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Effects of HIV-1 Tat and drugs of abuse on antiretroviral penetration inside different CNS cell types

Sulay H. Patel
Virginia Commonwealth University

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Effects of HIV-1 Tat and drugs of abuse on antiretroviral penetration inside different CNS cell types

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By,

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Virginia Commonwealth University
Richmond, Virginia
September 2018
Dedication

This dissertation is dedicated to my parents for their constant support, unstinting love, and numerous sacrifices. This endeavor would not have been possible without both of you. Love you!
Acknowledgements

This work would not have been possible without help and support of many people. First and foremost, I would like to thank my advisor Dr. MaryPeace McRae for giving me opportunity to work on this project. She had been a great mentor and I had learned lot from her, both personally and professionally. I would like to thank her for giving me the freedom in shaping my project according to my career interest. In her I see a great scientist and even better human being, something that had always inspired me to emulate and the best mentor someone could ask for.

Secondly, I would also like to thank my dissertation committee for providing valuable insights on potential roadblocks of some of the studies and in refining some of my experimental design. I would particularly like to thanks Dr. Sweet for discussions during drug transporter research group meeting, Dr. McClay for helping me with the statistics, and Dr. Hauser for providing his lab resources during the course of my study. I would like to give my special thanks to Dr. Slattum, past graduate program coordinator in DPOS, for taking personal interest in my project and being a constant source of motivation.

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Finally, I would like to thank my spiritual guru, param pujya bapa shree, for showing the right path to be a better person every other day.
ABBREVIATIONS

ABC – ATP binding cassettes
AIDS – Acquired immunodeficiency syndrome
ANI – Asymptomatic neurocognitive impairment
ANOVA – Analysis of variance
BBB – Blood brain barrier
BCA - Bicinchoninic Acid Assay
BCRP – Breast cancer resistant protein
bFGF – human basic fibroblast growth factor
cART – Combination antiretroviral therapy
CDC – Center for disease control
CNS – Central nervous system
CNT – Concentrative nucleoside transporter
CPE – CNS penetration effectiveness score
CRF – Circulating recombinant forms
CSF – Cerebrospinal fluid
DFN – Difference from nominal
DPBS – D Phosphate buffer solution
ESI – Electron spray ionization
EBM-2 – Endothelial Basal Media-2
ENT – Equilibrative nucleoside transporter
EthD-1 – Ethidium homodimer-1
FBS – Fetal bovine serum
FDA – Food and drug administration
GNDF – Glial derived neurotrophic factor
HAND – HIV-associated neurocognitive disorders
HAD – HIV associated dementia
HBMEC – Human brain microvascular endothelial cells
HBSS – Hank’s balanced salt solution
hCMEC/D3 – Human cerebral microvascular endothelial cells
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEXM1 – Hexamethylene bi-acetamide inducible protein-1
IDU – Injection drug use
LC-MS/MS – Liquid chromatography tandem mass spectrometry
LLOQ – Limit of quantification
LTR – Long terminal repeat
MAPK – Mitogen activated protein kinase
MATE – Multidrug and toxin extrusion protein
MND – Mild neurocognitive disorder
MMP – Matrix metalloproteinase
MRM – Multi reaction monitoring
MRP – Multidrug resistant protein
NNRTI – Non-nucleoside reverse transcriptase inhibitors
NRTI – Nucleoside reverse transcriptase inhibitors
NtRTI – Nucleotide reverse transcriptase inhibitors
OAT – Organic anion transporter
OCT – Organic cation transporter
PAF – Platelet activating factor
Papp – Apparent permeability
PDGF – Platelet derived growth factor
PBMC – Peripheral blood mononuclear cells
P-gp – P-glycoprotein
p-TEFb – positive transcription elongation factor-b
PIC – Pre-integration complex
QC – Quality control
Rh123 - Rhodamine
ROS – Reactive oxygen species
RSD – Relative standard deviation
RT – Room temperature
RTC – Reverse transcription complex
snRNP – small nuclear ribonucleao protein
TAT – Transactivator of transcription
TEER – Transendothelial electrical resistance
TNFα – Tissue Necrosis Factor- alpha
ULOQ – Ultra low limit of quantification
UPLC – Ultra pressure liquid chromatography
UNAIDS – United nation join program on HIV/AIDS
VEGF – Vascular endothelial growth factor
ZO – Zonula occludens
Abstract

EFFECTS OF HIV-1 TAT AND DRUGS OF ABUSE ON ANTIRETROVIRAL PENETRATION INSIDE DIFFERENT CNS CELL TYPES

By Sulay Patel B.Pharm, M.S

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2018

Major Director: Dr. MaryPeace McRae, Department of Pharmacotherapy and Outcome Sciences.

Human immunodeficiency (HIV) infection can result in neurocognitive deficits in about one-half of infected individuals. Despite excellent systemic effectiveness, restricted antiretroviral penetration across the blood-brain barrier (BBB) is a major limitation in fighting HIV infection within the central nervous system (CNS). Drug abuse exacerbates cognitive impairment and pathologic CNS changes in HIV-infected individuals. This work investigates the effects of the HIV-1 protein, Tat, and drugs of abuse on factors affecting drug penetration into the brain.

Firstly, an in vitro model of the blood-brain barrier was built to study effects of HIV-1 Tat and methamphetamine (Meth) on integrity and function of the BBB, in turn how HIV-1 Tat and meth will affect antiretroviral penetration into the brain. We found that co-exposure
HIV-1 Tat and Meth results in inhibition or impairment of P-glycoprotein activity at the BBB. Also, simultaneous inhibition of P-glycoprotein (P-gp) and Multidrug Resistant Protein -1 (MRP-1), by verapamil and MK-571 causes an increase in accumulation of atazanavir inside the primary human brain endothelial cells.

Secondly, we developed and validated the method for simultaneous determination of tenofovir, emtricitabine, and dolutegravir in cell extracts of CNS cells. This method was used to study how HIV-1 Tat and/or morphine affects antiretroviral penetration in CNS cells like human brain microvascular endothelial cells, human astrocytes, human microglia, and human pericytes. We found that in untreated cells, accumulation of antiretroviral drugs was higher in hCMEC/D3 cells compared to other CNS cell types. Also, HIV-1 Tat and/or morphine had no significant effect on antiretroviral penetration amongst these cell types. Overall, the rank order of intracellular accumulation observed in treated and untreated cells was dolutegravir > emtricitabine > tenofovir.
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INTRODUCTION

History and Origin of HIV-1 infection

Human immunodeficiency virus -1 (HIV-1) is a lentivirus, belonging to the retroviral family, and an etiologic agent for Acquired Immunodeficiency Syndrome (AIDS). It was first recognized in 1981 when young homosexual men were reported of having opportunistic infections (*Pneumocystis carinii* pneumonia, cytomegalovirus-associated retinitis, and cryptococcal meningitis) and rare malignancies (non-Hodgkin’s lymphoma and Kaposi’s sarcoma) (1). All the affected individuals showed significant reductions in the CD4 T lymphocyte counts, resulting in the impaired immune system. In 1983, Montaignier and colleagues at The Pasteur Institute isolated a virus from the lymph node of an asymptomatic patient, who had lymphadenopathy (2). During the same time, Gallo and colleagues at National Institute of Health (NIH) reported the isolation of a retrovirus from an AIDS patient and named it Human T-cell Leukemia Virus Type III, which was subsequently named as Human Immunodeficiency Virus (HIV-1) (3). HIV-1 comprises of four different subgroups of virus, namely, M group (Main group), which is the pandemic virus responsible for 98% of global viral isolates, Group O (Outlier group), Group N (non-M, non-O group), and Group P. HIV-1 from group M further consist of nine clades (A, B, C, D, E, F, J, and K) and 49 circulating recombinant forms (CRFs) (4). The HIV-1 virus is phylogenetically evolved from SIVcpzPtt from central chimpanzee subspecies, which is the source of human and gorilla infection. It is thought that cross-species transfer of HIV to human happened due to exposure to body fluids of the infected ape during bushmeat hunting (5).
Human Immunodeficiency Virus -2 (HIV-2) is related to HIV-1, but it differs considerably in virulence, transmission rates, and phylogenetic origin (6). HIV-2 is evolved from sooty mangabey monkeys, and it is endemic to West Africa (7). The scope of this work will focus on HIV-1 and HIV-1 related proteins.

**HIV-1 Transmission and Epidemiology**

HIV-1 is transmitted through the following major routes:

1) **Sexual Transmission:** Anal sex is the highest risk factor for transmitting HIV in both men and women (8). Vaginal sex has a comparatively lower risk, and oral sex has little or no risk for transmitting HIV (8). Sexual transmission can also lead to other sexually transmitted infections.

2) **Injection Drug Use (IDU):** Injection drug use and HIV are interlinked epidemics. In 2016, individuals who injected drugs accounted for 9% of all HIV diagnoses (9). Most of these transmissions occurred through exchanging needles and risky sexual behaviors under the influence of drugs (9). Recently in 2014, in Scott County, Indiana, there were instances of HIV-1 outbreak reported due to sharing of needles in people who abuse prescription opioids (10).

3) **Mother- to- Child Transmission:** Globally, about 1.8 million children are living with HIV (11). Of which, more than 90% of these children were either infected in utero, at birth or through feeding of breast milk (9). With the advent of antiretroviral drugs, perinatal HIV has significantly reduced. Mostly, mother to child transmission is observed in sub-Saharan countries mostly due to poor availability of antiretroviral drugs (12).

4) **Blood Transfusion:** Occasionally, HIV transmission is caused by blood transfusion.
According to a UNAIDS Data of 2018, 36.7 million people worldwide are living with HIV. There were 1.8 million new HIV infections reported in 2016 and 0.94 million deaths due to AIDS (11). According to the CDC HIV surveillance report, there are about 1.2 million people in the USA and dependent areas living with HIV in 2016. There were 38,500 new cases of HIV diagnoses were reported in 2016 (9). The highest rate of diagnoses and incidence were reported in southern states, mid-Atlantic, and California. African Americans, who represent 18% of the population, accounted for 44% of new HIV diagnoses, while Hispanics/Latinos, who represent 18% of the population, accounted for 25% new diagnoses. Men who have sex with men had 70% of new infections, 24% of new infections were in heterosexual individuals, and 6% were among people who inject drugs. These prevalence estimates illustrate significant disparity based on race and sexual orientation.

**HIV-1 Biology and Clinical Stages of HIV-1 Infection**

HIV-1 is a spherical virus with a nine kilobase genome consisting of *gag, pol, env, vit, vpu, vpr, tat, rev,* and *nef* genes (13). HIV-1 primarily infects cells with CD4 receptors. However, HIV-1 viral isolates were found in the cells, which do not requires CD4 receptors to infect cells (14). The life cycle of HIV-1 from its entry into the cell maturation can be explained through nine steps or stages as shown in (Fig 1).

**Step 1: Binding of gp120**

HIV-1 envelope protein is encoded by HIV-1 env gene (15). It is synthesized as a precursor protein, which is processed by host proteases into gp120 and gp41. The unprocessed protein has been designated as gp160 based on its molecular weight.
1 envelope protein comprises of heavily glycosylated trimers of gp120 (surface unit) and gp41 (transmembrane unit) heterodimers, which are non-covalently linked with each other. Surface glycoprotein (gp120) is responsible for receptor binding on the host cell membrane; it contains five conserved domains (C1-C5) and five variable loops (V1-V5) (16). Variable loops lie on the surface of gp120 and are thought to play a vital role in immune evasion and coreceptor binding. HIV-1 primarily infects CD4 receptor containing T cells and macrophages (17). Initially, interactions between several attachment factors and envelope proteins bring virus close to CD4 receptor and coreceptor on the host cell, increasing efficiency of viral infection. These interactions can be either non-specific, between envelope proteins and negatively charged cell surface heparan sulfate proteoglycans (18) or specific between envelope protein and α4β7 integrin and DC-SIGN (19, 20). These interactions lead to binding of gp120 to CD4 receptor containing target cells.

Step 2: Co-receptor Binding

Binding of HIV-1 envelope protein (gp120) to the CD4 receptor causes conformational changes in variable loops 1, 2 and repositioning of variable loop 3. Additionally, the binding of gp120 to the CD4 receptor causes the formation of a bridging sheet, which comprises of two double-stranded β sheets. Together with bridging sheets and changes in variable loop three aids in co-receptor binding (21). Variable loop 3 of gp120 binds to coreceptor CCR5 and CXCR4, which further leads to fusion of the virus with the host cell.
Figure 1: The Life Cycle of HIV-1 (Adapted from Ronaldson et al., Glia, 56 (16), 1711-1735, DOI: 10.1002/glia.20725)
cell membrane. In the brain, endothelial cells at the blood-brain barrier, astrocytes, and oligodendrocytes can be infected with HIV-1 in CD4 independent fashion, using the coreceptor (22).

Step 3: Fusion

Coreceptor binding exposes hydrophobic fusion peptide gp41, which inserts into the host cell membrane. Then, the fusion peptide gp41 from each trimer folds at the hinge region bringing an amino-terminal helical region (HR-N) and a carboxy-terminal helical region (HR-C) together to form a six-helix bundle (23). Formation of the six-helix bundle is the driving force in bringing the two membranes close to each other and resulting in pore formation. With the formation of the fusion pore, viral contents are released into the host cell cytoplasm. FDA approved fusion inhibitors, such as enfuvirtide, are peptides designed on the C-terminus sequence of gp41, which mimic the HR-C domain of gp41, and can bind and disrupt the intramolecular viral protein interaction preventing viral fusion with the host cell membrane (24).

Step 4: Reverse transcription

After viral entry and fusion, the viral capsid comprising of reverse transcriptase, integrase, and viral genomic RNA, is released into the cytoplasm (25). Immediately in the cytoplasm, there is the partial dissolution of the capsid, which releases HIV-1 RNA and the reverse transcriptase. The reverse transcriptase along with RNase H (Reverse Transcription complex) converts HIV-1 RNA into double-stranded DNA (26). This step processed by reverse transcriptase is highly prone to error, giving rise to many of the HIV mutations responsible for the drug resistance against reverse transcriptase inhibitors (26). After
completion of DNA synthesis, the reverse transcriptase complex matures into the pre-integration complex, comprising of linear viral DNA and proteins needed for viral integration in host DNA. This process is aided by the HIV-1 protein vpr encoded by vpr of HIV-1 genome (27). Reverse transcriptase, RNase, and integrase are encoded by pol of HIV-1 genome (28).

The nucleoside reverse transcriptase inhibitor (NRTI) class of drugs competitively inhibits reverse transcriptase by getting incorporated in the growing chain of viral DNA. This class of drugs lacks 3’ OH group, which is required for elongation of viral DNA, thus terminating viral DNA synthesis. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) acts at the allosteric site of the reverse transcriptase and brings conformational change in the catalytic site which prevents substrate attachment resulting in inhibition of the enzyme (25).

Step 5: Integration

After completion of viral DNA synthesis, viral DNA along with viral and cellular proteins required for integration are transported into the nucleus of the CD4 target cell. Inside the nucleus, two nucleotides are removed for 3’ end of the viral DNA creating sticky ends, which are integrated into the host genome. This process is catalyzed by the enzyme DNA integrase (28). Lastly, ligation of viral DNA into host DNA takes place to give a single DNA molecule (28). Integrase inhibitors, such as dolutegravir, raltegravir, and elvitegravir, act at this step of the viral life cycle by non-competitively inhibiting DNA strand transfer activity of integrase enzyme (25).

Step 6a and 6b: Transcription & Translation
After the integration of viral DNA into the host genome, viral genes are expressed in the host cell through transcription which initiates from a U3 promoter within the upstream Long Terminal Repeat (LTR) (29). This step requires HIV-1 Tat for efficient elongation (29). HIV-1 Tat is encoded by the HIV-1 tat gene. HIV-1 Rev, encoded by rev, and is responsible for nuclear transport of unspliced mRNA, whereas smaller transcripts are transported without the help of the Rev protein (25). This mRNA undergoes translation using host cell machinery to produce the viral structural, enzymatic and regulatory polyprotein precursors of Gag and Gag-pol (30). Structural proteins like viral capsid, matrix and nucleocapsid are synthesized as precursor polypeptide Gag from HIV-1 viral gene gag (25).

Step 7: Assembly and Budding

Both, Gag and Gag-pol precursor proteins multimerize through interactions of different domains along Gag and are targeted by N-terminal myristic acid to the inner leaflet of the plasma membrane (30). This allows Gag to preferentially associate at the plasma membrane, where they concentrate in lipid rafts that potentially serve as assembly platforms (31). At the same time, viral envelope proteins and viral genomic RNA are recruited at the plasma membrane. In this step, the HIV-1 protease cleaves viral proteins into smaller units, which assembles to form a mature virus that is now ready to infect other cells (31). Accumulation of 1500 to 2000 viral polyproteins beneath the plasma membrane induces curvature, leading to the formation of membrane coated spherical particles still attached to the plasma membrane. HIV-1 p6, which is part of Gag, is responsible for the final release of virus from the membrane by recruiting cellular Tsg101 protein and various associated factors that belong to the vesicular sorting pathway (31).
Steps 8 and 9: Release and Maturation of viral particles

The virus particles released in the previous step have immature morphology, characterized by a thin layer of circularly arranged Gag and Gag-Pol precursor polyproteins. HIV-1 viral protease cleaves these precursor polyproteins into their subcomponent. Due to this virus having characteristic conical inner cores are formed; ready to attack other cells (31). HIV protease inhibitors (atazanavir, lopinavir, ritonavir, darunavir)) competitively bind to HIV-1 protease and inhibits protease activity. HIV -1 protease is responsible for the cleavage of viral Gag and Gag-Pol precursors during viral maturation (25).

Clinical Stages of Infection

In the absence of antiretroviral therapy, HIV progress into three clinical stages (32) (Table 1):

Stage 1: Acute HIV Infection – It is characterized by flu-like illness lasting for a few weeks. It typically occurs during the first 2-4 weeks of HIV infection. Infected individuals have high viral titers during this phase, making them at higher risk of transmitting the virus.

Stage 2: Clinical Latency – In this phase, patients remain asymptomatic and viral replication rates are very low. In the absence of antiretroviral therapy, patients can remain in this phase for decades or can progress through it rapidly. At the end of this stage, viral loads rapidly multiply, and CD4 cell counts decline, resulting in immunodeficiency if left untreated.
Stage 3: Acquired Immunodeficiency Syndrome (AIDS) – It is the most severe form of illness defined by a marked reduction in CD4 cell count ( < 200 cells/mm) and resulting in much opportunistic illness. Lymph glands, chills, fever. Without the intervention of antiretroviral therapy survival rates are around three years.

Table 1: Stages of HIV-1 infection

<table>
<thead>
<tr>
<th>Stages of HIV-1 infection</th>
<th>CD4 counts</th>
<th>Symptoms</th>
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<tr>
<td>Stage -1</td>
<td>≥ 500 cells/µL</td>
<td>Acute HIV-1 infection and persistent generalized lymphadenopathy (PGL)</td>
</tr>
<tr>
<td>Stage - 2</td>
<td>200 – 499 cells/µL</td>
<td>Mostly asymptomatic for several decades if on cART.</td>
</tr>
<tr>
<td>Stage - 3</td>
<td>≤ 200 cells/µL</td>
<td>Opportunitistic infections and rare malignancies</td>
</tr>
</tbody>
</table>

Combination Antiretroviral Therapy

Zidovudine (AZT) is azidothymidine, nucleoside reverse transcriptase inhibitor, was first introduced as a treatment for HIV 1987 and proved to be beneficial o HIV patients.
Treatment with AZT was able to reduce mortality and opportunistic infections in AIDS patients by 8 to 24 weeks in an observational study (33). However, despite the early successes, therapy with AZT was marred with severe toxicities, such as bone marrow suppression, myalgia, and macrocytosis (33). In the mid-1990s, several other antiretroviral drugs were approved, which significantly improved the health outcomes of people living with HIV, transforming HIV into more of a chronic condition (34). With the advent of combination antiretroviral therapy regimen (cART), survival rates for individuals infected with HIV have increased to near normal lifespan in comparison to 11 months in a pre-cART era (35, 36).

Based on the mechanism of action, antiretroviral drugs are classified in the following categories (Table 2):

1) Nucleoside Reverse Transcriptase Inhibitors (NRTIs): NRTIs are phosphorylated by host cellular kinases, and they compete with endogenous nucleosides to get incorporated in growing chain of DNA. Once incorporated, NRTIs act as chain terminators by blocking the production of proviral DNA.

2) Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs): NNRTIs bind to the allosteric site of HIV-1 reverse transcriptase enzyme and form a hydrophobic pocket proximal to the active site resulting in reduced activity of the enzyme.

3) Protease Inhibitors (PIs): Protease inhibitors inhibit the action of HIV-1 protease, an enzyme which cleaves Gag and Gag-pol polyprotein precursor in the HIV-1 viral genome to generate matured active proteins.

4) Integrase Strand Transfer Inhibitors (INSTIs or Integrase Inhibitors): Integrase strand transfer inhibitors block the strand transfer step, which is required for the
integration of viral DNA into host cellular DNA. Integrase Inhibitors sequester magnesium cofactor ions of the enzyme and inhibits its activity.

5) Fusion Inhibitors: They are peptides which mimic HIV-1 fusion protein and prevent binding of HIV-1 to host cell. Enfurvitide mimics HIV-1 glycoprotein 41 (gp41).

6) CCR5 Antagonists: CCR5 Antagonist blocks the CCR5 coreceptor on the surface of host cells and prevents HIV entry into the cells.

7) Post Attachment Inhibitors: It is a monoclonal antibody which binds to the CD4 coreceptor on host cells and prevents attachment and entry of HIV into the cell.

8) Pharmacokinetic enhancers: They are used in treatment regimen to increase the effectiveness of drugs and improve pharmacokinetic properties.

The integrase strand transfer inhibitor based cART regimen recommended by HIV/AIDS guidelines, comprising of two nucleoside reverse transcriptase inhibitors (tenofovir, emtricitabine) and an integrase strand transfer inhibitor (dolutegravir) were used in this study (Table 3).

<table>
<thead>
<tr>
<th>Class of Drugs</th>
<th>HIV Medicines</th>
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Table 2: HIV medicines based on drug classes
<table>
<thead>
<tr>
<th>Category</th>
<th>Drugs</th>
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<tr>
<td>NRTIs</td>
<td>Abacavir, didanosine, emtricitabine, tenofovir (Nucleotide), tenofovir disoproxil fumarate (Nucleotide), tenofovir alfenamide (Nucleotide), lamivudine, zidovudine</td>
</tr>
<tr>
<td>NNRTIs</td>
<td>Efavirenz, etravirine, nevirapine, elvitegravir</td>
</tr>
<tr>
<td>PIs</td>
<td>Atazanavir, darunavir, fosampenavir, ritonavir, squinavir, tipranavir</td>
</tr>
<tr>
<td>INSTIs</td>
<td>Dolutegravir, Raltegravir, elvitegravir</td>
</tr>
<tr>
<td>Fusion inhibitors</td>
<td>Enfurvitide</td>
</tr>
<tr>
<td>CCR5 Antagonist</td>
<td>Maraviroc</td>
</tr>
<tr>
<td>Post-attachment inhibitors</td>
<td>Ibalizumab</td>
</tr>
<tr>
<td>Pharmacokinetic Enhancers</td>
<td>Cobicistat, Ritonavir</td>
</tr>
</tbody>
</table>

NRTI – Nucleoside reverse transcriptase inhibitor, NNRTI – Non-nucleoside Reverse transcriptase inhibitor, PIs – Protease inhibitors, INSTI – Integrase Strand Transfer Inhibitors
Table 3: Antiretroviral drugs to be examined in this study

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Class</th>
<th>Molecular Weight (g/mol)</th>
<th>Protein Binding</th>
<th>Drug Transporter Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenofovir</td>
<td>NtRTI</td>
<td>287.216</td>
<td>Low, 7%</td>
<td>MRP4 Substrate [36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OAT1&amp;3 Substrate [37]</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>NRTI</td>
<td>247.248</td>
<td>Low, &lt;4%</td>
<td>MATE1 Substrate [38]</td>
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<td></td>
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<td></td>
<td>MRP1 substrate (40) and Inhibitor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MRP2 &amp; MRP3 Inhibitor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P-gp inhibitor (1mM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CNT1, ENT2 [36, 37]</td>
</tr>
<tr>
<td>Dolutegravir</td>
<td>INSTI</td>
<td>419.385</td>
<td>High, &gt;99% bound to albumin</td>
<td>BCRP and P-gp, OCT-2 Substrate [41]</td>
</tr>
</tbody>
</table>

HIV-1 Structure and Proteins

The mature HIV-1 particle is about 100 nm in diameter. It consists of the outer lipid bilayer membrane called the envelope, which contains surface proteins (gp120) and transmembrane protein (gp41) product of the Env gene of the viral genome (34). Envelope proteins are responsible for the interaction with the CD4 receptor, which then results in viral entry into the host cell (23).

The inner surface of the viral membrane comprises of matrix shell has about 2000 copies of matrix protein (MA, p17). Conical capsid core found at the center of the virus has about 2000 copies of capsid protein (CA, p24). There are two copies of identical viral genomic RNA packed inside the capsid, which is stabilized by nucleocapsid (NA, p7) (35). These proteins (MA, CA, NA) are the cleavage products of p55, which is encoded by the Gag gene of the viral genome (35). Viral enzymes like reverse transcriptase, integrase, RNase H are found along with the viral RNA inside the capsid, which are encoded by the Pol HIV-1 gene (36).

HIV-1 has other regulatory proteins like Vif, Nef, Vpr that are packaged inside the virus particle. Alternatively, Tat, Rev, and Vpu proteins are not packaged inside the virus particle. Instead, they are synthesized inside the host cell (37). This work will focus mostly on HIV-1 Tat. Information of about other proteins is elaborately covered in the review by Faust et al. (38).

HIV-1 Tat

HIV-1 Tat (Transactivator of transcription), is a 14 kDa protein. Tat is also one of the first viral proteins to be synthesized by the host cell during the infection (43). After the
integration of viral DNA into the host genome, RNA II polymerase is recruited to the promoter region (5’ long terminal repeat) to start the transcription (25). Although several host transcription factors like NF-KB can bind to this region and increase viral gene expression, they mostly produce incomplete and short viral transcripts (44). To circumvent this, HIV-1 produces its transcription factor (HIV-1 Tat) to increase the fidelity of RNA II polymerase (45, 46).

HIV-1 Tat is an RNA binding transcription factor, which binds to TAR (Transactivator response element) located on viral RNA. After binding, it recruits host positive transcription elongation factor-b (p-TEFb) at the loop of 5’ end of viral RNA (47). Tat-P-TEFb complex phosphorylates RNA II polymerase resulting in expression of viral genome and post-integration events like maturation (48). P-TEFb consists of cdk-9 and cyclin T1 (Cyclin T1) subunits. In its inactive form, P-TEFb binds to 7SK snRNP (small nuclear Ribonuclear Proteins), where HEXIM1 (Hexamethylene bis-acetamide-inducible protein-1) protein inhibits the kinase activity of cdk-9. HIV-1 Tat competes with HEXIM1 for binding at this site, and in doing so releases p-TEFb from 7SK snRNP to be recruited at viral promoter for activation of RNA II polymerase (49).

HIV-1 Tat also alters the expression of cellular genes to promote viral replication. It induces transcription and secretion of chemokines (CXCL-10, CCL-2, CCL-3) in immature dendritic cells, resulting in recruitment of T-cell and monocytes (50). These events could amplify viral replication as these migrated cells can be further infected by HIV-1. Tat can bind to cellular promoters and increase expression of regulatory proteins like PP2A phosphatase leading to activation of proapoptotic pathways (51). HIV-1 Tat secreted extracellularly during infection can enter other cells through endocytosis (52).
Extracellular Tat has been implicated in various AIDS pathologies. For instance, extracellular Tat induces neovascularization responsible for tumor growth and metastasis in hypervascularized Kaposi’s sarcoma (53). HIV-1 Tat transactivates the genome of herpes virus, hepatitis C virus, and human Cytomegalovirus, facilitating opportunistic infections during AIDS (54–56). Lastly, HIV-1 Tat is neuro-inflammatory and plays a vital role in the neuropathogenesis of HIV-associated neurocognitive disorder (HAND) (57, 58). This document will mostly focus on the effects of HIV-1 about HAND.

**HIV-Associated Neurocognitive Disorder**

Approximately, 60% of individuals infected with HIV suffer from HIV associated neurocognitive disorder (HAND), which is a spectrum of neurocognitive impairment ranging from asymptomatic neurocognitive impairment to full-blown dementia (59). Despite aggressive use of combination antiretroviral therapy (cART), HIV viral loads persist in the central nervous system (CNS) (as measured in cerebrospinal fluid (CSF)) and the prevalence of mild to moderate neurocognitive impairment continues even after ten years of suppressive therapy (60). Based on severity, the HAND is classified into three groups: asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND) and HIV-associated dementia (HAD). In a post-cART era, asymptomatic to a mild form of the HAND are prevalent while the severe form (HAD) is rarely seen (61).

HIV-1 enters the brain soon after the infection (62, 63). It can either cross the blood-brain barrier (BBB) through transcytosis or through the “Trojan Horse” approach, where virus-infected immune cells migrate through BBB. Once inside the brain, virus sheds off from the immune cells principally monocyte-derived macrophages (MDMs), releases viral
proteins, and infects other cells in the brain (64, 65). In doing so, several chemokines and cytokines are released, which further increases the flux of immune cells into the brain through chemotaxis which results in the amplification of the infection (66). Inside the brain, HIV can infect many cell types like brain microvascular endothelial cells, glial cells (astrocytes, microglia), perivascular macrophages, and pericytes. There is scant evidence supporting HIV infection in neurons. Neurons lack CD+4 receptors but do express CCR5, CXCR4, CCR3 receptor required for HIV-1 infection. HIV-1 predominantly replicates in microglia and perivascular macrophages in CNS, while it infects other cells, but there is little or no replication (22). The infected cells secrete inflammatory cytokines and chemokines, which can contribute to neuronal injury and eventually neuronal death. There are two hypotheses regarding neuronal injury/death during HIV infection: (1) the direct injury hypothesis; HIV-1 Viral proteins like gp120, Tat, Vpr which are secreted during the infection are neurotoxic and that leads to neuronal injury and (2) the bystander effect hypothesis; which proposes neuronal injury/death are due to neuroinflammation caused by CNS infected cells (66, 67). Infected microglia and astrocytes release monocyte chemoattractant protein -1 (MCP-1, MCL-5, RANTES, CCL2), which is responsible for chemotaxis of MDMs into the brain via BBB (68). These MDMs can bring more virus inside CNS through Trojan horse approach and release viral proteins causing sustained inflammation in CNS. Microglia and astrocytes activated via feedback loop can further increase secretion of inflammatory from these
Figure 2: HIV-1 in the brain
cells. All these factors eventually contribute to neuronal injury or neuronal death (Figure 2).

This dissertation will focus on the effects of the HIV-1 Tat protein and drugs of abuse in different CNS cell types known to have an important role in the neuropathogenesis related to the development of HAND. The cell types used in this dissertation are brain microvascular endothelial cells, astrocytes, microglia, and pericytes.

**Human Brain Microvascular Endothelial cells**

Brain microvascular endothelial cells constitute the lining of the brain vasculature, which represents BBB. The blood-brain barrier forms the physical, metabolic and transport barrier between systemic circulation and CNS (69). The BBB is characterized by tight junction proteins (Claudin-5, Occludin, Junctional Adhesion molecules) which are responsible for the barrier properties of the BBB (67). These proteins are connected to the actin cytoskeleton via cytoplasmic proteins like zonula occludens -1, 2, 3 (ZO-1, 2, and 3). Tight junction proteins act as glue between endothelial cells in brain microvessels.

The passage of ions through BBB is restricted by tight-junction proteins, creating an ionic barrier which is vital for neural transmission and maintaining brain microenvironment (67). This charge barrier is measured as, Transendothelial electrical resistance (TEER), which experimentally indicates the integrity of monolayer of endothelial cells (70). BBB also contains γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (AP), and aromatic acid decarboxylase responsible for the metabolism of several drugs. Additionally, there are multiple transporters located on the luminal and abluminal side responsible for the influx or efflux of cargo, thereby having a precisely regulated system of permeability. This
selective permeability of solutes and ions helps maintain the ionic environment for the transduction of electrical signals in neurons.

**Effect of HIV-1 Tat on brain microvascular endothelial cells**

HIV-1 Tat enters the cell through absorptive endocytosis (43). Radio-labeled HIV-1 Tat injected intravenously in the mouse was found to be maximally distributed in the hippocampus, occipital cortex, and hypothalamus inside the brain, which is in congruence with HAND pathology observed in those parts of the brain in the Tat transgenic mouse model and post-mortem brain samples from HAND patients (71, 72). HIV-1 Tat is reported to decrease expression of tight junction proteins (claudin-5, occludin, and ZO-1) by up-regulating MMP-2 and 9, increased monocyte migration mediated via Rho activation (73), increased expression proinflammatory cytokines IL-6, IL-10, and TNF-α (74). Tat activates NF-κB and mitogen-activated phosphorylate kinase pathways leading to accumulation of TNF-α, which further up-regulates matrix metalloproteinase-9 (MMP-9) levels. Elevated levels of MMP-9 disrupts laminin and type IV collagen in brain microvasculature leading to increased permeability (75). HIV-1 Tat causes oxidative stress by releasing reactive oxygen species, which is amplified with TNF-α (76). HIV-1 Tat is also reported to increase expression of ABC family (P-glycoprotein) of transporters through a multitude of signaling pathways, which results in increased extrusion of antiretroviral drugs outside the brain (77–79)

**Human Astrocytes**

Astrocytes are derived from neuro-ectoderm embryonic lineage. Astrocytes provide a vital role in support of neurons and glial transmission (80). Potassium ions are released into
the extracellular space during neuronal activity, increasing the concentration of potassium (K⁺) ions and resulting in depolarization of neuronal membrane affecting axonal conduction and synaptic transmission. Astrocytes clear these potassium ions through potassium channels (Kir4.1) (81, 82). Astrocytes are responsible for the maintenance of the blood-brain barrier by secreting growth factor-like Vascular endothelial growth factor (VEGF), glial -derived neurotrophic factor (GNDF), and basic fibroblast growth factors (83). These factors promote the formation of tight junctions, the polarization of transporters, and inducing enzymes at the BBB (83).

**Effect of HIV-1 Tat on astrocytes**

One of the hallmarks of the pathology of HAND is astrocytosis, which is characterized by hypertrophy of astrocytes and increased expression of the glial fibrillary acidic protein (84). HIV-1 virus and viral proteins (HIV-1 Tat, gp-120) released due to HIV infection are responsible for astrocytosis. HIV-1 Tat is a potent activator of NF-κβ, it increases production of MCP-1 (CCL2), IL-6, and TNF-α in astrocytes via NF-κβ signaling pathway (85). Higher levels of MCP-1 are correlated with neurocognitive deficits due to HIV-1 infection (86). HIV-1 Tat also causes oxidative stress in astrocytes by releasing reactive oxygen species causing increased expression of adhesion molecules like VCAM-1/ICAM-1 in endothelial cells (83). Up-regulation of adhesion molecules leads to increases adhesion of monocyte-derived macrophages, leading to disruption of BBB.

Glutamate is a predominantly excitatory neurotransmitter in the brain. Removal of glutamate from the extracellular fluid is vital for the normal functioning of neurons. Glutamate is cleared mostly by neurons and astrocytes; however, it is reported that glutamate transporters on astrocytes are responsible for 90% of glutamate clearance
In vitro studies have demonstrated a reduction in the glutamate transporter (EAAT-1,2) in astrocytes in response to exposure to HIV-1, gp-120 and HIV-1 Tat (88, 89). Excitotoxic damage to neurons in HIV-1 infection is mediated by activation of the NMDA receptor by glutamate resulting in the intracellular accumulation of calcium ions (87).

Astrocytes respond robustly to viral proteins (HIV-1 Tat, gp120) and release inflammatory cytokines (glutamate, ROS, TNF-α, IFN-γ, and IL-6), which cause neuronal injury or death. Astrocytes are also considered to be latently infected with HIV, which makes CNS a reservoir of HIV (90). This dysregulation in astrocytes contributes significantly to the neuropathogenesis of HAND.

**Human Microglia**

Microglia are the site for productive replication of HIV in CNS. They are resident macrophages of the CNS derived from mesoderm (43). Perivascular macrophages seed the initial HIV-1 infection in CNS near the brain microvasculature, and the virus is transmitted through parenchyma towards microglia (22). Typically, microglia appear either in the resting or active state. The resting versus the active state of microglia can be differentiated by their morphology; cells are rounded in an active state and ramified in resting state (22). Activated microglia are absent in adult life but can be seen in developing the brain, or inflammatory conditions, such as HAND, within fully developed CNS (22). Infected microglia can release the virus, viral proteins (gp-120, tat, vpr), inflammatory cytokines (TNF-α, Platelet-activating factor (PAF), IL-1β), which can further activate uninfected microglia/astrocyte through positive feedback loop or can be directly neurotoxic (92). Despite microglia being a major site of productive replication, much of
the sustained inflammation, contributing to neurotoxicity is due to the response elicited by uninfected microglia (90). Even in patients with undetectable viral loads in their plasma, sustained inflammation is observed in microglia. This is a potential explanation for the prevalence of mild and asymptomatic forms of HAND even during suppressive antiretroviral therapy (92).

**Effects of HIV-1 Tat on microglia**

HIV-1 Tat induces expression of pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, and IL-6) via NF-KB signaling pathway (93). These cytokines can further activate astrocytes through a positive feedback loop or are directly neurotoxic. Additionally, HIV-1 Tat causes secretion MCP-1(CCL2), IL-8, IP-10, MIP-1α, MIP-1β and RANTES (CCL5) in human microglia, which can lead to chemotaxis of monocytes and leucocytes inside the brain (94). Overall, this milieu of inflammatory cytokines causes prolonged inflammation in CNS resulting in excitotoxic damage to neurons.

**Human Pericytes**

Pericytes are flat, undifferentiated, contractile cells that wrap around the basal lamina of the microvasculature (95). In an in-vitro study, it was demonstrated that co-culture of human pericytes with brain microvascular endothelial cells results in polarization of endothelial cells, suggested by proper localization of P-glycoprotein (96). Furthermore, studies in adult pericyte deficient mouse showed increased permeability to water and low-high molecular weight tracers. Pericytes maintain BBB by regulating expression of BBB specific genes and inducing polarization of astrocytic end-feet via PDGFRB activation (97). Additionally, in a study from the brains from HIV-infected patients as compared to
uninfected brain tissue, pericyte markers like α1-integrin, α1-smooth muscle actin were significantly reduced in HIV, even for those patients that had been on antiretroviral therapy, suggesting that HIV infection results in the loss of pericyte coverage irrespective of antiretroviral therapy (98). Upon exposure to TNF-α and IL-1β, upregulation of adhesion molecules was observed, which resulted in increased adhesion of monocytes. Pericyte inclusion into an in-vitro BBB model with endothelial cells resulted in enhanced monocyte migration as compared to the endothelial cell-only BBB model. (98).

**Effects of HIV-1 Tat on Pericytes**

HIV-1 Tat induces MAPK (Erk and JNK) pathways, which lead to nuclear localization of NF-κB and induction of PDGF-BB. Elevated levels of PDGF-BB results in loss of integrity of BBB due to movement of pericytes away from microvasculature (99).

**Drug Abuse and HAND**

Drug abuse and HIV are interlinked epidemics. Injection drug users are nine times at higher risk of getting HIV. Not only does substance abuse increases the risk of transmission, but it intrinsically alters neuropathogenesis of HIV. There is substantial evidence from laboratory-based and epidemiological studies suggesting drugs of abuse can exacerbate disease progression in HIV and also affects mortality and morbidity of HIV patients. This document will investigate the effects of drugs of abuse (methamphetamine and morphine) in brain endothelial cells, astrocytes, pericytes and microglia.
Methamphetamine

Methamphetamine is the second most abused drug in the world (100). Its co-exposure is frequently seen in HIV-infected patients, perhaps, due to risky sexual behavior caused under its influence and sometimes when injected through needles (101). Cognitive impairment caused by methamphetamine can lead non-adherence to medications worsening symptoms of HAND (102). Methamphetamine can enhance replication of HIV-1 by activating the promoter in microglia; an effect that can be synergized with HIV-1 Tat (103). It also causes disruption of blood-brain barrier by oxidative stress, excitotoxic damage by glutamate and release of inflammatory cytokines (104). The release of inflammatory cytokines by activated glia after methamphetamine exposure is linked to behavioral deficits in HAND (105). Methamphetamine effects on the BBB can be variable and often transient. There are several reports of methamphetamine exposure resulting in tight junction protein alterations, enhanced BBB permeability, diminished barrier tightness, and increased monocyte migration across the BBB (106, 107). However, other investigators have reported either no effects or transient effects on TEER, tight junction protein expression, and on paracellular permeability (108–111). We hypothesize that Meth alone or with HIV-1 Tat disrupts the BBB and alters antiretroviral penetration inside the brain.

Morphine

Morphine is an alkaloid obtained from seeds of the poppy, *papaver somniferum*. It exerts its pharmacological action of alleviating pain by acting as an agonist of µ-receptor. Activation of µ-receptor by morphine leads to closing of voltage-gated calcium channels,
stimulation of potassium efflux, and decrease the production of cAMP due to inhibition of adenylyl cyclase (112). Collectively, these events lead to decreased neuronal transmission and inhibition of neurotransmitter release (112). Morphine also regulates dopamine within nucleus accumbens predisposing it to abuse liability. A majority of action by morphine is exerted through μ-receptor, and to a lesser extent by κ or δ- opioid receptor (112). Despite having sufficient lipophilicity, morphine has a relatively low uptake into the brain (113). This low uptake of morphine can be explained by it being a substrate of P-glycoprotein (P-gp) located at the luminal side of the BBB (114). Expression of P-gp at BBB has been observed to alter during inflammation and other pathological conditions, which directly affects morphine uptake into the brain (114).

Injection drug users of opiates (morphine) have an increased risk of transmission of HIV. It is also reported that opiates fasten disease progression and exacerbate neuropathology in SIV models (115). Clinical studies in the post-cART era have found that HIV-1 infected individuals who abuse heroin (an acetylated derivative of morphine) showed significant neurocognitive deficits especially poor working memory and recall (116). These deficits persisted even after abstinence from heroin for long periods. Initially, morphine increases efflux of inwardly rectifying potassium current making neuron less excitable, however, from chronic exposure and tolerance neurons are decoupled from inwardly rectifying current resulting in increased excitability (113). Morphine provokes pronounced and robust inflammatory response in glia of HIV-1 infected individuals, especially astroglia. This response is spiraled by positive feedbacks loops formed between astrocytes and microglia resulting in sustained cytokine production (117). Overall, interactive effects of
prolonged inflammation by opiates on top of already existing inflammation due to HIV-1 infections leads to neurotoxicity exacerbating HAND.

**Effects of morphine on brain microvascular endothelial cells**

Brain microvascular endothelial cells express μ and opioid-related nociceptive opioid receptors. Endogenous opioids play an essential role in vascular development and differentiation (118). Activation of κ-opioid receptors are implicated in reduced vessel growth and differentiation, morphine being a mixed μ/κ opioid receptor agonist is likely to affect vessel growth and differentiation of endothelial progenitor cells (118). Mahajan et al. reported that treatment of brain microvascular endothelial cells with morphine causing reduction in TEER (119). TEER is a measure of blood-brain barrier integrity, as it measures the flux of ions through the barrier. Additionally, treatment of brain microvascular endothelial cells with HIV-1 Tat and morphine resulted further decrease in TEER values, demonstrating the additive effect (119). Treatment with morphine showed decreased expression of ZO-1 and occludin. However, the combined effects of Tat and morphine were not statistically significant compared to morphine alone (119). Morphine and Tat showed an additive effect on transmigration of PBMCs through BBB. These results suggest that morphine causes an increase in blood-brain barrier permeability, which is exacerbated by HIV-1 Tat (119). Clinically, it means more virus can invade the brain due to increased permeability resulting in worsening of HAND.

Morphine and Tat act synergistically to increase the production of inflammatory cytokines (TNF-α, IL-8) and intracellular calcium (121). These events can lead to activation of Myosin light chain kinases mediated by IP3 resulting in dysfunction of BBB and including a leakier barrier (119).
HIV-1 Tat and morphine were found to increase the expression of P-gp. P-gp is an efflux transporter located on the luminal side of blood-brain barrier (119). Many antiretroviral drugs are a substrate of P-gp, increased expression of P-gp results in more efflux of the drug outside the brain, the worsening prognosis of HAND.

**Effect of morphine on astrocytes**

Astrocytes express µ, κ, δ opioid receptors (120). Also, they have undeniable significance in neuronal support and glial transmission (118). There are reports of differential expression of opioid receptors on astrocytes on the basis of the cell cycle and development stages. There is also a regional difference in the expression of opioid receptors in astrocytes located in different parts of the brain (121). Overall, δ and κ opioid receptors are expressed at a much higher level than µ opioid receptor from astrocytes isolated from all parts of the brain (122). Cortical astrocytes had the highest level of total opioid receptors based on mRNA expression, as well as µ receptor expression (122).

Opiates drugs act by disrupting the ion and neurotransmitter homeostasis maintained by astrocytes in extracellular space, thereby causing neuronal injury and death. Opioids cause increases in intracellular concentration of calcium in astrocytes mediated IP3 pathway, which is synergistically increased by HIV-1 Tat, resulting in increased production of CCL2, CCL5, IL-6, and TNF-α. HIV-1 Tat and morphine cause increased production of RANTES and MCP-1 resulting in recruitment and activation of microglia and sustained inflammation (83). Morphine causes decrease expression of glutamate uptake transporter EAAT1 and EAAT2, affecting glutamate buffering capacity of astrocytes, in turn causing excitotoxic damage to neurons (118). HIV-1 Tat ± morphine can increase
replication of HIV, increased cytokine release and causes neurotoxicity. In a nutshell, morphine exacerbates neuropathogenesis of HAND in astrocytes.

**Effect of morphine on Microglia**

Microglia express µ, κ, δ opioids receptors. Microglia are one of the major sites for productive replication of the virus in CNS. Morphine has shown to cause microglial recruitment and activation (123). Morphine provokes major pathological events regarding HAND in microglia like an increase in inflammatory cytokine release, increase in oxidative stress, disruption of intracellular calcium homeostasis, and increased trafficking of monocyte-derived macrophages (124). Morphine is found to induce expression of CCR5 and CXCR4 receptors on microglia, which maybe partly due to secretion of chemokines (TNF-α, IL-2) that induces expression of chemokine receptor or decrease the production of chemokines which internalize these receptors in microglia (125). Collectively, morphine exacerbates the dysregulation of microglial functions, in an already compromised state due to productive infection of HIV-1.

**Effect of morphine on pericytes**

There were very few studies in current literature studying the effects of morphine on pericytes associated with brain micro-vessels. In an in-vitro study, morphine causes MAPK/ERK-mediated phosphorylation in pericytes mediated by the release of platelet-derived growth factor-BB from human umbilical vein endothelial cells, resulting in promotion of tumor angiogenesis and impairment of antiangiogenic therapy (122). It remains to be investigated the role of morphine in HIV-1 infected pericytes. Being a critical mediator for development and maintenance of blood-brain barrier, any inflammatory response from pericytes will have a reverberating response to the already disrupted BBB.
Antiretroviral penetration into the brain

The BBB limits antiretroviral drug penetration into the brain by acting as a physical impediment to the transport of drugs in CNS. It also possesses several efflux and uptake transporters, which are responsible for restricting antiretroviral drugs in the brain (Table 3). Once inside the brain, drug penetration into different CNS cells is further reduced by these transporters. This reduced penetration of antiretroviral drugs had caused the sub-therapeutic concentration of drugs in the brain leading to low-grade viral replication, CNS compartmentalization of the virus, drug resistance and irreversible CNS damage (126).

CNS penetration effectiveness (CPE) score measures drug penetration inside the brain; higher scores indicates more penetration in the brain (127). There had been a reduction in HIV RNA in CSF with the drug with higher CPE scores (61). Besides that, the utility of CSF as a surrogate marker for CNS drug exposure remains controversial. CSF drug concentration indicates the extracellular availability of the drug in the brain; however, it does not divulge information on intracellular drug concentration in brain parenchyma (128). In addition to that, there had been an inconsistent relationship between higher CPE score and clinical outcomes of HAND (61). There is a varying degree of efficacy of antiretroviral drugs in different cell types. For instance, NRTI's requires lower EC$_{50}$ in macrophages than lymphocytes due to low levels of endogenous nucleoside pool in macrophages compared to lymphocytes, which causes lower competition between drug and nucleoside in macrophages than in lymphocytes indicates differential efficacy of antiretroviral drugs (131, 132). Gray et al., showed EC$_{90}$ for NRTIs (Lamivudine, Stavudine, and Zidovudine) was many times higher than the achievable concentrations
in CSF (131). These results warrant a thorough investigation into antiretroviral drug penetration in different brain cell types and brain regions.

Table 3: Transporters expressed on cell type of interest

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Efflux Transporter Expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCMEC/D3 &amp; HBMEC</td>
<td>P-gp, MRP-1,2,4,5 and BCRP(132), MATE (low level in hCMEC/D3)(133)</td>
</tr>
<tr>
<td></td>
<td>MATE is not present in HBMEC(134)</td>
</tr>
<tr>
<td>Human Astrocytes</td>
<td>P-gp(135)-(136), MRP-1, 4, 5(137), BCRP(138)-(139), OCT-3 (140)</td>
</tr>
<tr>
<td>Human Pericytes</td>
<td>P-gp(135)</td>
</tr>
<tr>
<td>Human Microglia</td>
<td>P-gp(141), MRP-1(142)</td>
</tr>
</tbody>
</table>

hCMEC/D3- human Cerebral Microvascular Endothelial cells/D3, HBMEC- Human Brain Microvascular Endothelial Cells, P-gp – P-glycoprotein, MRP-1, 2, 4, 5 – Multidrug Resistant Protein 1, 2, 4, 5, BCRP-Breast Cancer Resistant Protein, OAT- Organic Anion Transporter, OCT – Organic Cation Transporter.
INTRODUCTION

Despite aggressive use of combination antiretroviral therapy (cART), HIV infection results in HIV-associated neurocognitive disorders (HAND) in about half of infected individuals (143–148). Neurocognitive dysfunction can include decreased attention and concentration, as well as deficits in information processing, memory, learning, and psychomotor speed. There may also be motor slowing, incoordination, and tremor (143, 149). Although the severity of the most severe form of HAND, HIV-associated dementia (HAD), has diminished in the post-cART era, the incidence of HAND and accompanying deficits in neurobehavioral function persist as HIV/AIDS evolves into a chronic disease (147, 150).

The neurocognitive impairment seen in HIV type 1 (HIV-1)-infected individuals in the post-cART era is thought to principally result from a loss in synaptic connectivity (150, 151). The synaptodendritic injury is likely caused by the combined release of “cellular toxins”, which includes excessive oxyradicals and increases in inflammatory cytokine and chemokine secretion, and “virotoxins” consisting of neurotoxic viral proteins such as Tat and gp120 (152, 153). HIV is thought to enter the brain through infected macrophages, which traverse the vascular endothelium associated with the blood-brain barrier (BBB) (74, 154), and take up residence within the perivascular space of the central nervous system (CNS). The infected perivascular macrophages can subsequently infect resident...
microglia (155–157), as well as astroglia (158, 159). Limiting the bidirectional trafficking of HIV-infected macrophages into the CNS and restricting HIV replication within CNS compartments is of critical importance in restricting the viral production proteins and limiting CNS inflammation that drives HAND (22, 160–162).

A key limitation in fighting HIV infection in the CNS results from the restricted penetration of antiretroviral drugs across the BBB despite excellent systemic effectiveness. The BBB is the primary barrier limiting the passage of substances from the blood into the brain. The BBB is a selective barrier composed of microvascular endothelial cells lining the brain microvessels. The endothelial cells are surrounded by a basal lamina, pericytes, and astrocytic endfeet, which contribute to the induction and maintenance of BBB properties (69, 163–167). The BBB has an active interface between the circulation and the CNS, restricts the free movement of substances between blood and CNS, and is critical in maintaining CNS homeostasis (168). This barrier function is maintained by two major mechanisms. A paracellular barrier is formed by tight junctions between endothelial cells and restricts movement of water-soluble compounds between adjacent cells. A transcellular barrier is mediated by transcytosis involving the trafficking of specific substances in endocytotic vesicles through endothelial cells and is also mediated by the movement of specific substances by specific transporters. Endothelial cells express various efflux transport proteins (such as P-glycoprotein (P-gp; gene symbol \(ABCB1\)), breast cancer resistance protein (BCRP; gene symbol \(ABCG2\)), and multidrug resistance proteins (MRPs; gene family symbol, \(ABCC\) family). These transporters limit CNS exposure by active expulsion of substances from brain endothelial cell cytoplasm back into the blood (168).
Drug abuse exacerbates cognitive impairment and pathologic CNS changes in HIV-infected persons. Experimental and clinical evidence indicates that HIV neuropathogenesis and neurocognitive deficits are exacerbated with co-exposure to methamphetamine (169–172). Methamphetamine induces HIV transcription, potentiates HIV viral protein-mediated oxidative stress pathways in the brain (173), and increases pro-inflammatory cytokines (174).

Individually, HIV-1, HIV viral proteins, gp120 and Tat, and methamphetamine are known to disrupt the BBB. Our lab has demonstrated increased leakiness of fluorescent tracer compounds into the brain in Tat transgenic mice (175). In vitro, Tat and gp120 treatment of endothelial cells results in decreases in several important tight junction proteins with resultant increases in endothelial monolayer permeability (176). HIV-1 and the viral proteins Tat and gp120 have complex and variable effects on drug efflux proteins within cells of the neurovascular unit. In rodent brain microvascular endothelial cells, Tat induces P-gp and Mrp1 expression and function in brain microvascular endothelial cells (78, 177, 178). However, in primary human astrocytes, HIV can downregulate P-gp expression (179, 180). Methamphetamine effects on the BBB can be variable and often transient. There are several reports of methamphetamine exposure resulting in tight junction protein alterations, enhanced BBB permeability, diminished barrier tightness, and increased monocyte migration across the BBB (106, 107). However, other investigators have reported either no effects or transient effects on transendothelial electrical resistance (TEER), tight junction protein expression, and on paracellular permeability (108–111). To date, there is limited information regarding the effects of methamphetamine and Tat co-exposure on the BBB. One study reports enhanced alterations in tight junction protein
expression in the brain endothelium, a breakdown of BBB integrity as shown by decreased TEER and enhanced monocyte migration upon co-exposure with Tat and methamphetamine (106). Although these limited studies provide some insight into how HIV-1 proteins and methamphetamine can alter the integrity of the BBB, it is not known how the molecular alterations in the BBB disrupt the function of the barrier. Specifically, it is uncertain the extent to which HIV-1 protein and methamphetamine-induced alterations in cART penetration result from disruptions to BBB integrity and/or drug transporter function. In the current study, the effects of Tat and methamphetamine exposure on factors affecting antiretroviral penetration, such as paracellular and transcellular barrier integrity and drug transporter function, were investigated in an in vitro model of the BBB.

Methods

METHODS

Materials and Reagents

Ascorbic acid, DMSO, human basic fibroblast growth factor (bFGF), hydrocortisone, lucifer yellow, Rhodamine 123 (Rh123), verapamil hydrochloride, were obtained from Sigma Aldrich (Saint Louis, MO). Propranolol was purchased Fisher Scientific (Pittsburg, PA). MK-571 was purchased from Cayman Chemical (Ann Arbor, Michigan). The following reagents were obtained through the AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (NIAID), National Institute of Health (NIH): atazanavir and lopinavir. [3H]-atazanavir and [3H]-propranolol was purchased from Moravek (Brea, CA). HIV-1 Tat1-86 was purchased from Immunodiagnostics, Inc (Woburn, MA). Endothelial basal medium (EBM-2) was purchased from Lonza (Basel, Switzerland);
Chemically defined lipid concentrate, penicillin-streptomycin, trypsin/EDTA, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Invitrogen (Waltham, MA); fetal bovine serum (FBS) from PAA The Cell Culture Company (Pasching, Austria); and Lithium chloride from Merck (Kenilworth, NJ).

Cells

hCMEC/D3 cells were grown in EBM-2 supplemented by Lonza's EGM2-MV Bullet Kit (Fisher, Waltham, MA, cat# NC9902887, which has 5% FBS plus growth factors. Additional supplementation with 10 mM HEPES, 1X penicillin/streptomycin and 1 ng/mL bFGF was also made, and this media was called growth media. After hCMEC/D3 were plated on transwells, the cells were grown in EBM-2 media, supplemented with 5% FBS, 1× penicillin/streptomycin, 1 ng/mL hydrocortisone, 5 μg/mL ascorbic acid, 0.05% chemically defined lipid concentrate, 10 mM HEPES, 1 ng/mL bFGF and 10 mM lithium chloride (subsequently referred to as transwell medium). Cells were grown in transwell media for 48 h, and then serum supplementation was decreased to 1.25% FBS.

Blood-Brain Barrier Model

Human brain microvascular endothelial cells, hCMEC/D3, were generously provided by Dr. Babette Weksler of Weill Cornell Medical College, Cornell University (New York, NY). This cell line was selected because it is well characterized, of cerebral origin, and is derived from human brain microvascular endothelium (132, 181). These cells express chemokine receptors, up-regulate adhesion molecules in response to inflammatory cytokines, and display characteristics of the BBB, including tight junctional proteins and the capacity to actively exclude drugs (181, 182).
hCMEC/D3 cells, between passages 25 and 35, were maintained at 37°C with 5% CO₂. To generate the BBB model, cells were seeded at a density of 15 × 10⁴ cells/well on the upper surface (the apical chamber) of 12-well transwell plates. The base of each transwell insert is configured with a polycarbonate membrane with 3.0 μm diameter pores (Corning, Fisher Scientific, Pittsburgh, PA) pre-coated with rat-tail collagen type I (Cultrex). Medium was replenished with fresh medium every other day for 4 days until functional studies were performed. To assess the integrity of the BBB, TEER was measured on the transwell membrane insert plates using EVOM2 chopsticks from World Precision Instruments (Sarasota, FL). Preliminary data from our lab demonstrates that the hCMEC/D3 cells form its tightest paracellular barrier by day 6 in vitro, as determined by TEER (Fig. 4A).

**Treatment**

The Tat concentration (100 nM) used in this study was based on published research (85, 124, 183–185) and is also within the range of concentrations detected in serum of HIV-infected individuals (186). A methamphetamine concentration of 10 µM was chosen based on preliminary screening of different concentrations (0.01, 0.1, 1, and 10 µM). This range was selected based on concentrations detected in the blood of methamphetamine users (187).

**Cell Viability**

LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies, Waltham, MA) was used to measure hCMEC/D3 viability when treated with Tat and methamphetamine. A working solution of 2 µM ethidium homodimer-1 (EthD-1) and 1 µM calcein-AM was made in DPBS. hCMEC/D3 cells were seeded on a sterile 24-well plate at 5 × 10⁴ cells/mL.
hCMEC/D3 cells were then treated with Tat and methamphetamine) for 24 h. 200 μL of working solution was added to each well for 30 min at RT. Fluorescent images were taken at excitation/emission wavelengths 495/515 nm to visualize calcein-AM (live cells) and 528/617 nm to visualize EthD-1 (dead cells). Ten images were taken at random for both calcein-AM and EthD-1 and analyzed using ImageJ software (NIH). The relative ratio of live/dead cells was quantified by evaluating the fluorescent pixels of green and red.

**Substrate Permeability**

Briefly, apical-to-basolateral flux (A-to-B; mimicking blood to brain flux across the BBB) of the compound was conducted as follows. Cell monolayers were pre-incubated with transport buffer (Hanks’ balanced salt solution (HBSS) with 25 mM D-glucose and ten mM HEPES, pH 7.2) for 30 min at 37°C. The apical (donor) compartment buffer was replaced with 0.5 mL of transport buffer containing substrate. The accumulation of the compound in the receiver (basolateral) compartment was assessed at intervals up to 60 min. For basolateral-to-apical flux (B-to-A; mimicking brain to blood flux across the BBB) flux, the substrate was added to the basolateral (lower) compartment, and samples were collected from the apical compartment at different time points. At each time point, 100 μL was sampled from the apical compartment. The same volume of transport buffer (without substrate) was added back to the apical compartment to keep the total volume within the compartment the same over the experiment. After the experiment, the amount of substrate accumulated within the receiver compartment was measured by the appropriate method (see specific sections below).
**BBB permeability**

HIV-1 Tat and methamphetamine effects on BBB permeability were determined as described above with some modifications. Lucifer yellow (100 μM, 0.521 kDa) was added to the apical compartment and sampled from the basolateral (receiving) compartment at different time points to assess the paracellular permeability. [³H]-propranolol flux across the BBB was used to measure any changes in non-efflux transporter-mediated transcellular transport. Propranolol (10 μM) crosses cells transcellularly, but its transport is not influenced by active transport proteins, such as the efflux transporter P-gp (188). Control values were determined by compound flux across the membrane inserts with no cells. At the conclusion of the experiment, the amount of lucifer yellow within the basolateral (receiver) compartment was measured using a 96-well fluorescence plate-reader (PHERAstar FS, BMG Labtech, Cary, NC) with appropriate excitation/emission wavelengths. [³H]-propranolol concentrations were measured using a liquid scintillation analyzer (Perkin-Elmer TriCarb 2800TR).

**Antiretroviral Drug Flux across the BBB.** HIV-1 Tat and methamphetamine effects on antiretroviral flux across the BBB was determined as described above. The antiretroviral drugs atazanavir and lopinavir were used in this study. A-to-B flux of both antiretroviral compounds was measured. [³H]-atazanavir and lopinavir concentrations were measured using liquid scintillation, and LC-MS/MS-based method developed by Wang et al., respectively (189).

**Cellular Accumulation Experiments.** hCMEC/D3 cells were grown to confluence in a 6-well tissue culture plate. Transport protein functional activity was analyzed by measuring the intracellular accumulation of the substrates Rh123 (5 μM) and atazanavir
(100 μM). hCMEC/D3 cells were treated with Tat (100 nM) and methamphetamine (10 μM) for 24 h. The cells were pre-incubated with HBSS buffer containing the P-gp inhibitor verapamil (100 μM) and/or the MRP-1 inhibitor MK-571 (30 μM) for 30 min at 37°C (temperature maintained throughout the experiment). Cells were washed with HBSS and incubated with dosing solution containing Rh123 with and without inhibitor for one h. At 60 min, each well was washed twice with ice-cold HBSS and cells were lysed using NP40 buffer and 25× CPI. At the conclusion of the experiment, the intracellular content of Rh123 was measured using a fluorescence plate reader with appropriate excitation/emission wavelengths. Atazanavir concentrations were measured using a liquid scintillation analyzer. Intracellular drug accumulation was normalized to protein content for each well.

**Western Blotting**

Protein expression was analyzed by immunoblotting, using standard techniques. hCMEC/D3 cells were treated for 24 h with Tat and methamphetamine, cells were lysed, total protein was harvested, and protein concentrations were measured by Pierce BCA protein assay. Twenty μg of cell lysate was loaded on 12% Mini-Protean TGX gel (Bio-Rad, Hercules, CA) for all proteins, except ZO-1 where 7.5% Mini-Protein TGX gel was used. Following electrophoresis and transfer to PVDF, the membrane was blocked in 5% nonfat milk solution and incubated overnight in appropriate primary antibody. Antibodies used include mouse anti-β-actin (1:4000, Sigma Aldrich, cat. A1978), mouse anti-P-glycoprotein (C219, 1:200 dilution, Covance, Princeton, NJ, cat. SIG-38710, rat anti-MRP-1 (1:50 dilution, Kamiya Biomedical Company, Seattle, WA, cat. MC201) and rabbit anti-ZO-1 (1:500) dilution, Life Technologies, Cat 402200). Blots were then incubated for at RT for 1 h with the horseradish peroxidase-conjugated appropriate secondary
antibodies [anti-mouse (1:20,000), anti-rabbit (1:20,000) or anti-rat (1:4000)]. Signals were enhanced using chemiluminescence using SuperSignal West Dura System (Pierce/Thermo Fisher Scientific) and detected by exposure to ChemiDoc system (Bio-Rad). The chemiluminescence signal intensity was quantified using the ImageLab™ software (Bio-Rad). All protein expression data are expressed as relative density, which was obtained by the ratio of the absolute density of transporter to that of β-actin.

Quantitative PCR methods

cDNA was prepared from 300 ng RNA per sample using the iScript kit (Bio-Rad) according to the manufacturer’s instructions. Pre-designed TaqMan assays were obtained from Life Technologies for ABCB1 (Hs00184500_m1) and ABCC1 (Hs01561483_m1). Quantitative PCR (qPCR) analysis was performed on a QuantStudio3 (Life Technologies) using three duplicates per sample, with five biological replicates per condition, for both target and the endogenous control, human TBP (TATA-box binding protein, Life Technologies). Each plate also included a serially-diluted standard sample in addition to no template controls.

Data Analysis

Transport of compounds of interest are expressed in terms of apparent permeability (Papp) and is described by the following equation

\[ Papp = \frac{dX}{dt} \times A \times C_o \]

where \( \frac{dX}{dt} \) is mass of the transported compound (X) transported over time (t), A is the surface area of the membrane insert, and \( C_o \) is an initial concentration in the donor compartment (190).
Statistics

All the data was represented as mean ± SEM. TEER values, apparent permeability, and western blots were compared by one-way ANOVA using Tukey’s post hoc test. Rh123 flux from apical to basolateral compartments (Papp\textsubscript{A-to-B}) and the basolateral to apical compartments (Papp\textsubscript{B-to-A}) were compared by using two-way repeated measures ANOVA with Tukey’s post hoc test. Intracellular accumulation of Rh123 and atazanavir with and without transporter inhibitors were compared by two-way ANOVA using Tukey’s post hoc test. The statistical analysis was performed in GraphPad Prism software, version 7.0. \( p \) values < 0.05 were considered significant for all comparisons.

RESULTS

Cell Viability Studies

These studies were done using the LIVE/Dead™ assay to exclude the possibility that any of the treatments might be cytotoxic to hCMEC/D3 cells under our experimental conditions (Figure 3). Cells treated with a cytotoxic level (70%) of ethanol served as a positive control for dead cells, while untreated cells served as positive control for live cells. Our results showed that none of the treatments caused hCMEC/D3 cell death: Live cells: Control cells 97 ± 0.7%, Tat 97 ± 1.6%, Meth 99 ± 0.4%, Tat + Meth 98 ± 0.5% and; Dead cells: control 3 ± 0.7%, Tat 3 ± 1.6%, Meth 1 ± 0.4%, Tat + Meth 2 ± 0.5% (Figure 3A).

Tat and Methamphetamine Effects on Factors Affecting BBB Integrity

Transendothelial Electrical Resistance (TEER)
Tight junctions form an impervious barrier between brain endothelial cells that hamper free movement of ions. This charge difference is a measure of TEER. Higher TEER values are indicative of a tighter barrier. TEER values were measured on consecutive days, and the highest values were on days five and six, 44.9 ± 5.1 Ω * cm² to 52.5 ± 4.7 Ω * cm² (mean ± SEM), respectively (Figure 4A). Based on this observation, all the experiments involving vectorial transport on transwell inserts were done on day 5, and cells treated with Tat, methamphetamine, or Tat + methamphetamine on day four (24 h before transport experiment). There was a significant decrease in TEER values in the Tat only group (34 ± 2.4 Ω * cm²) as compared to control (42 ± 3.2 Ω * cm²; p < 0.05), suggesting impairment of barrier integrity upon Tat exposure. However, TEER values for methamphetamine and Tat + methamphetamine treatments were not statistically significant from controls (Figure 4B).

**Tight Junction Expression**

ZO-1 is tight-junction associated protein which anchors tight junction to the actin cytoskeleton. It is responsible for maintaining integrity and tightness of the barrier (191). Changes in expression of ZO-1 may lead to alteration of paracellular permeability across blood-brain barrier (192). We measured the effect of Tat and methamphetamine on the expression of ZO-1 protein in hCMEC/D3 cells via western blot (Figure 4C). Exposure to HIV-1 Tat reduced ZO-1 expression by (58 ± 16%, p = 0.2137), methamphetamine significantly reduced expression of ZO-1 (40 ± 18%, p = 0.0465), and HIV-1 Tat + methamphetamine co-exposure also significantly reduced expression of ZO-1 (26 ± 15%, p = 0.0132), as compared with controls (Figure 4C).
Figure 3: Tat and methamphetamine effects on cell viability.  

**A.** The green portion of the bar represents the percentage of live cells, and the red portion represents the percentage of dead cells. Data are represented as a mean percentage of live or dead cells ± SEM from n = 3 independent experiments; each experiment was performed in duplicate. **B.** Fluorescent images of cells labeled with calcein-AM (Live, Green) and ethidium homodimer (EthD-1) (Dead/dying, Red). Treatments (24 h) do not cause significant toxicity in endothelial cells as compared with vehicle-treated controls.
Figure 4: Transcend thelial electrical resistance (TEER).  

A. TEER values were measured each day in culture to establish optimal days for conducting the experiments (n = 3-8 experiments). Maximal TEER (value) was achieved on day 6.  

B. Tat alone significantly reduced TEER values across the hCMEC/D3 cell barrier (*p < 0.05 vs. control); mean ± SEM from n = 8-10 independent experiments per group; each experiment consisted of duplicate or triplicate samples.  

C. Western blot of ZO-1 tight junction-associated protein and β-actin used as loading control. Each bar represents density ratio of ZO-1 to β-actin expressed as percent control, mean ± SEM. Methamphetamine alone or combined Tat and methamphetamine significantly reduced expression of ZO-1 in hCMEC/D3 cells as compared to vehicle-treated controls (*p < 0.05).
P-glycoprotein is expressed and functional in hCMEC/D3 cells

P-gp expression in the hCMEC/D3 cells of the BBB was confirmed by western blot (Figure 5A), and P-gp function was assessed using two approaches. First, intracellular accumulation of the P-gp substrate, Rh123 was determined in cells grown on standard, 24-well culture dishes (not a transwell format). To determine the role of P-gp in Rh123 accumulation, the experiments were performed under control conditions (no inhibitor) or in the presence of verapamil, a P-gp inhibitor. Inhibition of P-gp-mediated efflux of Rh123 by verapamil resulted in a 78% increase in the cellular accumulation of Rh123 ($p < 0.05$) (Figure 5B). Second, the bidirectional transport of Rh123 was studied in the BBB model in which hCMEC/D3 cells were grown on transwell membrane inserts. Apical to basolateral (A-to-B; representing blood to the brain) transport and basolateral to apical (B-to-A; representing brain to blood) transport of Rh123 was measured at 15, 30, 45 and 60 min. Apparent permeability ($P_{app}$) was calculated for transport in both directions (Figure 5C). The $P_{app}$ of Rh123 in the B-to-A direction ($5.27 \times 10^{-3}$ cm/min) was two-fold greater than apparent permeability in the A-to-B direction ($2.88 \times 10^{-3}$ cm/min), consistent with the active efflux of Rh123 in the B-to-A direction (Figure 5C).

Tat and Methamphetamine Effects on Flux across the BBB

Paracellular Flux

Lucifer yellow (LY, 100 µM) traverses the BBB via the paracellular route (193). Control $P_{app}$ values from our lab are consistent with those reported by others (194). hCMEC/D3 cells were treated with Tat, methamphetamine or Tat + methamphetamine 24 h before
experiment (195). Lucifer yellow Papp in the A to B direction was determined. No significant changes were observed in Papp values following 24 h exposure to Tat, methamphetamine, or Tat + methamphetamine (Table 4).

**Transcellular Flux**

Propranolol, a basic lipophilic drug that traverses the barrier via transcellular transport was added on to the upper chamber. Propranolol is not a known substrate for efflux transporters (196). The transcellular barrier in our model (as measured by, Papp of propranolol, $2.28 \times 10^{-3}$ cm/min) was lower than in previously reported studies ($4.5 \times 10^{-3}$ cm/min), suggesting the barrier was less permeable to propranolol than what was reported by others (197). Papp was calculated for all the treatments. No significant changes were observed in Papp values for propranolol following 24 h exposure to Tat, methamphetamine, or Tat + methamphetamine, as was expected (Table 4).

Lopinavir and atazanavir are protease inhibitors used in the treatment of HIV-1 infections. Penetration of these drugs to the brain is limited by extrusion of these drug through efflux transporters expressed on the luminal side of BBB. We studied antiretroviral drug transport in our *in vitro* model of BBB. The cells were exposed to Tat and/or methamphetamine for 24 h and apparent permeability of these drugs was calculated to study relative penetration of these drug across BBB in the presence of treatments. None of the treatments caused significant changes in net permeability of atazanavir or lopinavir in the transwell format (Table 4).
Figure 5: P-glycoprotein expression and function. **A.** Western blot demonstrating the expression of P-glycoprotein in hCMEC/D3 cells, with β-actin used as loading control. Caco-2 and P-gp-overexpressing MDCK cells were used as positive controls. Jurkat cells were used as the negative control for P-gp expression. **B.** P-gp-mediated intracellular accumulation of rhodamine 123 (Rh123) ± the P-gp inhibitor verapamil. Verapamil significantly increases the intracellular accumulation of Rh123. **C.** The flux of Rh123 is asymmetric in the BBB model, demonstrating greater transport of Rh123 in the B-to-A direction as compared to the A-to-B direction, consistent with active efflux mechanisms. Data are presented as the mean ± SEM from n = 3 independent experiments; each experiment was performed in triplicate; (*p < 0.05).
### Table 4: Apparent Permeability from (apical to basolateral side) (Papp) cm/min

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control (SEM)</th>
<th>Tat (SEM)</th>
<th>Meth (SEM)</th>
<th>Tat + Meth (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atazanavir</td>
<td>5.64×10^{-4} (1.16×10^{-4})</td>
<td>6.08×10^{-4} (1.45×10^{-4})</td>
<td>6.41×10^{-4} (1.86×10^{-4})</td>
<td>4.86×10^{-4} (1.42×10^{-4})</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>6.20×10^{-3} (2.41×10^{-3})</td>
<td>5.57×10^{-3} (2.24×10^{-3})</td>
<td>8.33×10^{-3} (3.79×10^{-3})</td>
<td>9.72×10^{-3} (4.88×10^{-3})</td>
</tr>
<tr>
<td>Propranolol</td>
<td>2.28×10^{-3} (3.26×10^{-4})</td>
<td>2.04×10^{-3} (2.36×10^{-4})</td>
<td>1.92×10^{-3} (1.48×10^{-4})</td>
<td>1.93×10^{-3} (1.36×10^{-4})</td>
</tr>
<tr>
<td>Lucifer Yellow</td>
<td>1.11×10^{-3} (6.94×10^{-5})</td>
<td>1.15×10^{-3} (7.82×10^{-5})</td>
<td>1.18×10^{-3} (1.16×10^{-4})</td>
<td>1.19×10^{-3} (8.56×10^{-5})</td>
</tr>
</tbody>
</table>

Table 4: Apparent permeability (Papp) of substrates/drugs measured from their passage from upper to lower chamber (A-to-B) of transwell system in hCMEC/D3 cells treated with Tat, methamphetamine, or Tat plus methamphetamine for 24 h. Propranolol is a lipophilic drug and a marker for transcellular transport, while Lucifer yellow is a marker for paracellular transport. Atazanavir and lopinavir are the protease inhibitors used in the treatment of HIV infection. Papp is represented as the mean ± the standard error of mean (SEM), *p <0.05, for n = three independent experiments done in triplicate. There was no statistically significant difference in Papp for any antiretroviral drugs in hCMEC/D3 cells treated with Tat, methamphetamine, or combined Tat and methamphetamine when compared to controls.
Intracellular Drug Accumulation

Rhodamine123

Rh123 passively diffuses into the hCMEC/D3 cells and is then subject to being expelled from the cell by the efflux transporter P-gp. When hCMEC/D3 cells were treated with the P-gp inhibitor verapamil, Rh123 accumulation within the cell increased significantly ($p < 0.0002$) (Figure 5B). Next, Rh123 accumulation in the cells treated with Tat and/or methamphetamine was studied to assess the effects of treatment of P-gp mediated efflux activity. When compared to control, only the Tat + methamphetamine experimental group showed a significant increase in intracellular accumulation of Rh123 ($^*p = 0.0182$) (Fig. 5). This suggests that Tat + methamphetamine co-exposure may influence P-gp expression or function within our BBB model. To further evaluate the influence of Tat and methamphetamine on P-gp, similar experiments were performed with Rh123 but also in the presence of the P-gp inhibitor, verapamil (Figure 6). As would be expected in cells with functional P-gp, inhibition of P-gp by verapamil decreased efflux of Rh123 and resulted in significant increases in Rh123 intracellular accumulation in the control group ($^*p = 0.0004$). The increase in Rh123 within the cell upon P-gp inhibition was also observed with the Tat only ($^*p = 0.0085$) group (Figure 6), again consistent with the finding that exposure to Tat alone does not alter P-gp function. Verapamil inhibition did not alter the intracellular accumulation of Rh123 in the methamphetamine, and Tat + methamphetamine group, suggesting that P-gp function was already impaired after exposure to these treatments.
Atazanavir

The apparent permeability studies described above, did not demonstrate a significant difference in net atazanavir flux across the membrane in either the A-to-B direction (control $2.23 \times 10^{-3}$ cm/min, Tat $2.22 \times 10^{-3}$ cm/min, methamphetamine, $2.23 \times 10^{-3}$ cm/min, Tat + methamphetamine $2.28 \times 10^{-3}$ cm/min) (Table 4) or B-to-A (data not shown). However, many antiretroviral drugs, including atazanavir, are substrates of transporters such as P-gp and MRPs. In order to assess the role of efflux transporters in drug accumulation inside hCMEC/D3 cells, and effects of Tat and/or Meth on the function of P-gp and MRPs, intracellular accumulation of atazanavir was studied in hCMEC/D3 cells in a non-transwell format in vitro.

Intracellular accumulation of atazanavir was not significantly different across Tat and/or methamphetamine treatment groups (Figure 7). Thus, accumulation of atazanavir was examined in the presence of P-gp and MRP inhibitors (verapamil and MK-571, respectively), individually as well as both inhibitors together. Surprisingly, neither P-gp inhibition alone nor MRP inhibition alone resulted in significant changes in atazanavir intracellular accumulation (Figure 7). To test whether P-gp or MRP transporters might compensate for one another if either alone is inhibited, we simultaneously inhibited both transporter classes. Interestingly, when P-gp and MRPs (verapamil + MK-571) were both inhibited, there was a significantly higher accumulation of atazanavir compared to groups lacking the inhibitors ($p < 0.05$) suggesting atazanavir is shunted to the alternative efflux transporter if either P-gp or MRPs are blocked. Therefore, the maximum accumulation of atazanavir was observed when both P-gp and MRPs are inhibited concurrently (Figure
Irrespective of whether P-gp and/or MRPs are inhibited, however, neither Tat nor methamphetamine had effects on the overall accumulation of atazanavir.

**Effects of Tat and/or Methamphetamine on protein/ gene expression of P-gp and MRP**

We assessed the effects of Tat and/or methamphetamine on P-gp and MRP1 gene and protein expression by qPCR and immunoblotting. All qPCR experiments performed within expected parameters and standard curves showed good linear relationships between serial dilutions (min $R^2 > 0.989$). Using the untreated samples as a reference, P-gp expression in hCMEC/D3 cells showed no significant changes following treatment with Tat, methamphetamine, and Tat + methamphetamine. Similarly, MRP1 showed no significant change following treatment with Tat, methamphetamine, and Tat + methamphetamine (data not shown).

Similarly, no statistically significant differences in the expression of P-gp or MRP1 proteins between the experimental groups was observed (Figure 8). Therefore, no changes in mRNA or protein expression of P-gp or MRP1 were observed after 24 h exposure of hCMEC/D3 cells to Tat, methamphetamine, or Tat + methamphetamine.
Figure 6: Tat and methamphetamine effects on Rhodamine123 intracellular accumulation. Effects of 24 h exposure to Tat and/or methamphetamine on P-gp mediated Rh123 accumulation. Open circles and solid circles represent the accumulation of Rh123 in the absence and presence of verapamil, respectively. In the absence of verapamil (open circles) there was significantly higher accumulation in Tat + methamphetamine group when compared to control or compared to Tat only group, consistent with an impairment of P-gp (and thus less efflux of Rh123 out of the cell) upon exposure to Tat + methamphetamine. Inhibition of P-gp by verapamil (solid circles) significantly increased Rh123 accumulation in control and Tat only group, which would be expected for cells with functional P-gp. All symbols represent intracellular accumulation of Rh123 (nanomol/mg protein); mean ± SEM from n = 5 independent experiments; each experiment was performed in triplicate (*p < 0.05, verapamil vs. the identical treatment without verapamil, #p < 0.05, groups treated without verapamil).
Figure 7: Atazanavir intracellular accumulation studies. Effect of 24 h exposure of Tat and/or methamphetamine on atazanavir accumulation in hCMEC/D3 cells under control conditions (open circle), inhibition of P-gp by verapamil (solid circle), inhibition of MRPs by MK-571 (open square), and inhibition of both P-gp and MRPs by verapamil + MK-571 (inverted triangle). Treatment with Tat and/or methamphetamine did not affect atazanavir intracellular accumulation under any conditions (± inhibitors). However, regardless of the treatment group, when P-gp and MRP-1 were inhibited together, atazanavir intracellular accumulation was significantly increased as compared to the same groups with no inhibitor (*), MK-571 (#), and verapamil (⊗). These data suggest that inhibition of a single transporter is not sufficient to alter the intracellular accumulation of atazanavir, perhaps because the substrate shunts from the inhibited transporter to the functional transporter. Symbols represent atazanavir accumulation in mean ± SEM for different inhibitor conditions (open circle-no inhibitor, open square-MK-571, solid circle-verapamil, inverted triangle-MK-571+verapamil; mean ± SEM from n= 3 independent experiments per group; each experiment performed in triplicate (*p < 0.05, no inhibitor vs verapamil + MK-571, #p < 0.05, MK-571 vs verapamil + MK-571, ⊗p < 0.05, verapamil vs verapamil + MK-571)
Figure 8: Immunoblots of P-gp and MRP-1. Western blots of P-gp (A) and MRP-1 (B) from the cell lysate of hCMEC/D3 cells exposed with Tat and/or methamphetamine for 24 h. Treatments had no significant effect on the expression of P-glycoprotein (P-gp) or Multidrug Resistant Protein-1 (MRP-1). Each bar represents the mean ± SEM of the relative density of the drug efflux protein (absolute density of P-gp or MRP-1 to the absolute density of β-actin) for n = 4 independent experiments (*p < 0.05).
DISCUSSION

The purpose of these studies was to investigate the effects of HIV-1 Tat and methamphetamine on factors affecting antiretroviral penetration, such as paracellular and transcellular pathways of drug flux across the BBB. We hypothesized that Tat and methamphetamine exposure would result in BBB breakdown and that the loss of integrity would disrupt antiretroviral drug flux. To address these questions, an in vitro BBB model was used to explore the effects of treatment on different aspects of paracellular and transcellular transport. Since both Tat, and to lesser extent methamphetamine, can be intrinsically cytotoxic, we assessed the effects of treatments acutely before breakdown of the cellular barrier. Methamphetamine effects on the BBB are complex, and further investigations of temporally related changes may be necessary. Additionally, treatment effects on antiretroviral penetration are mediated by multiple transport systems, and this redundancy may help abrogate Tat- or methamphetamine-induced alterations in a single transport protein. Overall, our results demonstrate that in combination Tat and methamphetamine affect aspects of barrier integrity without affecting the net flux of paracellular compounds, and also may alter key aspects of transcellular transport.

Effects of Tat and methamphetamine on paracellular integrity
Following 24 h exposure to Tat, TEER values (a measure of BBB tightness or integrity) were significantly decreased. The data complement findings by Mahajan et al., which found reductions in TEER in Tat-treated primary human brain microvascular endothelial cells co-cultured with astrocytes (106). Methamphetamine exposure (regardless of Tat) resulted in significant decreases in ZO-1 protein expression in this model. Interestingly, no effects of Tat or methamphetamine on lucifer yellow (a paracellular marker) flux were
noted, despite reports that methamphetamine disrupts the BBB. The published reports of methamphetamine effects on the BBB are often inconsistent. This is likely due to the highly varied model systems and experimental protocols used in these studies. Furthermore, many of the studies used methamphetamine concentrations that are more than the plasma concentrations found in typical drug abuse (198). The use of extremely high concentrations may result in direct neurotoxic stress which, in turn, could lead to BBB breakdown (199, 200). Our studies used 10 μM concentrations, which is within the range of plasma concentrations measured from methamphetamine abusers (0.1-11.1 μM) (187) and we demonstrated lack of toxicity at this concentration using the LIVE/DEAD assay. Other factors that contribute to the complexity of interpretation of methamphetamine effects on the BBB is that methamphetamine exposure times vary widely between studies. In one study of high dose (30 mg/kg) methamphetamine exposure in mice, barrier permeability to the paracellular compound Evans blue was altered, albeit transiently; increase observed at 24 h, but not 72 h (109). In another study using primary rat brain microvascular endothelial cells, investigators observed that TEER values initially decreased but recovered over the four h following methamphetamine exposure (for both 1 μM and 50 μM methamphetamine) (108). In the same study, the lateral continuity of the tight junction proteins VE-cadherin, occludin, claudin-5, and ZO-1 was maintained following methamphetamine (1 μM) exposure, albeit only 2- and 6-h time points were reported (we observed decreases following 24 h exposure). However, increases in vesicular transport, unrelated to paracellular transport, were noted for cells treated with 1 μM, but not 50 μM, methamphetamine (108). In brain capillaries from methamphetamine-treated mice, transient increases in claudin-5 and occludin, but not
ZO-1 protein, were demonstrated by western blot at 3 h and returned to baseline levels by 24 h (110). Lastly, decreases in the expression of tight junction proteins at 12 h and 24 h (10 μM) without changes in paracellular permeability (111), and decreases in TEER without changes in tight junctions for up to 72 h also have been reported (106). Collectively, these studies provide evidence for potentially transient and complex interactions of methamphetamine on the BBB, and that the effects of methamphetamine on the BBB are related to dose, exposure duration, and specific experimental model used. Given the variability among published studies, future studies should further explore the temporal nature of this complex interaction and should consider examining in intact systems in vivo to increase the physiological and translational relevance.

**Effects of Tat and methamphetamine on transcellular flux**

Propranolol crosses the barrier transcellularly, but is not a substrate for drug efflux proteins (58). Tight junction modulation often alters paracellular flux but does not always confer changes in transcellular transport (201, 202). Therefore, neither tight junction protein modulation nor changes in drug efflux proteins were expected to alter the flux of propranolol in these experiments. Indeed, we observed no changes in propranolol flux across the barrier in response to exposure to Tat- and/or methamphetamine. P-gp is a major drug efflux protein responsible for extrusion of substrates from the brain back into the blood. Several antiretroviral drugs used in the treatment of HIV are substrates for P-gp, limiting brain exposure to these medications, and other antiretrovirals are inducers or inhibitors of P-gp (203–210). Tat- and/or methamphetamine-induced modulation of P-gp expression or function may affect the ability of these antiretrovirals to cross the BBB. The inability of antiretroviral drugs to enter the CNS, in turn, would limit the ability of the therapeutic regimen to reduce viral loads within the brain.
Using Rh123 as a prototypical P-gp substrate (211), the effects of Tat and methamphetamine on Rh123 flux were measured in our model system. Tat exposure did not affect the overall accumulation of Rh123 into cells lacking the inhibitor. In the presence of the known P-gp inhibitor verapamil, Rh123 accumulated in Tat-treated cells (73% increase) and by 93% in controls. Taken together, these data suggest that P-gp was functioning normally and that the transporter was unaffected by exposure to Tat alone. Tat exposure (up to 12 h) was previously shown to increase P-gp expression and function in primary mouse brain microvascular cells (177). Differences between our results and those reported by Hayashi et al. might result from differences in the Tat protein used (Hayashi used Tat1-72 versus Tat1-86 in our study), Tat concentration, or the duration of exposure, as well as differences in the origins of the cells used in the BBB model. Exposure to Tat + methamphetamine resulted in the increased cellular accumulation of the P-gp substrate, Rh123, in brain microvascular endothelial cells. This suggests that P-gp was inhibited or impaired P-gp function within these cells. When the Tat + methamphetamine-exposed group was treated with verapamil, no additional accumulation of Rh123 was observed (Fig. 5), suggesting that P-gp function was maximally impaired after exposure to methamphetamine. The changes in P-gp function in these experiments were not accompanied by significant alterations in gene or protein expression at 24 h (Fig. 5). Others have reported methamphetamine exposure increasing P-gp protein expression (at 3 h), but its effects at other time points and the functional consequences thereof have not been reported (110). Other amphetamines, including MDMA, have been shown to have inhibitory effects on P-gp activity but effects of methamphetamine itself on P-gp function were not reported (212). Given the transient
nature of methamphetamine effects on other properties of the BBB discussed above, further investigation into the effects of methamphetamine exposure on drug efflux transporters, such as P-gp, is warranted.

Atazanavir is a protease inhibitor used in the treatment of HIV and is a known substrate for P-gp and several MRPs (213). To evaluate potential effects of Tat and methamphetamine on brain penetration of antiretroviral drugs, we examined the effects of Tat and methamphetamine on atazanavir flux across the BBB using the transwell system as well as atazanavir accumulation within cells. Tat and methamphetamine did not result in changes in atazanavir flux under either experimental system, nor were there significant alterations in gene or protein expression of MRP1 or P-gp. Prior experiments using Rh123 indicated that P-gp function (but not expression) was impaired in the presence of methamphetamine. Therefore, to verify the role of P-gp and the MRPs on the flux of atazanavir in our system, additional cellular accumulation studies were performed in the presence of verapamil and/or MK-571 (an MRP inhibitor). When single inhibitors were used (either verapamil or MK-571), no significant changes in intracellular atazanavir accumulation were observed. However, for all treatment groups, simultaneous inhibition of P-gp and MRP resulted in significant increases in atazanavir intracellular accumulation as compared to the same groups with no either no inhibitor or single transport inhibition (Fig. 6). These data suggest that inhibition of a single transporter is not sufficient to alter the intracellular accumulation of atazanavir, perhaps because the redundancy of two efflux transport proteins allows for substrate shunting from the inhibited transporter to the functional transporter.
Taken together, these data support previous reports that atazanavir is a substrate for both P-gp and MRP, but that neither Tat nor methamphetamine significantly alter the net flux of atazanavir efflux in our system. Another potential explanation for the seeming discrepancy between effects of Tat and methamphetamine on P-gp-mediated transport of Rh123 and atazanavir can potentially be explained by differences in uptake mechanisms for each substrate. At the concentrations used in these studies, Rh123 enters the cell primarily by passive diffusion (214), whereas atazanavir cellular uptake has been shown to be mediated by organic anion transporting polypeptide (OATP) uptake transporters as well (215). Therefore, Tat and/or methamphetamine exposure in our system would not be expected to alter Rh123 entry into the cell; but may, indeed, alter OATP-mediated uptake of atazanavir. Future studies should explore the effects of Tat and methamphetamine on OATP and on OATP-mediated antiretroviral transport.

Understanding the role of efflux transporters may have implications beyond restricting access to the CNS. P-gp can also be expressed by microglia and astrocytes—the principal resident cell types that are infected within the CNS—suggesting that even if a drug such as atazanavir enters the CNS, it may be difficult to achieve therapeutic concentrations within HIV-infected cells in the CNS (179, 216). Moreover, P-gp expression in rat astroglia can be downregulated by HIV gp120 resulting in a net decline in P-gp function (179, 180). By contrast, others report that HIV Tat can upregulate P-gp in brain endothelial cells (177, 178), which may occur before onset of the more cytotoxic effects of Tat through the release of inflammatory intermediaries such as cytokines or oxyradicals, or prior to activation of cell death pathways (153). The evidence infers that
the effects of HIV on efflux transporters are complex and may differ depending on the particular HIV protein and transporter involved, and the duration of exposure. Collectively, our investigations indicate that HIV-1 Tat and methamphetamine alter aspects of BBB integrity and antiretroviral flux across the barrier. Dysregulation of drug transport proteins occurs, and the resultant net effects on the transport of an antiretroviral drug into the brain are dependent on the specific transport system involved. The interactive effects of Tat and methamphetamine on drug transport are complex and likely related to the particular antiretroviral agent involved, and its dose and exposure duration. Future directions should include a thorough investigation on the temporal effects of HIV and methamphetamine on other drug transporters such as the MRPs, as well as the uptake transporters that have a role in antiretroviral entry into the cell.
Simultaneous determination of intracellular concentrations of tenofovir, emtricitabine, and dolutegravir in the human brain microvascular endothelial cells using liquid chromatography-tandem mass spectrometry

INTRODUCTION

According to the Joint United Nations Program on HIV/AIDS (UNAIDS) report of 2018, 36.9 million people globally are living with Human Immunodeficiency Virus (HIV) (9). In the early years of the HIV epidemic, post-diagnosis survival rates were measured in months. With the advent of combination antiretroviral therapy (cART), patients are better able to achieve and maintain plasma viral suppression, and survival rates of individuals living with HIV have reached a near normal lifespan (35). However, even for patients who can achieve viral suppression within the blood, HIV persists within the central nervous system (CNS). The persistence of HIV and the resultant chronic inflammation likely contribute to the development of neurocognitive deficits experienced by many persons infected with HIV (60, 61, 217). Approximately, 50-60% of individuals infected with HIV suffer from HAND, which is a spectrum of neurocognitive impairment ranging from asymptomatic neurocognitive impairment to full-blown dementia (218–220).

Poor brain penetration of antiretroviral drugs likely contributes to the persistence of HIV within the brain (221). Antiretroviral penetration into the brain is mediated, in part, by the blood-brain barrier (BBB), which restricts the entry of the drugs into the central nervous system (CNS), and by uptake and efflux transport proteins that are expressed at the luminal side of BBB (126). Depending on the physicochemical properties of the drug or substrate, efflux transport proteins can either facilitate or impede xenobiotic entry into the brain (222). Furthermore, the net flux of drugs into the cytoplasm of individual cell types within the CNS is also mediated by transport proteins, which are integral membrane
proteins expressed at the cell surface. Drug efflux proteins, such as P-glycoprotein and Breast Cancer Resistance Protein (BCRP) are transport systems that can actively extrude substrates, including several antiretroviral drugs, out of the cell on which they are expressed (13, 223); thereby limiting the overall intracellular concentrations of antiretroviral drugs (224). In contrast, uptake transporters, when located on the luminal side of the BBB facilitate the movement of substrates across the BBB and into individual cells (225).

Human brain microvascular endothelial cells, the primary cellular constituent of the BBB, form the lining of brain microvessels. Through the expression of tight junction proteins, these cells form a tight intercellular barrier that restricts the entry of toxins, and other foreign substances, including xenobiotics, across the BBB, and into the brain (226). Structural and functional deficits in brain microvascular endothelial cells are critical in advancing the neuropathogenesis of HIV (227). These cells are the site of entry of free circulating virus, as well as the entry point for HIV infected monocytes, to pass through the barrier and enter the brain (64, 228). Moreover, human brain microvascular endothelial cells may also be a site of HIV replication (229–231). In this study, we developed and validated an assay for simultaneous quantification of three antiretroviral drugs and applied this method in the quantification of antiretroviral concentrations within an immortalized human brain microvascular endothelial cell line (hCMEC/D3). The hCMEC/D3 cell line has been widely used as a model of primary human brain microvascular endothelial cells and in the construction of an in vitro BBB model (132, 194, 232, 233). The hCMEC/D3 cells mimic key aspects of primary endothelial cells and
display characteristics of the BBB, including the expression of tight junction proteins, the uptake and efflux transport proteins, the expression of chemokine receptors (including HIV-1 co-receptors), and they also can regulate the expression of cellular adhesion molecules in response to inflammatory cytokines (132).

Because HIV enters and replicates within infected cells, intracellular drug concentration is important factor in determining the efficacy of an antiretroviral drug. Several antiretroviral drug classes including the nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-NRTIs (NNRTIs), integrase inhibitors, and protease inhibitors (PIs) act on intracellular targets. As discussed above, the intracellular accumulation of antiretroviral drugs is dependent on several factors, including drug transport protein expression and activity, which varies among different cell types (131, 234–236). There is also evidence of tissue-specific differences in the penetration of antiretroviral drugs within cerebral cortical, female genital tract tissue, and lymphoid compartments when compared to blood, which may affect the efficacy of antiretroviral therapy (237, 238). Accordingly, the goal of this study was to develop an assay to simultaneously measure the intracellular concentration of multiple antiretroviral drugs within a clinically relevant drug regimen.

For this study, the cART regimen of a nucleoside reverse transcriptase inhibitor (NRTI), emtricitabine, a nucleotide reverse transcriptase inhibitor (NtRTI), tenofovir, and an integrase strand inhibitor, dolutegravir, was used (Figure 9). This regimen is currently one of the recommended initial regimens for most people with HIV (239). These drugs have previously been quantified, individually or simultaneously, in either human plasma or peripheral blood mononuclear cells or mouse plasma (240–244). To best of our
knowledge, this is the first study quantifying the intracellular concentrations of these three antiretroviral drugs simultaneously in human brain microvascular endothelial cells.

Figure 9: Chemical structure of tenofovir, emtricitabine, and dolutegravir

METHODS

Chemical and reagents

Tenofovir and emtricitabine were obtained from the United States Pharmacopeia (Rockville, MD). Dolutegravir was purchased from TLC PharmaChem (Ontario, Canada). For cellular accumulation studies, the following drugs were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Tenofovir, Emtricitabine [(-) FTC]. Dolutegravir was purchased from Sigma Aldrich. Tenofovir-d6 and emtricitabine-13C15N2 were obtained from Toronto Research Chemical Inc. (Ontario, Canada). Dolutegravir-d5 was purchased from BDG Synthesis (Wellington, NZ). Methanol, acetonitrile with 0.1% formic acid, and 2-propanol (HPLC grade) were obtained from Honeywell Chemicals (Muskegon, MI). Ultrapure water was collected from Milli-Q® (Molsheim, France) integral water purification system. 6-well plates used in these studies were from Corning Inc. (Corning, NY, USA). Human brain microvascular endothelial cells,
hCMEC/D3, were generously provided by Dr. Babette Weksler of Weill Cornell Medical College, Cornell University (New York, NY). Endothelial basal media (EBM-2) and the EGM2-MV Bullet kit were purchased from Lonza (Basel, Switzerland). Gibco® HBSS (Hank’s Balanced Salt Solution), penicillin-streptomycin (10,000 U/mL), HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] was obtained from Thermo Fisher Scientific (Waltham, MA). The EGM2-MV Bullet kit contained human endothelial growth factor (hEGF), insulin-like growth factor (IGF), human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), hydrocortisone, ascorbic acid, FBS, GA-1000 (gentamycin and amphotericin). 0.45 µm MultiscreenHTS filter plate was purchased from Millipore (Millipore sigma, USA).

**Cell culture**

hCMEC/D3 were grown in EBM-2 media supplemented with EGM2-MV bullet kit at 5% CO₂, 37°C and 95% relative humidity. This medium was called growth medium for hCMEC/D3 cells. Cells seeded on 6-well plates were grown on EBM-2 media supplemented with 1.25% FBS, 5 mL of penicillin/streptomycin solution (10,000 U/mL), 5 mL of 10 mM HEPES and 1.25 mL of 0.5 ng/mL bFGF. This media was referred to as maintenance media.

**Chromatography**

The HPLC system consisted of an Agilent 1260 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA), a Shimadzu, System Controller, SCL-10A Vp, Pump, LC 10AD Vp Solvent Degasser (DGU14A, Shimadzu, Columbia, MD, USA), and an CTC PAL autosampler (Zwingen, Switzerland). The analytical column was XBridge C18, 50 x 2.1 mm, 5µm (Waters Corp, Milford, MA, USA). The column temperature was maintained
at 25 °C. Mobile phase A was water and mobile phase B was 0.1 % formic acid in acetonitrile. The elution pump (Agilent 1260) gradient was: 0–1.0 min 0.0% B at 0.4 mL/min flow, 1.1–3.0 min from 50% to 90% B at 0.4 mL/min flow rate, 3.1–7.0 min 90% B at 0.8 mL/min flow rate and 7.1–10.0 min 0.0% B at 0.8 mL/min flow rate. The makeup pump (Shimadzu) ran an isocratic gradient at 50:50 mobile phase A: mobile phase B and at a flow rate of 0.2 mL/min. The switching valve program was: 0-1.8 min at position A (loading sample into column and desalting), 1.8-3.0 min at position B (elution to MS source) and 3.0 to 10.0 min at position A (column flushing and re-equilibration). Injection volume was 50 µL and total run time was 10.0 min.

**Mass spectrometric conditions**

The Mass Spectrometer was an AB Sciex API 4000 system (Applied Biosystems Sciex, Ontario, Canada) using Analyst 1.6.3 software, which was operated in the electrospray ionization (ESI) positive multiple reaction monitoring (MRM) modes. The MS/MS parameters were; ions source temperature (500.0 °C), ion spray voltage (5000 V), electron multiplier CEM (2400 V), Collison gas flow CAD (8.0), curtain gas flow CUR (25.0), nebulizer gas floe NEB/GS1 (45.0), turbo ion spray gas AUX/GS2 (45.0), deflector potential DF (-150), pause time 95 ms). Mass transitions and optimized MRM parameters are shown in (Table 5).
Table 5: Mass spectrometric parameters

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Q1</th>
<th>Q3</th>
<th>DP</th>
<th>CE</th>
<th>EP</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenofovir</td>
<td>288.2</td>
<td>175.9</td>
<td>64</td>
<td>36.00</td>
<td>10</td>
<td>12.07</td>
</tr>
<tr>
<td>Tenofovir-d6</td>
<td>294.3</td>
<td>182.2</td>
<td>64</td>
<td>37.11</td>
<td>10</td>
<td>10.07</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>248.1</td>
<td>130.0</td>
<td>36.74</td>
<td>19.09</td>
<td>10</td>
<td>8.98</td>
</tr>
<tr>
<td>Emtricitabine-13C 15N2</td>
<td>251.3</td>
<td>133.0</td>
<td>36.75</td>
<td>15.07</td>
<td>10</td>
<td>8.98</td>
</tr>
<tr>
<td>Dolutegravir</td>
<td>420.1</td>
<td>277.0</td>
<td>81.97</td>
<td>37.95</td>
<td>10</td>
<td>20.59</td>
</tr>
<tr>
<td>Dolutegravir-d5</td>
<td>425.3</td>
<td>277.2</td>
<td>83</td>
<td>36.70</td>
<td>10</td>
<td>19.78</td>
</tr>
</tbody>
</table>

Q1 = Quadrapole mass filter 1 (Q1) and 3 (Q3), DP = Declustering potential (Volts)
CE = Collision Energy (Volts), EP = Entrance Potential (Volts), CXP = Collision Cell Exit Potential

**Cell Extract Preparation**

hCMEC/D3 cells were grown in T75 flasks as described above. At 90% confluency, cells were washed twice with 9.5 mL of ice-cold Hank’s Balanced Salt Solution (HBSS), and cells were lysed with a 15 min ice-cold methanol (9.5 mL) incubation followed by scraping of the cells. The resulting cell extracts were stored at -80°C.
**Stock Preparation**

Stock solutions of tenofovir, tenofovir-d6, emtricitabine, emtricitabine-$^{13}$C$_6$, $^{15}$N$_2$ were prepared in ultrapure water at a concentration of 100 µg/mL. Dolutegravir, dolutegravir-d5 stock solutions were prepared in DMSO at a concentration of 100 µg/mL. Subsequent dilutions were made in water for spiking calibration standards and quality control samples. All stock solutions were stored at 4-8°C in amber colored glass bottles.

**Preparation of Calibration Standards and Quality Control (QC) samples**

Samples were vortex mixed for 1.0 min at 750 rpm then centrifuged for 5 min at 4696 x g. Two hundred microliters of each sample were aliquoted into a 96-well plate (VWR® Microplate, pyramid bottom, 2 mL well volume) and 20 µL of 50 ng/mL working internal standard solution was added. Sample plate were vortex mixed for 5 min at 750 rpm and centrifuged for 15 min at 6102 x g, and 150 µL of supernatant was collected, filtered through a 0.45 µm multiscreen HTS filter plate (centrifuged for 5 min at 3500 x g) and dried under a steady stream of nitrogen gas at 45°C. The dried extracts were reconstituted with 150 µL of ultrapure water, mixed for 10 min at 1200 rpm. Moreover, centrifuged again for 15 minutes at 6102 x g. Lastly, 50 µL of the final extract was injected into LC-MS/MS.

**Method Validation**

The developed LC-MS/MS method was validated for linearity, precision, and accuracy according to the FDA bioanalytical guidance for these parameters (245).
Linearity and lower limit of quantification (LLOQ)

Cell lysate calibration curves were constructed by plotting peak area ratio of each analyte to its corresponding labeled internal standard versus standard concentrations. Ten calibration standards in duplicates over five validation runs were constructed using a linear 1/concentration squared weighted least-squares regression algorithm. The lower limit or quantitation (LLOQ) was defined as the lowest concentration on the calibration curve with precision (calculated as percent relative standard deviation; % RSD) and accuracy (calculated as percent difference from nominal; % DFN) within ± 20%. For the rest of the levels on calibration curve precision and accuracy (as defined above) should be within ± 15%.

Selectivity

Six blank cell extracts from hCMEC/D3 cells at six different passages were processed in the same fashion as samples (see sample preparation). The selectivity of this assay was assessed by comparing LLOQ level and blank cell extracts. To assess for possible interference from blank cell matrix, no peaks in blank cell extracts should be greater than 20% response of LLOQ.

Accuracy and Precision

Accuracy and precision were evaluated using six QC levels (LLOQ, Low-QC, MedLow QC, Med-QC, MedHigh-QC, and High-QC). Intra-day accuracy and precision were determined by analyzing six replicates at each QC level in a single assay. Inter-day accuracy and precision were determined by analyzing three replicates of each QC level except LLOQ in five independent runs. To validate the dilution integrity of above the
ULOQ samples; dilution QC samples (200 ng/mL) were diluted threefold and analyzed in six replicates in one validation run. Accuracy and precision within ± 20% for LLOQ and ± 15% for other samples of their nominal concentration was considered acceptable.

**Recovery**

Extraction recovery of target analytes from cell lysate at low, mid and high QC levels was assessed by comparing extracted analytes responses (pre-extracted spiked samples) to analytes responses spiked into extracted blank cell extract samples (post-spiked samples) which was considered 100% recovery.

**Post-Extraction Addition experiment (Matrix Factor)**

Matrix Factor was evaluated by comparing analytes responses spiked into extracted blank cell lysate samples (post-spiked samples) to analyte responses in an external solvent (water).

**Stability Studies**

Post-preparative extract stability was evaluated at low and high QC levels. QC samples were processed with a calibration curve and stored in the autosampler at 4°C for seven days, then re-analyzed and processed against fresh extracted calibration curve, Analyte stability in frozen matrix was determined by analyzing three replicates of low QC and high QC samples that had been stored at -80°C for 120 days. For all stability experiments; precision and accuracy should be within ± 15%.
**Application of the validated method**

The hCMEC/D3 cells were grown on 6-well plates. At 90% confluency, the cells were washed thrice with 1.0 mL Hank’s balanced salt solution (HBSS). The cells were treated with individual drug solutions of tenofovir, emtricitabine, or dolutegravir. Each drug was prepared in a 100 mM stock solution in water except dolutegravir, which was diluted in DMSO. All subsequent working solutions were made in water, and the final concentration of DMSO for dolutegravir was less than 0.1%. The final treatment concentrations for each drug were 1436 ng/mL, 1236 ng/mL, 2097 ng/mL (all corresponding to 5 µM) and 2871 ng/mL, 2473 ng/mL, 4194 ng/mL (corresponding to 10 µM), respectively. Additionally, cells were also treated with two different combinations of tenofovir, emtricitabine, and dolutegravir in the same incubation solution. In the first combination antiretroviral solution, all antiretrovirals were at a final concentration of 5 µM, and in the second combination, all antiretrovirals were at a final concentration of 10 µM. The concentrations used in these studies were based on the maximum serum concentration (Cmax) of drug obtained from clinical studies (246–248). Each treatment group was incubated for one hour before harvest and sample preparation. Upon harvest, the antiretroviral-containing incubation solutions were removed from each well and cells were quickly washed three times with 1.0 mL of ice-cold HBSS. Cells were lysed by adding 1.0 mL of methanol to the cells, which was allowed to sit on ice for 15 minutes. Cell extract was collected by scraping the cells with a cell scraper. Two hundred microliters of the extract were collected in a 96-well plate, and post-processing of the extract was similar as mentioned in sample preparation.
The cell lysate was used to calculate protein concentration by Pierce™ BCA protein assay. Intracellular drug accumulation was expressed as the amount of drug accumulated per µg of total protein per hour.

Software

Analyst® was used to process data collected from mass-spectrometer. Statistical analyses were performed using GraphPad Prism (V7) (San Diego, California)

RESULTS

Linearity

Linearity was obtained over a concentration range of 0.100-100 ng/mL for all analytes; The average correlation coefficient \( (r^2) \) from five validation runs weighted by \( 1/x^2 \) was found to be 0.996 (0.172 %RSD), 0.994 (0.145 %RSD), 0.994 (0.124 %RSD) for tenofovir, emtricitabine and dolutegravir, respectively. Precision (%RSD) of back-calculated standards were within 9.29%, 10.4 %, and 13.07% for tenofovir, emtricitabine and dolutegravir, respectively and accuracy (%DFN) were within ±5.43%, ±11.81%, ±2.39% for tenofovir, emtricitabine and dolutegravir, respectively.

Selectivity

Six separate isolations of blank cell lysate collected from passages 26-32 of hCMEC/D3 cells were extracted according to the sample preparation procedure for any potential interferences at the mass transitions and expected retention times of target analytes. No significant chromatographic peak greater than 20% of the mean LOQ response was detected for all analytes.
Accuracy and precision

Inter-day precision and accuracy were found to be within ± 11.69% (Table 6). Intra-day precision and accuracy were within ± 13.33% (Table 7) for all analytes.

Table 6: Inter-day accuracy and precision (n=15)

<table>
<thead>
<tr>
<th></th>
<th>0.3 ng/mL</th>
<th>1.0 ng/mL</th>
<th>2.0 ng/mL</th>
<th>50 ng/mL</th>
<th>75 ng/mL</th>
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<tbody>
<tr>
<td>Tenofovir</td>
<td>%RSD</td>
<td>7.25</td>
<td>6.33</td>
<td>7.68</td>
<td>10.36</td>
</tr>
<tr>
<td></td>
<td>%DFN</td>
<td>1.64</td>
<td>1.29</td>
<td>4.38</td>
<td>3.33</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>%RSD</td>
<td>6.73</td>
<td>3.84</td>
<td>3.06</td>
<td>3.67</td>
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<tr>
<td></td>
<td>%DFN</td>
<td>7.12</td>
<td>4.90</td>
<td>7.25</td>
<td>3.11</td>
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<tr>
<td>Dolutegravir</td>
<td>%RSD</td>
<td>11.69</td>
<td>6.61</td>
<td>6.47</td>
<td>6.83</td>
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<tr>
<td></td>
<td>%DFN</td>
<td>-0.19</td>
<td>2.05</td>
<td>3.93</td>
<td>6.44</td>
</tr>
</tbody>
</table>

%RSD = Percent Relative Standard Deviation, %DFN = Percent Difference From Nominal concentration

Table 7: Intra-day accuracy and precision (n=6)

<table>
<thead>
<tr>
<th></th>
<th>0.1 ng/mL</th>
<th>0.3 ng/mL</th>
<th>1.0 ng/mL</th>
<th>2.0 ng/mL</th>
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<tbody>
<tr>
<td>Tenofovir</td>
<td>%RSD</td>
<td>13.13</td>
<td>5.53</td>
<td>5.65</td>
<td>5.70</td>
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</tr>
<tr>
<td></td>
<td>%DFN</td>
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<td>-6.80</td>
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<tr>
<td>Emtricitabine</td>
<td>%RSD</td>
<td>4.56</td>
<td>5.75</td>
<td>2.32</td>
<td>1.87</td>
<td>2.41</td>
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<tr>
<td></td>
<td>%DFN</td>
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<td>10.00</td>
<td>3.60</td>
<td>2.75</td>
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<tr>
<td>Dolutegravir</td>
<td>%RSD</td>
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<td>2.98</td>
<td>1.96</td>
<td>1.72</td>
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<td></td>
<td>%DFN</td>
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<td>0.00</td>
<td>-8.00</td>
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<td>1.67</td>
</tr>
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</table>

%RSD = Percent Relative Standard Deviation, %DFN = Percent Difference From Nominal concentration

Dilution integrity
The intra-assay precision and accuracy were within 9.00% and ± 3.90%, respectively, for diluted QC samples for all analytes.

**Extraction recovery**

Extraction recoveries of all analytes from cell lysate were calculated by comparing pre-extraction spiked samples and post-extraction spiked samples at 2.0, 50 and 75 ng/mL (n=3). Extraction recoveries for tenofovir, emtricitabine, and dolutegravir were found to be consistent across different concentration levels; 84.89 (1.18 %RSD), 86.76 (2.50 %RSD), and 87.33 (3.86 %RSD), respectively.

**Matrix Factor**

Post-extraction addition experiment was conducted to evaluate matrix effects at 2.0, 50 and 75 ng/mL; by comparing post-extraction spiked matrix samples to matrix free-samples prepared at the same concentrations. Results showed that matrix effects were within 88.82 (4.88 %RSD), 100.16 (3.09 %RSD), and 104.64 (2.52 %RSD) for tenofovir, emtricitabine, and dolutegravir, respectively.

**Stability studies**

All stability studies showed results within ± 15%; post-preparative extract stability was found to be stable for seven days at 2-8 °C, and long-term matrix stability was demonstrated for 120 days at -80 °C.

**Application of the method**

Tenofovir, emtricitabine, and dolutegravir were incubated individually and together as a cocktail at both 5 µM and 10 µM for one hour at 37°C to allow time for drug uptake into
the cells. Following the one hour incubation, the intracellular concentrations of each drug within the cells was measured using the LC-MS above/MS method. Amount of drug accumulated with the cells was normalized by the protein content of each well. We were able to quantify tenofovir, emtricitabine and dolutegravir in the cell lysate of hCMEC/D3 cells treated antiretroviral drugs treated individually and in within a commonly clinically used cART cocktail (Figure 10). Individual drug and cocktail concentrations were used at both 5 µM and 10 µM (Table 8). Intracellular concentrations of tenofovir, emtricitabine, and dolutegravir were determined in hCMEC/D3 using this method.

DISCUSSION

Although several methods have been developed to determine antiretroviral drugs individually or simultaneously in human plasma or peripheral blood mononuclear cells (PBMCs), to our knowledge this is the first reported method to simultaneously determine the regimen tenofovir, emtricitabine, dolutegravir within brain cells. Prathipathi et al. determined this regimen along with elvitegravir and rilpivirine in mouse plasma, however, they also did not analyze this regimen in PBMCs or other cellular matrices (243). cART regimens pose an analytical challenge of determining all the drugs simultaneously due to the differences in physicochemical properties of the drugs. In the regimen above, NRTIs (tenofovir, emtricitabine) are hydrophilic, while dolutegravir is lipophilic (222).

A simple extraction procedure involving protein precipitation using 100% methanol was applied. Raw cell extract was centrifuged, the cell pellet utilized for protein assay, and the supernatant was processed further for LC-MS/MS analysis (Refer to 2.8). Several analytical columns were used to optimize target analytes separation including: X bridge® (C18, 50 X 2.1mm, 5 µM), Phenomenax® (C18, 50 X 2.1mm., 5 µM), Atlantis® (C18, 50
X 2.1mm., 5 µM), Acquity (UPLC® BEH C18 50 x 2.1 mm, 1.7 µm). These columns were compared in terms of peak shape and resolution. X-bridge® (C18, 50 X 2.1mm, 5 µM) demonstrated excellent peak shape and chromatographic resolution for all analytes (Figure 2). Sub-2 µm UPLC columns are known to provide better separation. However, high back-pressure is the main disadvantage of this column, and no superior benefits were observed with the UPLC column compared to the 5 µm C18 column. Several solvent system combinations including water, methanol, and acetonitrile with 0.1% formic acid were attempted. The best peak shape for all analytes was observed with a slow gradient using water (as mobile phase A) and 0.1% formic acid in acetonitrile (as mobile phase B). Room temperature and elevated column temperatures were also tested, and room temperature was found to be convenient for this separation. Several autosampler rinse solutions were evaluated to eliminate any assay-related carryover (especially for dolutegravir). Different combinations of methanol, ethanol, isopropanol with water and formic acid were evaluated; 90:10:0.5 (v/v/v) of ethanol/water/formic acid was the autosampler rinse solution that eliminated carryover and was chosen for the final method.

Our newly developed method was applied to measure intracellular concentrations of tenofovir, emtricitabine, and dolutegravir in a human brain endothelial cell line (hCMEC/D3). We were able to quantify the concentrations of tenofovir, emtricitabine, and dolutegravir in the hCMEC/D3 cells. Rank order for the accumulation of each drug inside the hCMEC/D3 cells was dolutegravir > emtricitabine > tenofovir. This pattern can be explained by physicochemical properties of each drug. Poor accumulation of tenofovir is explained by its dianionic form at physiological pH responsible for poor bioavailability, and
activity against HIV-1 in a cell-based assay (249). Emtricitabine is a polar moiety with a LogP value of -1.40, making it less permeable inside the cells (222). Dolutegravir is sufficiently lipophilic with a LogP value of 1.10, thus potentially providing a rationale for why it had the highest accumulation among these drugs (222).

Furthermore, drug accumulation inside cells can be influenced significantly by uptake and efflux transporters. The two uptake transporters that are known to mediate tenofovir entry into cells; the organic anion transport proteins 1 and 3 (OAT1; SLC22A6 and OAT3; SLC22A8, respectively). Although OAT3 is known to be expressed at the human BBB, neither OAT1 nor OAT3 are expressed by the hCMEC/D3 cells (38, 250). This could be an explanation for the low intracellular concentrations of tenofovir within these cells. Tenofovir is also a substrate of the efflux transporter multidrug resistant protein-4 (MRP-4; ABCC4), which could further limit intracellular tenofovir concentrations by extruding the drug from the cell (38). hCMEC/D3 cells are reported to have a similar expression level of MRP-4 as that in isolated human brain microvessels (133) and therefore MRP-4 may be further limiting the overall accumulation of tenofovir within hCMEC/D3 cells. Emtricitabine is a substrate of the uptake transporter, multidrug and toxin extrusion protein-1 (MATE1: SLC47A1), however, they were found at a low level in hCMEC/D3 cells (133). Dolutegravir is the substrate of P-glycoprotein (P-gp; ABCB1) and breast cancer resistant protein (BCRP; ABCG2), both of these transporters are expressed and functional in hCMEC/D3 cells and limits intracellular accumulation (42). However, experimental manipulation of these potential interactions of drugs and uptake/efflux transporters was beyond the scope of this study. Finally, this method was successfully applied to estimate intracellular concentrations of tenofovir, emtricitabine and dolutegravir.
in endothelial cells (hCMEC/D3). Besides endothelial cells, HIV-1 infects astrocytes, microglia, and pericytes in the CNS. Intracellular concentration of antiretroviral drugs within these cell types are scantly reported in the literature, but it may be important to ascertain anti-HIV-1 activity of the drugs inside each CNS cell type. Thus, future studies will be done to assess intracellular antiretroviral concentrations in human pericytes, human astrocytes, and human microglia.

In this study, we developed a bioanalytical assay with high sensitivity and specificity to simultaneously measure tenofovir, emtricitabine, and dolutegravir in cell extracts of hCMEC/D3 cells. The method was successfully validated according to FDA guidance and applied to determine uptake of tenofovir, emtricitabine, and dolutegravir inside hCMEC/D3 cells.
Table 8: Intracellular accumulation of antiretroviral drugs/ treatment regimen

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentration Drugs</th>
<th>5µM</th>
<th>10µM</th>
<th>5µM</th>
<th>10µM</th>
<th>5µM</th>
<th>10µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Picomoles/mg of protein</td>
<td>Picomoles/mg of protein</td>
<td>Conc. (ng/mL)</td>
<td>Conc. (ng/mL)</td>
<td>Protein Conc. (mg/mL)</td>
<td>Protein Conc. (mg/mL)</td>
</tr>
<tr>
<td><strong>Individual Drugs</strong></td>
<td></td>
<td>5.91 ± 1.74</td>
<td>5.32 ± 1.07</td>
<td>0.32 ± 0.08</td>
<td>0.64 ± 0.17</td>
<td>0.22 ± 0.08</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>Tenofovir</td>
<td></td>
<td>19.74 ± 3.13</td>
<td>16.95 ± 4.66</td>
<td>1.18 ± 0.10</td>
<td>2.44 ± 0.44</td>
<td>0.26 ± 0.05</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td></td>
<td>74.84 ± 9.84</td>
<td>250.49 ± 45.45</td>
<td>14.19 ± 0.62</td>
<td>48.57 ± 5.04</td>
<td>0.46 ± 0.05</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>Dolutegravir</td>
<td></td>
<td>2.63 ± 0.86</td>
<td>5.15 ± 1.11</td>
<td>0.32 ± 0.07</td>
<td>0.64 ± 0.15</td>
<td>0.46 ± 0.06</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td><strong>Drugs in combination</strong></td>
<td></td>
<td>9.29 ± 1.89</td>
<td>23.36 ± 4.56</td>
<td>1.01 ± 0.11</td>
<td>2.40 ± 0.21</td>
<td>0.46 ± 0.06</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>Tenofovir</td>
<td></td>
<td>81.09 ± 18.51</td>
<td>266.90 ± 40.99</td>
<td>14.67 ± 1.41</td>
<td>46.63 ± 2.71</td>
<td>0.46 ± 0.06</td>
<td>0.43 ± 0.06</td>
</tr>
</tbody>
</table>

Intracellular accumulation of each antiretroviral drug in the brain endothelial cell line, hCMEC/D3 cells. hCMEC/D3 cells were incubated with 5 or 10µM of dolutegravir, emtricitabine, or tenofovir or the cocktail of 5 or 10 µM of each drug given as a three-drug combination (cART). Data are expressed as mean ± SEM (For drug accumulation in picomoles/µg of protein, and ng/mL, the protein concentration in µg/mL) for n=3 independent experiments.
Figure 10: LC-MS/MS chromatograms of (A) Blank cell lysate matrix, (B) Blank cell lysate matrix spiked with labeled internal standards (Tenofovir-d6, Emtricitabine-13C6, 15N2 and Dolutegravir-d5) and (C) 0.1 ng/mL (LLOQ) of Tenofovir, Emtricitabine and Dolutegravir (D) intracellular accumulation inside hCMEC/D3 samples.
Effect of HIV-1 Tat and Morphine on antiretroviral penetration in different CNS cell types

Introduction

In 2016, approximately 36.7 million people are living with HIV (9). Nearly half of the HIV-infected individuals experience HIV-associated neurocognitive disorder (HAND) (218, 251). Clinical presentation of HAND can range from severe (HIV-Associated Dementia) to mild neurocognitive disorder (MND) or asymptomatic neurocognitive impairment (ANI) (252). Substance abuse and HIV are interlinked epidemics, and substance abuse is also one of the significant comorbidities in HAND (253). Heroin (an opioid) is one of the most commonly used illicit drugs in HIV patients, and it is one of the major drugs of abuse in Injection Drug users (IDUs) (254, 255). Recently, prescription opioid abuse through injection had led to outbreaks of HIV (10). Opioid abuse worsens HIV progression by effecting host immune functions causing opportunistic infections (256), increasing virus entry into immune cells and replication therein (257), increases trafficking of infected immune cells into CNS (257). CNS infection of HIV causes immune activation of microglia, astrocytosis, and chronic inflammation, which are hallmarks of the neuropathogenesis of HAND (258, 259).

Despite aggressive use of cART (combination antiretroviral therapy), HIV viral loads persist in the central nervous system (CNS) (as measured in cerebrospinal fluid (CSF)) and prevalence of mild to moderate neurocognitive impairment continues even after many
years of suppressive therapy (60, 222, 260–262). Persistence of viral loads (as measured from CSF) in CNS despite the use of cART, the CNS is a sanctuary for the virus and latent infection in CNS (221, 263, 264). Viral persistence within the CNS suggests that the concentrations of antiretroviral drugs inside the brain are sub-therapeutic, resulting in low level of ongoing replication of the virus in the CNS. The viral proteins released from infected cells cause chronic inflammation, leading to the development of HAND (158, 265, 266). Patients on cART regimens with high CNS penetration effectiveness (CPE) have reduced viral loads in the CNS (as measured in CSF), but the correlation between CPE and clinical outcomes in HAND is not clearly understood (261, 267). Since most antiretroviral drugs (protease inhibitors, nucleoside reverse transcriptase inhibitors (NRTI), and integrase strand transfer inhibitors (INSTI), have their targets located inside the host cell; intracellular concentrations are key in determining the efficacy of antiretroviral drugs (222).

HIV-1 Tat (Transactivator of Transcription) is a regulatory protein, which induces HIV-1 gene expression during transcription initiation and elongation. Cells infected by HIV-1 secrete HIV-1 Tat, which then easily permeates into other cells, including neurons, through adsorptive endocytosis, affecting the viability and functions of neurons (43). Tat causes direct neurotoxicity through an increase in intracellular calcium resulting in excitotoxic damage (268), caspase activation leading to apoptosis (269), and release of proinflammatory cytokines (270). Infected monocytes and perivascular macrophages predominantly seed HIV infection in CNS (271, 272), releasing the virus and viral proteins (including HIV-1 Tat), which in turn activates glial cells (uninfected and infected). The activated glial cells then release inflammatory cytokines (59, 273, 274), increase
glutamate release (275), and cause neuronal dysfunction and death. HIV-1 Tat upregulates P-gp (P-glycoprotein) expression and activity (276), which is responsible for efflux of antiretroviral drugs at the blood-brain barrier (223). Opioids, especially, morphine produces a robust inflammatory response in the CNS, on top of already existing inflammation due to HIV-1 infections, exacerbating HAND. Morphine disrupts blood-brain barrier resulting in trafficking of infected immune cells in the brain (119), increases expression of chemokine receptors on microglia and astrocytes (277, 278), promotes the release of pro-inflammatory cytokines from glial cells (85, 253), and causes neurotoxicity. Morphine is a substrate of P-gp, and it is known to induce expression of P-gp, which reduces penetration of antiretroviral drugs that are substrate of P-gp (279–281). Co-exposure of morphine and Tat synergistically increases blood-brain barrier (BBB) permeability (106), increases caspase-3 activity leading to apoptosis in murine oligodendrocytes (282), upregulates pro-inflammatory cytokines in CNS cell types (human astrocytes, microglia, and brain microvascular endothelial cells) (119, 283, 284), and increases microglial activation and migration. These events cause chronic inflammation, which affects neural function and viability underlying HAND. Interactive effects of HIV-1 Tat and morphine may alter antiretroviral drug penetration in HIV infected CNS cells via efflux or uptake transporters, leading to sub-therapeutic concentrations of antiretroviral drugs in the brain. However, the effects of Tat and morphine on antiretroviral drug penetration inside CNS cell types have not been substantially investigated in the current literature.

In this study, we measured drug concentration in primary human brain microvascular endothelial cells, human astrocytes, human microglia, human pericytes, and immortalized
human cerebral microvascular endothelial cells (hCMEC/D3). Each of these cell types has a distinct role in the neuropathogenesis of HAND, namely, human brain microvascular endothelial cells (HBMEC) line the small cerebral vessels forming the Blood-Brain Barrier (BBB), through which free virus or virus-infected monocytes enter CNS (285). The BBB also restricts the entry of xenobiotics, including antiretroviral drugs, into the brain via tight junctions and drug transporters (efflux and uptake). Inside the CNS, productive replication of virus occurs in perivascular macrophages and microglia, while astrocytes, pericytes, and brain microvascular endothelial cells are sites of non-productive replication of the virus. Pericytes, astrocytes, brain microvascular endothelial cells, are part of neurovascular Unit (NVU). Pericytes wrap around small cerebral vessels and are responsible for differentiation (286) and stabilization of capillaries (287), and maintenance of BBB (288, 289). HIV infection in pericytes causes the release of inflammatory cytokines, disrupts BBB, increasing the flux of virus in the brain (231). Astrocytes are also part of the NVU and are responsible for maintenance of the BBB through the release of several factors like sonic hedgehog (shh) (290), basic fibroblast growth factor and angiopoietin-1 (291), and glial-derived neurotrophic factor (GNDF) (292). HAND is mediated-glially due to the presence of all types of glial cells (astroglia, microglia, oligodendroglia, and ependymal cells) throughout CNS (117). Also, glia (infected and uninfected) are responsible for neuronal injury and death of through the release of inflammatory cytokines and neurotoxins.

There is evidence of differential efficacy of the antiretroviral drug in different cell types. Asachop et al. reported the lower intracellular concentrations of antiretroviral drugs in human microglia when compared to human lymphocytes, resulting in significantly higher
EC$_{50}$ values in human microglia (237). Similarly, Gray et al. demonstrated that antiretroviral drugs have significantly lower efficacy in CNS cell types when compared to peripheral mononuclear blood cells and monocyte-derived macrophages (131). This evidence suggests that the efficacy of antiretroviral drug significantly changes in different cell types, which may be mediated by differential accumulation of drugs into these cells. However, intracellular accumulation of antiretroviral drugs within different brain cell types in the setting of HIV and drug abuse is scantly reported in the current literature. To address this gap, we hypothesize there is differential intracellular penetration of the cART regimen consisting of tenofovir, emtricitabine, and dolutegravir (a regimen recommended by recent guidelines issued by Department of Health Human Services (239)), in different CNS cell types (primary HBMECs, astrocytes, microglia, pericytes, and the immortalized cell line, hCMEC/D3), when treated with HIV-1 Tat and/or morphine.

**Methods**

**Chemical and reagents**

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Tenofovir, Emtricitabine [( - ) FTC], Dolutegravir. Tenofovir-d6 and emtricitabine-13C 15N2 were obtained from Toronto Research Chemical Inc. (Ontario, Canada). Dolutegravir-d5 was purchased from BDG Synthesis (Wellington, NZ). Methanol, acetonitrile with 0.1% formic acid, 2-propanol (HPLC grade) was obtained from Honeywell Chemicals (Muskee, MI). 6-well plates were used from Corning Inc. (Corning, NY, USA). Human brain microvascular endothelial cells, hCMEC/D3, were generously
provided by Dr. Babette Weksler of Weill Cornell Medical College, Cornell University (New York, NY). Endothelial basal media (EBM-2) and the EGM2-MV Bullet kit were purchased from Lonza (Basel, Switzerland). The EGM2-MV Bullet kit contained human endothelial growth factor (hEGF), insulin-like growth factor (IGF), human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), hydrocortisone, ascorbic acid, FBS, GA-1000 (gentamycin and amphotericin). 0.45 µm multiscreen HTS filter plate was purchased from Millipore (Millipore sigma, USA). Primary Human pericytes and astrocytes, along with pericyte and astrocyte medium was purchased from Science Cell, Inc., (Carlsbad, CA). Primary human microglial cells and primary human microglial complete serum media were obtained from Celprogen, Inc. (Torrance, CA). Primary human brain endothelial cells, complete classic basal media, cultureboost™, Passage reagent Group 1,2,3 (PRG®), were purchased from Cell Systems, Inc. (Kirkland, WA). Recombinant HIV-1 Tat was obtained from ImmunoDx, Inc. (Woburn, MA). Ultrapure™ water was obtained from Quality Biologicals, Inc. (Gaithersburg, MD). GIBCO® Hanks Balanced Salt Solution (HBSS), Rat Tail Collagen Type 4, Pierce BCA Protein Assay kit were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA).

**Cell Culture**

hCMEC/D3 were grown in EBM-2 media supplemented with EGM2-MV bullet kit at 5% CO₂, 37°C and 95% relative humidity (termed growth medium). Cells seeded on 6-well plate were grown on EBM-2 media supplemented with 1.25% FBS, 5 mL of penicillin/streptomycin solution (10,000 U/mL), 5 mL of 10 mM HEPES and 1.25 ml of 0.5 ng/mL bFGF (termed maintenance medium). Primary human astrocytes were grown in
astrocyte medium consisting of 500 mL of basal media, 10 mL of FBS, 10 mL of astrocyte growth supplement, and 5 mL of penicillin/streptomycin solution at 5% CO₂, 37°C and 95% relative humidity. Cells were used from passage 8-13. Primary human pericytes were grown in pericyte medium consisting of 500 mL of basal media, 10 mL of FBS, 10 mL of pericyte growth supplement, and 5 mL of penicillin/streptomycin solution at 5% CO₂, 37°C and 95% relative humidity. Cells were used up to 10 passages. Primary human microglia was grown in primary human microglia complete growth media with serum at 5% CO₂, 37°C and 95% relative humidity. Cells were propagated from passage 2 to passage 10.

Primary human astrocytes, pericytes, and microglia were seeded on T75 flasks and 6-well plate pre-coated with 0.5 mg/mL of poly-L-Lysine in 0.1 M sodium borate buffer at pH 8.4. hCMEC/D3 cells were plated on collagen-coated T75 flasks, and 6-well plate coated. With 0.15 mg/mL of Rat Tail Collagen IV solution overnight and washes three times with D-PBS (Dulbecco’s Phosphate buffer solution). Primary human microvascular endothelial cells were coated with Attachment Factor™ for 5s and aspirated before seeding. These cells were grown in complete classic basal media supplemented by CultureBoost™until passage 7-9. Passage reagent Group 1,2,3 (PRG®) from Cell Systems, Inc., were used for subculturing and passaging.

**Treatment**

When cells reached 90% confluency, they were treated with Tat (100nM) and morphine (500 nM) for 24 h. A stock solution of HIV-1 Tat and Morphine were made in ultra pure water. After 24 h, antiretroviral accumulation over 1 h was measured. Stock drug concentration for tenofovir and emtricitabine were prepared in water at 5 mM and 10 mM,
while dolutegravir was prepared in 100 mM stock in DMSO. The final concentration of working stock solution was made to 5 µM, and 10 µM for each drug in water, the final concentration of DMSO were less than 0.01% in final working working solution.

Concentrations of drugs were decided based on Cmax of the drug in clinical trials (246–248, 293). Experiments were performed in two designs. In the first design, treatment includes only antiretroviral drugs, where each cell type was treated with an individual drug (tenofovir, emtricitabine, dolutegravir) and combined ARV (all together) at 5 µM and 10 µM for 1 hour at 37°C at 5% CO₂. This experimental design had no HIV-1 Tat and morphine treatment. In the second experimental design, each cell type was treated with HIV-1 Tat, morphine, and Tat + morphine for 24 h, and then treated with combined ARV at 5 µM and 10 µM for 1 h.

**Intracellular accumulation of antiretroviral drugs**

Each cell type was allowed to grow to 90% confluency on six-well plates. At the end of treatments (described above) for each experimental design, cells were washed thrice with 1 mL of ice-cold HBSS. Then 1 mL of ice-cold methanol was added on each well of the plate and allowed to sit on the ice for 15 minutes. Methanolic cell extract was prepared by thoroughly scrapping the cells with scrapper on ice. The resultant solution was collected and centrifuged at 7500 rpm. 200 µL of supernatant from each sample was aliquoted into a 96-well plate, and 20 µL of 50 ng/mL is working internal standard solution was added. Samples were vortex mixed for 5.0 minutes at 750 rpm and centrifuged for 15 minutes at 5000 rpm. 150 µL of supernatant was collected, filtered through a 0.45 µm multiscreen HTS filter plate (centrifuged for 5 min at 3500 rpm) and dried under a steady stream of nitrogen at 45°C. The dried extracts were reconstituted with 150 µL of water,
mixed for 10 minutes at 5000 rpm and re-centrifuged again for 15 minutes at 5000 rpm. 50 µL of the final extract was injected for LC-MS/MS analysis (refer Chapter 3). The pellet containing cell debris was incubated with 50 µl lysis buffer (NP40+complete protease inhibitor) for 5 minutes on ice. Cell pellets containing lysis buffer were sonicated and analyzed for protein content using the BCA Protein Assay.

**Statistical Analysis**

Two way ANOVA with Tukey's post hoc test was used for all the analyses. A p value less than or equal to 0.05 was considered statistically significant. GraphPad Prism Software (San Diego, California) was used for statistical analyses.

**Results**

**Intracellular accumulation of individual and combined antiretroviral drugs in untreated CNS cell types**

To determine differential accumulation of antiretroviral drugs in primary human brain endothelial cells, primary human astrocytes, primary human pericytes and immortalized human brain microvascular endothelial cells (hCMEC/D3), each cell type was treated with tenofovir, emtricitabine and dolutegravir individually and in a three-drug combination at both 5 µM and 10 µM for 1 h. In all the cell types, intracellular accumulation in (picomoles/µg of protein) was in the following order: dolutegravir > emtricitabine > tenofovir (Table 9). This trend was consistent when cells were treated both as individual agents and also in when given in a combination of antiretroviral drugs, at both concentrations. hCMEC/D3 cells had a significantly higher accumulation of tenofovir (p=0.00001), emtricitabine (p=2.89e-08) in comparison with other cell types that were
treated either individually or in a combination of antiretroviral drugs. Intracellular accumulation of dolutegravir was higher in hCMEC/D3 than other cell types, but it was not statistically significant ($p=0.0767$) (Table 9). The intracellular concentrations of each drug (given as individual drug or in combination) were significantly higher for the concentration of 10 µM as compared to 5 µM for emtricitabine ($p=0.0001$) and dolutegravir ($p=8.88e^{-7}$), as would be expected. However, the intracellular concentration of tenofovir were higher at 5 µM than 10 µM, although this did not reach statistically significance ($p=0.0867$) (Table 9). Intracellular accumulation at a loading concentration of 10 µM would be double that of 5 µM provided there is a linear relationship between drug accumulation and loading dose. Experimentally, such relationships were not observed and may be due to the involvement of drug transporters (uptake or efflux), whose activities might become saturated above a certain concentration. Overall, these results demonstrate that intracellular accumulation of tenofovir, and emtricitabine either individually or in combination, was significantly higher in hCMEC/D3 (immortalized cell line) than primary cell types. The trend for intracellular concentration across all cell types, and loading concentrations was of dolutegravir > emtricitabine > tenofovir.

**Intracellular accumulation of combined antiretroviral drugs in CNS cell types treated with HIV-1 Tat and/or morphine**

Intracellular accumulation of combined antiretroviral drugs at loading concentrations of 5 µM and 10 µM were estimated in all the cell types after exposure with 100 nM of Tat and/or 500 nM of morphine for 24 h. Consistent with trends from intracellular
accumulation in untreated cells, the rank order of intracellular accumulation was
dolutegravir > emtricitabine > tenofovir. Intracellular accumulation of antiretroviral drugs
treated with loading concentration of 5 μM was significantly different than with 10 μM
loading concentration in all the cell types, with the treatment of Tat and morphine (Table
10 A, B, C). There were no significant differences in intracellular accumulation of
antiretroviral drugs after treatment with Tat and morphine in any of the cell types tested
(Table 10 A, B, C).

Discussion

The antiretroviral regimen comprised of tenofovir, emtricitabine and dolutegravir is one of
the recommended regimens from Department of Health and Human Services for
treatment of both for adult and adolescent patients infected by HIV (294). Currently, this
treatment regimen is being used in several randomized clinical trials, namely, DOLPHIN1
and VESTED - evaluating efficacy in pregnant women, INSPIRING – evaluating efficacy
in opportunistic TB infection, ADVANCE – evaluating efficacy for the first line of treatment
(295). Once-daily dosing and greater half-life in tissue than in plasma make it preferred
regimen for HIV-1 infection (296). Passage of these drugs into the brain is restricted by
the BBB, because of their physicochemical properties and because these ARVs are
substrate for efflux transporters located on the luminal side of blood-brain barrier. Poor
penetration of these drugs into the brain is believed to one of the reasons for viral
persistence inside the brain (297).
The questions addressed by the present study were (1) is there a differential accumulation of antiretroviral drugs, individually and in combination, in different CNS cell types and, (2) is there a differential accumulation of combined antiretroviral drugs in different CNS cell types when treated with HIV-1 Tat and/or morphine. Our results indicated that intracellular accumulation of tenofovir, emtricitabine, and dolutegravir, individually or in combination at a loading concentration of 5 µM and 10 µM in untreated cells, was significantly higher in hCMEC/D3 cell line (immortalized) when compared to the other primary cells from the CNS. There were also no significant changes in ARV accumulation upon exposure to Tat and/or morphine within the different CNS cell types. Overall, the trends of intracellular antiretroviral drug accumulation in untreated or treated cells, either individually or in combination, at a loading concentration of 5 µM and 10 µM, was found to be dolutegravir > emtricitabine > tenofovir. Rimawi et al. investigated penetration of these same antiretroviral drugs (tenofovir, emtricitabine, and dolutegravir) through the placental barrier and observed similar trends in intracellular accumulation of these ARVS within placental tissue (298). The study reported herein is, to our knowledge, the first to determine intracellular concentrations of two NRTI (tenofovir and emtricitabine) and an INSTI (dolutegravir) in different CNS cell types.

We wanted to determine intracellular accumulation of antiretroviral drugs, both individually and in combination at clinically relevant concentrations (5 µM and 10 µM) in different CNS cell types. The results demonstrate that, irrespective of whether the antiretroviral drugs were dosed individually or in combination, there was a significantly higher accumulation of tenofovir and emtricitabine within hCMEC/D3 cells when
compared to the accumulation within the other cell CNS types. Dolutegravir intracellular concentrations, however, were not significant in hCMEC/D3 compared to other cell types.

The result observed herein may be attributed to differences in physicochemical properties of each drug, as well as the affinity for, and activity of, uptake and efflux transporters within each cell type. For example, tenofovir is a substrate of the uptake transporters OAT1, OAT3, and the efflux transporter MRP-4 (38). OAT1 and OAT3 have been detected at low levels in hCMEC/D3 cells (133); while in primary human microvascular brain endothelial cells only OAT3 is expressed (134). Furthermore, OAT1 and OAT3 are absent in glial cells or pericytes (140). These transporter expression patterns are consistent with the observed findings of higher accumulation of tenofovir in hCMEC/D3 cells compared to other cell types. Furthermore, MRP4, which is responsible for efflux of tenofovir out of the cell, is expressed in hCMEC/D3 (133), primary brain endothelial cells (299), astrocytes (137) but not reported to be present in pericytes or microglia (142, 135). Emtricitabine is a substrate of the MATE1 (Multidrug and Toxic Compound Extrusion1) transporter (39), which is responsible for extruding drug outside the cell and is not expressed in hCMEC/D3 (133, 300), and expressed in primary brain microvessels (301), and there is no evidence in current literature for its presence in glia or pericytes. MATE1 expression profiles might explain the significant accumulation of emtricitabine in hCMEC/D3 cells when compared to primary brain microvascular endothelial cells. Dolutegravir is a substrate of P-gp and BCRP; however, cellular accumulation of dolutegravir in hCMEC/D3 cells was not significantly different when compared to other primary CNS cell types. hCMEC/D3 is an immortalized cell line derived from human brain microvascular endothelial cells that might
have different genotypic and phenotypic features than primary cells. This difference might explain the significantly higher intracellular accumulation of antiretroviral drugs in hCMEC/D3 cells compared to primary cells. hCMEC/D3 cells are routinely employed in BBB studies because primary human brain microvascular endothelial cells are difficult and costly to acquire (302). However, variations caused by the immortalization process to generate hCMEC/D3 might result in differences in protein expression between the immortalized cell and the primary brain endothelial cells.

We aimed to determine the effects of Tat and/or morphine on combined antiretroviral penetration inside these CNS cell types. Contrary to our hypothesis, we found that exposure of Tat and/or morphine had no significant effect on the intracellular accumulation of antiretroviral drugs. The treatment time point of 24 h was based on pro-inflammatory cytokines released from cells on exposure with Tat and morphine from previous studies (85, 119). These inflammatory cytokines regulate expression of drug transporters, (180, 303–305) which, in turn, may affect intracellular drug accumulation. Future studies including concentration and time-dependent effects of Tat and/or morphine on the release of pro-inflammatory cytokines from each of the cell type will thoroughly investigate treatment effects on intracellular accumulation, and significant changes if any. Antiretroviral drugs were incubated for 1 h based in order to achieve quantifiable concentrations intracellularly. Also, previous studies reported the same period to study the cellular accumulation of antiretroviral drugs (38, 232).

Moreover, NRTIs need to be converted to their triphosphorylated metabolite to be pharmacologically active. This intracellular transformation requires about 16-18 h of time (306). Thus, an expanded time course of intracellular drug accumulation might
significantly alter the intracellular accumulation of antiretroviral drugs. Bosquet et al. used 20 h incubation of tenofovir, emtricitabine and efavirenz for determining intracellular accumulation and studying modulation of ABC (ATP Binding Cassette) transporters by antiretroviral drugs (307). They reported significant differences in accumulation of each drug within PBMC. Therefore, further studies investigating longer incubation times of antiretroviral drugs, and concentration and time-dependent studies measuring pro-inflammatory cytokines, would provide better insights into intracellular drug accumulation.

Our findings show that the overall trend in the rank order of intracellular accumulation of antiretroviral drug is consistent throughout the study, dolutegravir had the highest accumulation, while tenofovir had lowest, accumulation, and emtricitabine had intermediate accumulation. The intracellular accumulation of the antiretroviral drug in all the cell types was consistent with CNS penetration effectiveness score for each drug, dolutegravir > emtricitabine > tenofovir (127, 308). Recent reports link dolutegravir with neuropsychiatric adverse events in different populations (309–311). Our findings report higher accumulation of dolutegravir in all the cell types after 1-hr of incubation; however, chronic exposure may lead to higher accumulation inside the cells leading to cellular toxicity, causing CNS side effects. This trend may be partially explained by the physicochemical properties of drugs. Dolutegravir has highest due to lipophilicity (log P 1.11) of the drugs tested and, therefore, it would be predicted to have the highest intracellular accumulation (222). Emtricitabine has a polar moiety, making it less permeable than dolutegravir, which would limit its penetration into the cells (222). Based on physicochemical properties, it could be predicted that tenofovir would have the lowest accumulation because it would be anionic at physiological pH (312). It must be recognized
that drug accumulation is dependent on more than just its physicochemical properties. Affinity for, and activity of, uptake and efflux transporters as well as transporter and metabolism mediated interactions with other drugs, may also modulate intracellular drug exposure. Some of the aforementioned physicochemical properties are correlated to the substrate specificity of transporters (313–315). Also, the treatment regimen studied herein is reported to have limited to no potential drug-drug interactions at drug transporters (316, 317). Expression of transporters in CNS cells is regulated by nuclear receptors (318), xenobiotic activation of nuclear receptors and subsequent induction of expression of the transporter is a complex multi-stage process, requiring incubation time of several days (41). The incubation time of antiretroviral drugs for one hour is likely insufficient to alter expression of drug transporters. Thus, physiochemical properties, as well as drug transporter involvement, provides the rationale behind the overall trend in accumulation amongst three drugs in this study. This accumulation pattern observed in this study is also reflected in the CNS efficacy of each drug. Protein-free IC$_{50}$ values of tenofovir, emtricitabine, and dolutegravir were reported to be 201.6 ng/mL, 70 ng/mL, 0.2 ng/mL, respectively (222).

In conclusion, our results indicated that Tat and morphine had no significant impact on the intracellular accumulation of antiretroviral drugs. hCMEC/D3, an immortalized cell line had significantly higher accumulation of drugs compared to primary cell types.
Table 9: Intracellular accumulation (picomoles/mg of protein) of 5µM of individual and combined antiretroviral drugs in untreated cells

<table>
<thead>
<tr>
<th></th>
<th>5 µM of individual drugs</th>
<th>5 µM of combined drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tenofovir</td>
<td>Emtricitabine</td>
</tr>
<tr>
<td>HBMEC</td>
<td>2.46 ± 1.02</td>
<td>2.84 ± 0.96</td>
</tr>
<tr>
<td>Microglia</td>
<td>0.75 ± 0.19</td>
<td>5.63 ± 1.54</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>2.33 ± 0.89</td>
<td>12.88 ± 6.49</td>
</tr>
<tr>
<td>Pericytes</td>
<td>0.96 ± 0.29</td>
<td>6.79 ± 2.77</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>5.17 ± 0.99</td>
<td>19.50 ± 4.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>10 µM of individual drugs</th>
<th>10 µM of combined drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tenofovir</td>
<td>Emtricitabine</td>
</tr>
<tr>
<td>HBMEC</td>
<td>3.99 ± 0.99</td>
<td>8.88 ± 1.94</td>
</tr>
<tr>
<td>Microglia</td>
<td>1.99 ± 0.35</td>
<td>10.67 ± 2.18</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>2.94 ± 0.75</td>
<td>17.31 ± 7.50</td>
</tr>
<tr>
<td>Pericytes</td>
<td>2.40 ± 0.75</td>
<td>9.15 ± 1.48</td>
</tr>
<tr>
<td>*hCMEC/D3</td>
<td>4.85 ± 0.65</td>
<td>21.04 ± 3.78</td>
</tr>
</tbody>
</table>
Table 9A: Intracellular accumulation (picomoles/µg of protein) of 5µM of individual and combined antiretroviral drugs in untreated cells. Each value of Intracellular accumulation is represented in mean ± SEM of picomoles/µg of protein. Human cerebral microvascular endothelial cell line/D3 has significantly higher accumulation for tenofovir and emtricitabine compared to other cell types with individual drug or in combination, and at either 5 µM or 10µM. Each value represents intracellular accumulation in picomoles/µg of protein expressed as mean ± SEM for n=6 independent experiments.
Table 10 A: Intracellular accumulation of tenofovir from 5 and 10 µM of combined antiretroviral drugs in different CNS cell types. Intracellular accumulation is represented as mean ± SEM picomoles/mg of protein for n=6 independent experiments. There were no statistically significant differences between treatments, however, drug accumulation between 5 µM and 10 µM were significantly different.

<table>
<thead>
<tr>
<th>Tenofovir</th>
<th>Control</th>
<th>Tat</th>
<th>Mor</th>
<th>Tat + Mor</th>
<th>Control</th>
<th>Tat</th>
<th>Mor</th>
<th>Tat + Mor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBMEC</td>
<td>0.69 ± 0.25</td>
<td>0.95 ± 0.23</td>
<td>2.08 ± 1.11</td>
<td>1.16 ± 0.42</td>
<td>1.85 ± 0.33</td>
<td>3.39 ± 0.98</td>
<td>2.87 ± 0.46</td>
<td>1.27 ± 0.64</td>
</tr>
<tr>
<td>Microglia</td>
<td>0.65 ± 0.23</td>
<td>0.83 ± 0.23</td>
<td>0.80 ± 0.29</td>
<td>0.69 ± 0.12</td>
<td>1.03 ± 0.12</td>
<td>0.79 ± 0.04</td>
<td>0.93 ± 0.06</td>
<td>0.89 ± 0.16</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>2.08 ± 0.59</td>
<td>1.96 ± 0.61</td>
<td>1.43 ± 0.35</td>
<td>1.21 ± 0.20</td>
<td>3.36 ± 1.69</td>
<td>3.93 ± 0.65</td>
<td>3.60 ± 0.33</td>
<td>3.21 ± 0.36</td>
</tr>
<tr>
<td>Pericytes</td>
<td>1.08 ± 0.30</td>
<td>0.81 ± 0.18</td>
<td>1.11 ± 0.23</td>
<td>1.46 ± 0.29</td>
<td>2.83 ± 1.07</td>
<td>3.02 ± 1.57</td>
<td>1.59 ± 0.45</td>
<td>1.76 ± 0.51</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>0.40 ± 0.09</td>
<td>0.24 ± 0.03</td>
<td>0.25 ± 0.03</td>
<td>0.21 ± 0.04</td>
<td>0.82 ± 0.15</td>
<td>0.68 ± 0.10</td>
<td>1.38 ± 0.60</td>
<td>0.86 ± 0.15</td>
</tr>
</tbody>
</table>
Table 10 B: Intracellular accumulation of emtricitabine from 5 and 10 µM of combined antiretroviral drugs in different CNS cell types. Intracellular accumulation is represented as mean ± SEM picomoles/mg of protein for n=6 independent experiments. There were no statistically significant differences between treatments.

<table>
<thead>
<tr>
<th>Emtricitabine</th>
<th>5 µM of combination antiretroviral drugs</th>
<th>*10 µM of combination antiretroviral drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tat</td>
</tr>
<tr>
<td>HBMEC</td>
<td>3.26 ± 0.44</td>
<td>2.89 ± 0.72</td>
</tr>
<tr>
<td>Microglia</td>
<td>4.99 ± 0.89</td>
<td>7.26 ± 0.77</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>3.61 ± 0.68</td>
<td>3.72 ± 0.89</td>
</tr>
<tr>
<td>Pericytes</td>
<td>4.10 ± 1.16</td>
<td>3.22 ± 0.75</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>1.89 ± 0.13</td>
<td>1.79 ± 0.14</td>
</tr>
</tbody>
</table>
Table 10 C: Intracellular accumulation of dolutegravir in picomoles/mg of protein in treated cells

<table>
<thead>
<tr>
<th>Dolutegravir</th>
<th>5 µM of combination antiretroviral drugs</th>
<th>*10 µM of combination antiretroviral drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tat</td>
</tr>
<tr>
<td>HBMEC</td>
<td>47.76 ± 7.98</td>
<td>35.83 ± 5.42</td>
</tr>
<tr>
<td>Microglia</td>
<td>5.97 ± 1.61</td>
<td>12.00 ± 3.47</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>12.06 ± 3.35</td>
<td>13.48 ± 4.22</td>
</tr>
<tr>
<td>Pericytes</td>
<td>13.19 ± 4.39</td>
<td>10.64 ± 3.33</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>7.88 ± 0.92</td>
<td>6.20 ± 0.57</td>
</tr>
</tbody>
</table>

Table 10C: Intracellular accumulation of dolutegravir from 5 and 10 µM of combined antiretroviral drugs in different CNS cell types. Intracellular accumulation is represented as mean ± SEM picomoles/mg of protein for n=6 independent experiments. There were no statistically significant differences between treatments. however, drug accumulation between 5 µM and 10 µM were significantly different.
Conclusions & Future Directions

Collectively, our investigations indicate that HIV-1 Tat and methamphetamine alter aspects of BBB integrity without affecting net flux of paracellular compounds. Tat and methamphetamine may also affect several aspects of transcellular transport. Tat and methamphetamine containing treatment inhibited or impaired P-glycoprotein-dependent rhodamine123 efflux in hCMEC/D3 cells, without affecting P-glycoprotein or MRP-1 protein expression levels. Despite impaired P-glycoprotein dependent transport with Tat and methamphetamine exposure, atazanavir failed to accumulate within the cells. Because atazanavir is a substrate for two efflux transporters (P-glycoprotein and MRP-1) within the BBB, it is likely that the redundancy allows atazanavir to be shunted through the opposing transporter when the first is impaired. Indeed, single transporter inhibition did not result in significant increases in atazanavir accumulation, but simultaneous inhibition of efflux transporters increased atazanavir accumulation in the BBB cells.

A simple and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for simultaneous determination of tenofovir, emtricitabine, and dolutegravir in cell lysates of an immortalized human brain microvascular endothelial cell line (hCMEC/D3) was developed and validated. Analytes were separated on a reverse phase C18 column using H2O and acetonitrile with 0.1% formic acid mobile phases. The analytes were detected using positive electrospray ionization mode with multiple reaction monitoring (MRM). The assay was linear in the concentration range of 0.1-100 ng/mL for all analytes. Intra- and inter-assay precision and accuracy were within ± 13.33% and ± 11.69%, respectively.
The validated method was applied to determine the intracellular concentrations of the antiretroviral regimen consisting of tenofovir, emtricitabine, and dolutegravir in five different brain cell types: primary human brain microvascular endothelial cells, primary human microglia, primary human astrocytes, primary human pericytes, and the immortalized brain microvascular endothelial cell line, hCMEC/D3 cells. Intracellular antiretroviral drug accumulation, either individually or in combination was found to be significantly higher in hCMEC/D3 cells compared to other cell types. These cells were treated with Tat and/or morphine. After 24 h, intracellular accumulation was quantified. No significant differences in antiretroviral drugs accumulation were observed between treatment groups. Overall, rank-order of intracellular accumulation of antiretroviral drugs in different CNS cell types in picomoles/µg of protein was found to be dolutegravir > emtricitabine > tenofovir.

Future studies will include incorporating treatments (Tat and/or Morphine) for the expanded period, increasing the incubating period of the antiretroviral drugs, and single round infectivity assays to determine the efficacy of antiretroviral drugs in several cell types in presence or absence of morphine. Additionally, we plan to develop and validate the LC-MS/MS method mentioned above in mouse plasma and brain tissue homogenates of a mouse. This method will be used in studying antiretroviral concentration in different parts of brains of Tat transgenic mice as well as in a live virus (EcoHIV) rodent model.
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