655.
348. Wenning, L.A., et al., *Lack of a significant drug interaction between raltegravir and tenofovir*. Antimicrob Agents Chemother, 2008. **52**(9): p. 3253-8.
349. Kulkarni, R., et al., *The Combined Anti-HIV-1 Activity of Emtricitabine and Tenofovir with the Integrase Inhibitors Elvitegravir or Raltegravir Show High Levels of Synergy In Vitro*. Antimicrob Agents Chemother, 2014.
350. Abel, S., et al., *The effects of cotrimoxazole or tenofovir co-administration on the pharmacokinetics of maraviroc in healthy volunteers*. Br J Clin Pharmacol, 2008. **65 Suppl 1**: p. 47-53.
351. Andrews, E., et al., *Assessment of the pharmacokinetics of co-administered maraviroc and raltegravir*. Br J Clin Pharmacol, 2010. **69**(1): p. 51-7.
352. Talameh, J.A., N.L. Rezk, and A.D. Kashuba, *Quantifying the HIV-1 integrase inhibitor raltegravir in female genital tract secretions using high-performance liquid chromatography with ultraviolet detection*. J Chromatogr B Analyt Technol Biomed Life Sci, 2010. **878**(1): p. 92-6.
353. Anton, P.A., et al., *RMP-02/MTN-006: A phase 1 rectal safety, acceptability, pharmacokinetic, and pharmacodynamic study of tenofovir 1% gel compared with oral tenofovir disoproxil fumarate*. AIDS Res Hum Retroviruses, 2012. **28**(11): p. 1412-21.
354. Massud, I., et al., *Lack of prophylactic efficacy of oral maraviroc in macaques despite high drug concentrations in rectal tissues*. J Virol, 2013. **87**(16): p. 8952-61.
355. Dobard, C., et al., *Postexposure protection of macaques from Vaginal SHIV infection by topical integrase inhibitors*. Sci Transl Med, 2014. **6**(227): p. 227ra35.
356. Richardson-Harman, N., et al., *Dose-response relationship between tissue concentrations of UC781 and explant infectibility with HIV type 1 in the RMP-01 rectal safety study*. AIDS Res Hum Retroviruses, 2012. **28**(11): p. 1422-33.
357. Cost, M., et al., *Characterization of UC781-tenofovir combination gel products for HIV-1 infection prevention in an ex vivo ectocervical model*. Antimicrob Agents Chemother, 2012. **56**(6): p. 3058-66.
358. Haaland, R.E., et al., *UC781 microbicide gel retains anti-HIV activity in cervicovaginal lavage fluids collected following twice-daily vaginal application*. Antimicrob Agents Chemother, 2012. **56**(7): p. 3592-6.
359. Dezzutti, C.S., et al., *Development of HIV-1 Rectal-Specific Microbicides and Colonic Tissue Evaluation*. PLoS One, 2014. **9**(7): p. e102585.
360. Pirrone, V., et al., *Combinatorial Approaches to the Prevention and Treatment of HIV-1 Infection*. Antimicrobial Agents and Chemotherapy, 2011. **55**(5): p. 1831-1842.
361. Belec, L., et al., *Combinatorial prevention of HIV transmission in women: the case for a vaginal microbicide*. Future Microbiology, 2011. **6**(7): p. 731-737.
362. Moss, J.A., et al., *Pharmacokinetics and Preliminary Safety Study of Pod-Intravaginal Rings Delivering Antiretroviral Combinations for HIV Prophylaxis in a Macaque Model*. Antimicrob Agents Chemother, 2014. **58**(9): p. 5125-35.