In vitro and in vivo regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: implications in HIV-1 neuropathogenesis and its treatment

by

Tamima Ashraf

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
Leslie Dan Faculty of Pharmacy
University of Toronto

© Copyright by Tamima Ashraf, 2015
In vitro and in vivo regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: implications in HIV-1 neuropathogenesis and its treatment

Tamima Ashraf
Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto
2015

Abstract

Cognitive impairment remains highly prevalent in human immunodeficiency virus-1 (HIV-1) infected patients due to viral replication and associated brain inflammation. One obstacle to effective treatment is poor penetration of antiretroviral drugs across blood-brain barrier (BBB) and into HIV-1 brain cellular targets (microglia, astrocytes) due to functional expression of efflux transporters [P-glycoprotein (P-gp), Multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP)]. Identifying therapeutic compounds that are not substrates of these transporters but target signaling pathways involved in inflammation may benefit treatment of HIV-associated neurological complications. The aim of this PhD project was: i) to investigate signaling pathways involved in the regulation of Mrp1 and P-gp in cultured rat/human astrocytes triggered with HIV-1 glycoprotein 120 (gp120) or cytokines, ii) to implement and characterize an in vivo model of gp120-associated brain inflammation, iii) to assess the efficacy of chloroquine, minocycline and simvastatin in reversing brain inflammation in vivo and iv) to elucidate key signaling pathways involved in gp120-associated brain inflammation in vivo. We demonstrated that gp120-associated TNF-α release resulted in increased Mrp1 functional expression in primary cultures of rat astrocytes and both c-jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) pathways were involved. In human astrocytes, we demonstrated decreased P-gp expression following exposure to HIV-1/gp120/interleukin-6 (IL-6) and involvement of NF-κB signaling in P-gp downregulation. In our in vivo model, gp120 administration resulted in a significant increase in inflammatory
markers in different brain regions and in cerebrospinal fluid (CSF). Administration of anti-inflammatory compounds partially or completely reduced upregulation of these markers. Activation of mitogen-activated protein kinases (MAPKs) was also observed both in vitro and in vivo which was attenuated by the anti-inflammatory compounds. Our data demonstrate that: i) gp120 generates an inflammatory response and alters expression of efflux transporters both in vitro and in vivo, in part, through an interaction with MAPK pathway and ii) anti-inflammatory agents could partially/completely reverse this response suggesting that they could serve as a promising novel therapeutic approach for HIV-1-associated brain inflammation.
Acknowledgments

I am grateful to Dr. Reina Bendayan, my thesis supervisor, for her tremendous support, encouragement and guidance throughout my graduate studies. Thank you for giving me the opportunity to pursue my doctoral studies under your supervision. I have learnt a lot from you and had a great experience in your laboratory.

I would like to sincerely thank my committee members Drs. Stephane Angers, Peter Pennefather, Lyanne Schlichter and Sukriti Nag for their continuous support, constructive criticism and guidance throughout my Ph.D. studies. I am truly grateful to all of you.

A special thanks to Dr. Patrick Ronaldson, a former Ph.D. and postdoctoral fellow in our laboratory, who I had the opportunity to work with during the first few months of my Ph.D. studies. Thank you for sharing your time, knowledge and expertise and providing me with the necessary training with the astrocyte work.

Thanks to Dr. Carolyn Cummins and Rucha Patel for their help with the qPCR analysis.

I thank Dr. Michelle Farrugia and Dr. Yuri Persidsky for their help with the human fetal astrocyte work.

I thank Dr. Jeffrey Henderson for collaborating with us and providing me with the opportunity to learn immunohistochemistry in his facility.

My sincere thanks to the Graduate Department of Pharmaceutical Sciences for their generous support and approval of several fellowship awards.

Thanks to all the members of the Bendayan lab, past and present: Tianna Huang, Kevin Robillard, Gary Chan, Olena Kis, Anu Shah, Nilasha Banerjee, Dr. Maria De Rosa, Arpit Shah, Monika Zhang, Donald Wang, Amy Kao and Billy Huang. A special thanks to Dr. Tozammel Hoque for his assistance in various projects and all his encouragements throughout the past few years. I sincerely thank Dr. Wenlei Jiang, Dr. Chiping Wu, Dr. Min Rui and Blake Ziegler for all their help with the animal and capillary work.

I would also like thank my friends from the faculty- Lilia Magomedova, Celine Lacroix and Monica Patel for their constant encouragement and support. I wish you all the best in your future endeavors.

My deepest gratitude to my parents, my brother and my in-laws for always being there for me. My heartfelt thanks to my mother, Anowara Begum, for being a constant source of courage and inspiration. Thank you for teaching me how to always remain positive.

Lastly, I would like to thank my significant other, Mirza Ridwanul Haque, for being the greatest source of support and encouragement. Thank you for being there for me during the ups and downs. It would not have been possible without you.
Table of Contents

Table of Contents

Acknowledgments................................................................................................................. iv
Table of Contents.................................................................................................................. v
List of Tables ........................................................................................................................ xi
List of Figures ........................................................................................................................ xii
List of Appendices ................................................................................................................ xv
List of Abbreviations ............................................................................................................. xvi
Chapter 1 ................................................................................................................................. 1
  1 Human immunodeficiency virus-1 (HIV-1) pathogenesis .................................................. 1
    1.1 HIV-1 epidemiology ....................................................................................................... 1
    1.2 HIV-1 life cycle .............................................................................................................. 2
    1.3 Treatment of HIV-1 infection ......................................................................................... 4
      1.3.1 Entry inhibitors ....................................................................................................... 4
      1.3.2 Fusion inhibitor ....................................................................................................... 5
      1.3.3 NRTIs .................................................................................................................... 5
      1.3.4 NNRTIs .................................................................................................................. 6
      1.3.5 Integrase inhibitors ............................................................................................... 6
      1.3.6 PIs ......................................................................................................................... 6
    1.4 Brain barriers and parenchymal cellular compartments ................................................. 9
      1.4.1 Blood-brain barrier (BBB) ..................................................................................... 9
      1.4.2 Pericytes ............................................................................................................... 10
      1.4.3 Brain parenchymal cellular compartments ......................................................... 10
      1.4.4 Blood-cerebrospinal fluid barrier (BCSFB) ......................................................... 12
1.5 Brain HIV-1 infection and HIV-1-associated neurological disorders ........................................... 13
  1.5.1 HIV-1-associated neuropathogenesis ................................................................. 13
  1.5.2 HIV-associated neurocognitive disorders (HAND) ........................................... 18
1.6 Treatment obstacles of brain HIV-1 infection: brain permeability of antiretroviral drugs ................................................................. 20
1.7 ATP-binding cassette (ABC) transporters ................................................................. 23
  1.7.1 P-glycoprotein (P-gp) ...................................................................................... 26
  1.7.2 Multidrug resistance-associated proteins (MRPs) ........................................... 29
  1.7.3 Breast cancer resistance protein (BCRP) ......................................................... 35
  1.7.4 Regulation of ABC transporters during HIV-1 infection ................................... 38
1.8 Potential adjuvant therapy ......................................................................................... 39
  1.8.1 Minocycline ...................................................................................................... 40
  1.8.2 Chloroquine .................................................................................................... 41
  1.8.3 Simvastatin ..................................................................................................... 42
1.9 Signaling pathways ................................................................................................. 43
  1.9.1 NF-κB ............................................................................................................. 44
  1.9.2 MAPKs .......................................................................................................... 45
  1.9.3 Regulation of ABC transporters by MAPK and NF-κB .................................... 50
  1.9.4 Effect of anti-inflammatory compounds on MAPK and NF-κB ...................... 51
1.10 In vitro and in vivo models to study drug transport and HIV-associated brain inflammation ................................................................. 52
  1.10.1 In vitro systems ............................................................................................. 52
  1.10.2 In vivo models ............................................................................................... 53
2 Goal ......................................................................................................................... 55
3 Rationale ................................................................................................................... 55
4 Hypotheses ................................................................................................................ 57
Chapter 2

6 Regulation of Mrp1 by TNF-α in cultured glial cells: involvement of NF-κB and JNK signaling pathways

6.1 Abstract

6.2 Introduction

6.3 Materials and methods

6.3.1 Materials

6.3.2 Cell culture

6.3.3 Gp120/cytokine Treatments

6.3.4 Immunoblot Analysis

6.3.5 Quantitative PCR

6.3.6 Enzyme-linked immunoabsorbant assay (ELISA)

6.3.7 Functional studies

6.3.8 Data Analysis

6.4 Results

6.4.1 Effect of cytokines on Mrp1 protein expression

6.4.2 Functional studies

6.4.3 Role of NF-κB on cytokine release and Mrp1 protein expression

6.4.4 Role of JNKs on Mrp1 functional expression

6.4.5 Effect of HIV-196ZM651 gp120 and TNF-α on Mrp1 gene expression

6.5 Discussion

6.6 Acknowledgements

Chapter 3

7 Regulation of P-gp by HIV-1 in primary cultures of human fetal astrocytes (HFAs)
11.3 Permeability of antiretroviral drugs during HIV-1 gp120-associated brain inflammation in vivo .......................................................... 175

12 Conclusion .................................................................................. 176

References ..................................................................................... 177

Appendices .................................................................................... 219

List of publications ........................................................................ 231

Copyright Acknowledgements ....................................................... 232
List of Tables

Table 1-1 Preferred and alternative antiretroviral regimens for antiretroviral therapy-naive patients (From Ashraf T, Robillard K, Chan G and Bendayan R. Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders. 2013. Current Pharmaceutical Design. 20: 1543-1563) ................................................................. 7

Table 1-2 Comparison of CSF to plasma ratios of antiretroviral drugs in HIV-infected patients. Values are reported as median and/or the range of CSF: plasma (from Ashraf T, Robillard K, Chan G and Bendayan R. Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders. 2013. Current Pharmaceutical Design. 20: 1543-1563)..... 22

Table 1-3 CNS expression/localization of ABC transporters implicated in antiretroviral drug transport (From Ashraf T, Robillard K, Chan G and Bendayan R. Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders. 2013. Current Pharmaceutical Design. 20: 1543-63)................................................................. 37

Table 6-1 ELISA analysis of TNF-α secretion in cultured astrocytes treated with HIV-196ZM651 gp120................................................................................................................................. 82

Table 7-1 Pro-inflammatory cytokine secretion in primary cultures of HFAs treated with HIV-196ZM651 gp120................................................................................................................................. 109
List of Figures

Figure 1-1 HIV-1 life cycle................................................................. 3

Figure 1-2 HIV-1- associated pathogenesis in the brain.............................. 17

Figure 1-3 Localization of ABC transporters at the BBB and BCSFB ................ 24

Figure 1-4 Localization of of ABC transporters in astrocytes, microglia and neurons .......... 25

Figure 1-5 Membrane topology of P-gp .................................................. 27

Figure 1-6 Membrane topology of MRP1/Mrp1. ........................................ 30

Figure 1-7 Membrane topology of BCRP ................................................... 35

Figure 1-8 The MAPK pathway ............................................................... 46

Figure 6-1 Effect of cytokines on Mrp1 protein expression in primary cultures of rat astrocytes 70

Figure 6-2 Effect of cytokine neutralizing antibodies on Mrp1 protein expression in primary cultures of rat astrocytes treated with HIV-196ZM651 gp120 .......................................................... 73

Figure 6-3 Effect of 24 h TNF-α exposure on the cellular retention of BCECF, a fluorescent Mrp substrate, by cortical rat astrocyte monolayers ................................................................. 75

Figure 6-4 Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and SN50, a peptidic NF-κB inhibitor ............................................................... 78

Figure 6-5 Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and BAY 11-7082, a pharmacological NF-κB inhibitor .......................................................... 80

Figure 6-6 Expression of Mrp1 in primary cultures of rat astrocytes treated with TNF-α and SN50, a peptidic NF-κB inhibitor ............................................................... 84

Figure 6-7 Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and SP600125, a pharmacological JNK inhibitor ....................................................... 86
Figure 6-8 Expression of Mrp1 in primary cultures of rat astrocytes treated with TNF-α and SP600125, a pharmacological JNK inhibitor... 89

Figure 6-9 Effect of 24 h gp120 or TNF-α exposure on the cellular retention of BCECF, a fluorescent Mrp substrate, by cortical rat astrocyte monolayers... 91

Figure 6-10 Effect of gp120 or TNF-α exposure on Mrp1 mRNA expression in primary cultures of rat astrocytes... 93

Figure 6-11 Proposed mechanism of NF-κB and JNK signaling in glial cells during an HIV-1 associated inflammatory response... 99

Figure 7-1 Immunoblot analysis of CXCR4 and CCR5 in primary cultures of HFAs... 110

Figure 7-2 Immunoblot and densitometric analysis of P-gp in primary cultures of HFAs after exposure to (A) either CCR5-tropic HIV-1 ADA or CCR5/CXCR4 dual-tropic HIV-1 89.6 viral isolates, (B) 1.0 nM gp120 or (C) IL-6 (0.5 ng/ml or 10ng/ml)... 112

Figure 7-3 Accumulation of [3H]digoxin by HFAs in the presence or absence of gp120... 113

Figure 7-4 Immunoblot and densitometric analysis of P-gp in primary cultures of HFAs treated with 1.0nM gp120 in the presence of 0.5µg/ml IL-6 neutralizing antibody (NAB)... 115

Figure 7-5 Immunoblot and densitometric analysis of P-gp in primary cultures of HFAs treated with (A) 1.0nM gp120 or (B) IL-6 (0.5 or 10ng/ml) in the presence of NF-κB inhibitory peptide, SN50 (1.0 μM)... 117

Figure 8-1 Effect of gp120 on the mRNA levels of (A) IL-1β, (B) iNOS and (C) TNF-α in different brain regions of ICV administered gp120 (500ng) rats along with a CCR5 antagonist, maraviroc (MVC)... 135

Figure 8-2 ELISA analysis of (A) IL-1β and (B) TNF-α secretion in the CSF of gp120 (500ng) administered rats in the presence of Maraviroc (MVC)... 137
Figure 8-3 (A) Immunohistochemical and (B) immunoblotting (upper panel) and densitometric analysis (lower panel) of GFAP in hippocampus of ICV administered gp120 rats compared to saline treated animals. ........................................................................................................ 139

Figure 8-4 Immunoblot (upper panel) and densitometric analysis (lower panel) of drug efflux transporters (P-gp, Mrp1 and Bcrp) in (A) frontal cortex, (B) hippocampus and (C) striatum of ICV administered gp120 rats. ........................................................................................................ 143

Figure 8-5 Effect of gp120 on the mRNA levels of (A) IL-1β, (B) iNOS and (C) TNF-α in different brain regions of ICV administered gp120 rats receiving simultaneous intraperitoneal injection of chloroquine or minocycline. ........................................................................................................ 146

Figure 8-6 ELISA analysis of (A) IL-1β and (B) TNF-α secretion in the CSF of gp120 (500ng) administered rats receiving simultaneous intraperitoneal injection of chloroquine or minocycline or simvastatin. ........................................................................................................ 148

Figure 8-7 Protein (upper panel) and densitometric analysis (lower panel) of signaling kinases in the hippocampal tissue of gp120 administered rats. ........................................................................................................ 152

Figure 9-1 A schematic summarizing gp120-mediated regulation of cytokine secretion and drug efflux transporters as well as effect of anti-inflammatory compounds in vitro or in vivo. ....... 171
List of Appendices

Appendix A......................................................................................................................219

Appendix B......................................................................................................................220
List of Abbreviations

Aβ, amyloid-β
AA, arachidonic acid
ABC, ATP-binding cassette
AIDS, acquired immunodeficiency syndrome
ANOVA, analysis of variance
BBB, blood-brain barrier
BCECF, 2',7'-bis- (2-carboxyethyl)-5- (and-6)- carboxyfluorescein
BCRP, breast cancer resistance protein
BCSFB, blood-cerebrospinal fluid barrier
CAR, constitutive androstane receptor
cART, combination antiretroviral therapy
CHARTER, CNS HIV Anti-Retroviral Therapy Effects Research
CNS, central nervous system
CPE, CNS penetration effectiveness
CSF, cerebrospinal fluid
CYPs, cytochrome P450 enzymes
DMEM, Dulbecco’s modified Eagle’s medium
DMSO, dimethyl sulfoxide
EAAT, Excitatory amino acid transporter
EDTA, ethylenediaminetetraacetic acid
EGTA, ethylene glycol tetraacetic acid
ELISA, Enzyme-linked immunoabsorbent assay
ESCRT, Endosomal sorting complex required for transport
ERKs, extracellular regulated kinases
FBS, fetal bovine serum
FITC, fluorescein isothiocyanate
FIV, feline immunodeficiency virus
GFAP, glial fibrillary acidic protein
GSH, glutathione
GSSG, glutathione disulfide
Gp120, glycoprotein 120
HAART, highly active antiretroviral therapy
HAD, HIV-associated dementia
HAND, HIV-associated neurocognitive disorder
HBSS, Hank’s balanced salt solution
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFA, human fetal astrocyte
HIV-1, human immunodeficiency virus-1
HIVE, HIV-1 encephalitis
HO-1, heme oxygenase-1
HRP, horseradish peroxidase
ICAM, intracellular adhesion molecule
ICV, intracerebroventricular
IFN, interferon
IκB, inhibitor of NF-κB
IKK, IκB kinase
IL-1β, interleukin-1β
IL-6, interleukin 6
iNOS, inducible nitric oxide synthase
JAK-STAT, Janus kinase/signal transducers and activators of transcription
JNKs, c-jun N-terminal kinases
LPS, lippopolysaccharide
LTC4, leukotriene C4
MAPK, mitogen-activated protein kinase
MCP-1, monocyte chemoattractant protein-1
MDR, multidrug resistance
MIF, macrophage migration inhibitory factor
MIP, macrophage inflammatory protein
MRP, multidrug resistance-associated protein
mtDNA, mitochondrial DNA
NBD, nucleotide binding domain
NF-κB, nuclear factor-κB
NNRTI, non-nucleoside reverse transcriptase
NO, nitric oxide
NRTI, nucleoside reverse transcriptase
P38Ks, P38 kinases
PBMCs, peripheral blood mononuclear cells
PBS, phosphate buffer saline
PIs, protease inhibitors
P-gp, p-glycoprotein
PPAR, peroxisome proliferator-activated receptor
PMEA, 9-(2-phosphonomethoxyethyl)adenine

PMSF, phenylmethylsulfonyl fluoride

PVDF, polyvinylidene difluoride

PXR, pregnane X receptor

qPCR, quantitative polymerase chain reaction

RIPA, radioimmunoprecipitation assay buffer

ROS, reactive oxygen species

SCID, severe combined immunodeficiency

SD, standard deviation

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM, standard error of mean

SIV, simian immunodeficiency virus

Tat, transactivator of transcription

TMD, transmembrane domain

TNF-α, tumor necrosis factor-α

VCAM, vascular adhesion molecule

Vpr, viral protein R

ZO-1, zona occluden-1
Chapter 1

Background

1  Human immunodeficiency virus-1 (HIV-1) pathogenesis

1.1  HIV-1 epidemiology

HIV-1 is an enveloped retrovirus that debilitates the immune system by infecting immune cells (i.e., T-cells, monocytes or macrophages) and ultimately causes acquired immunodeficiency syndrome (AIDS) in human body (Barre-Sinoussi et al., 1983; Popovic et al., 1984). According to the Joint United Nations Program on HIV/AIDS (UNAIDS), about 35.3 million people were living with HIV-1 infection worldwide in 2012. In the same year, 1.6 million people died from AIDS-related causes. Although the number of individuals newly infected with HIV-1 continues to decrease globally, approximately 2.3 million new infections have been reported in adults (2 million) and children (260,000) in 2012. Sub-Saharan Africa remains the most affected area (25 million infected individuals) by HIV-1. However, the incidence rate in this region has decreased significantly since 2001 due to more availability and accessibility of antiretroviral therapy. In West and Central Africa, HIV-1 prevalence remains comparatively low, whereas, the epidemics in Eastern Europe and Central Asia (1.3 million) are dramatically increasing. HIV-1 infected individuals also constitute significant populations in South and South East Asia (3.9 million) and in Latin America (1.5 million). In Western and Central Europe as well as North America, the incidence rate has changed very little since the last decade and availability of antiretroviral therapy has significantly reduced the AIDS-related mortality. An estimated 20,000 AIDS-related deaths and 48,000 new infections have been reported in North America in 2012. About 1.3 million people are currently living with HIV-1 infection in North America (UNAIDS, 2013). Heterosexual transmission remains the primary mode of HIV-1 transmission globally and intravenous drug use (i.e., needle sharing) or homosexual transmission are other significant mechanisms for HIV-1 acquisition.

Reproduced with permission from the copyright owner
1.2 HIV-1 life cycle

HIV belongs to the lentiviral subgroup of the family *Retroviridae* and similar to other lentiviruses, it is capable of infecting non-dividing cells such as T–cells, monocytes or macrophages. Two variants of HIV have been identified- HIV-1 and HIV-2. HIV-1 is responsible for the global HIV epidemic, whereas, HIV-2 is predominantly found in West Africa. HIV-1 has a 9kb genome that encodes nine genes- *gag, pol, env, vif, vpu, vpr, tat, rev* and *nef*.

In its native form, the viral envelope spike is a heterotrimer that is formed by three gp120 molecules attached to three gp41 molecules. The surface glycoprotein 120 (gp120) and the transmembrane protein gp41 expressed on the surface of the virus are encoded by the viral *env* gene. Gp120 is a heavily glycosylated protein that contains five conserved domains (C1-C5) and five variable domains (V1-V5). Structurally, gp120 has three main regions- the inner domain, the outer domain and the bridging sheet. The inner domain is conserved between HIV strains. The outer domain is heavily glycosylated and contains three of the five variable loops (V3-V5). The bridging sheet contains the other two variable loops and contributes to CD4 receptor and chemokine co-receptor binding. Entry of HIV-1 into host cells is initiated by binding of HIV-1 gp120 with CD4 receptor. CD4 is a transmembrane glycoprotein that is expressed by monocytes, macrophages and subsets of T-cells and dendritic cells. The gp120-CD4 binding induces a conformation change that exposes the co-receptor binding site and allows the binding of gp120 to chemokine co-receptor/co-receptors expressed on host cells. The co-receptors most frequently used by HIV-1 *in vivo* have been determined to be CCR5 and CXCR4. CCR5 and CXCR4 are both G-coupled receptors that contain an extracellular N-terminal tail, three intracellular and extracellular loops and a C-terminal cytoplasmic tail. Co-receptor selectivity decides viral tropism. Based on cellular tropism, HIV-1 can be divided into three groups: macrophage (M) tropic, T-cell (T) tropic and dual tropic. Dual tropic viruses can infect both macrophage and T-cell lines. M-tropic strains utilize CCR5 chemokine receptor to enter host cells, whereas, T-tropic viruses use CXCR4 chemokine receptor. Mutations to CCR5 gene (CCrndelta32) can alter the susceptibility to HIV-1 infection. The gp120-chemokine receptor binding allows the hydrophobic N-terminus of the gp41 to insert into the target cell membrane. The helically heptad repeats (HRs) of the gp41 ectodomain form a six helix bundle that leads to the close proximity of the host cell membrane and viral membrane. Following fusion, the viral core is released into the...
host cell. The viral single-stranded RNA is then immediately transcribed into double-stranded DNA by the viral reverse transcriptase and RNAse H. HIV-1 integrase then allows the integration of the viral genetic material into the host cell genome. The subsequent transcription, translation, post-translational modification and maturation precede the release of the new virions from infected cells (Bukrinskaya, 2004) (Figure 1-1). Viral gag plays a major role in the assembly of viral particles and budding of new virions (Meng and Lever, 2013). Gag has four domains- matrix, nucleocapsid, capsid and p6. Gag binds to viral RNA using nucleocapsid domain which is then trafficked to the membrane where gag multimerizes. During the budding process, gag uses the host cellular endosomal sorting complex required for transport (ESCRT) system. The C-terminal domain of gag interacts with ESCRTI and directs gag to ESCRTIII for budding. During the budding process, the immature virions acquire the viral envelope complex and HIV-1 protease cleaves the precursor viral proteins (i.e., gag-pol precursor) to yield mature HIV-1 (Meng and Lever, 2013).

The sustained viral replication continues during infection and the virus migrates to different tissues and organs using the circulatory system.

**Figure 1-1 HIV-1 life cycle.** The viral entry is initiated by the binding of viral envelope spike, a protein complex comprised of gp120 and gp41, to the CD4 receptor that triggers a conformational change and allows the subsequent binding of gp120 to the HIV-1 co-receptor (i.e., CCR5 and CXCR4). Binding of gp120 to the co-receptor causes the fusion of viral and cell
membrane allowing the release of viral genetic material into the cell cytoplasm. The immediate transcription of the viral RNA into double-stranded DNA by the viral reverse transcriptase allows the integration of the viral genetic material into the host cell genome. The subsequent transcription of the genome results in the production of HIV-1 genomic RNA as well as viral mRNA that is translated into proteins. The protein precursors are cleaved by viral protease into functional proteins that assemble at the plasma membrane of the host cell. Once released, the new virions undergo maturation before becoming infectious (from Ashraf T, Robillard K, Chan G and Bendayan R. Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders. 2013. Current Pharmaceutical Design. 20: 1543-1563)

1.3 Treatment of HIV-1 infection

Six different classes of antiretroviral drugs are currently used in HIV-1 treatment- entry inhibitor, fusion inhibitor, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitor and protease inhibitors (PIs). These drugs target different phases of HIV-1 life cycle (Sierra-Aragon and Walter, 2012). Due to the presence of viral reservoir in resting memory CD4 positive T-cells, viral eradication remains difficult. Other viral sanctuary sites are the central nervous system (CNS), testis and gut-associated lymphoid tissue. Table 1-1 summarizes recommended preferred and alternative antiretroviral regiments for the initial therapy of treatment-naive HIV patients. A brief description of different classes of antiretroviral drugs is provided below.

1.3.1 Entry inhibitors

Entry inhibitor targets viral binding to the host cell membrane. Entry inhibitors are developed as receptor antagonists that can interact with the host cell co-receptor and prevent the binding of viral gp120. Maraviroc is the first approved CCR5 antagonist and is only effective against R5 tropic viruses (Perry, 2010). Maraviroc is generally prescribed in combination with NRTIs. Interestingly, individuals carrying CCR5 mutant alleles are partially resistant to R5 tropic viral infection indicating the importance of co-receptors in viral infectivity (Liu et al., 1996). Other entry inhibitors that have been tested but discontinued are aplaviroc and vicriviroc (Caseiro et al., 2012; Nichols et al., 2008). Cenicriviroc is currently in development (Lalezari et al., 2011; Marier et al., 2011). Although the CXCR4 antagonist AMD3100 has been successful in early phase
studies, its use as antiviral treatment was terminated due to toxicity (Hendrix et al., 2000). Several attachment inhibitors (i.e., BMS-488043, BMS-663068) have also been developed, although none of these inhibitors are used clinically (Hanna et al., 2011; Nettles et al., 2012). These inhibitors block the interaction of CD4 with gp120. A post-attachment inhibitor, ibalizumab (TNX-355) has also been developed that prevents the CD4 bound gp120 from interacting with chemokine coreceptor (Pace et al., 2013).

1.3.2 Fusion inhibitor

Fusion inhibitors are designed to bind to the helical region within gp41 and prevent the conformational changes required for membrane fusion. To date, efuvirtide (T-20) is the only approved fusion inhibitor. Efuvirtide binding to HR1 prevents the association of HR1 with HR2 regions in gp41 and blocks membrane fusion. Sifuvirtide is another fusion inhibitor that has shown antiviral activity in early phase human studies (He et al., 2008; Liu et al., 2011).

1.3.3 NRTIs

NRTIs are nucleoside analogs that compete with nucleosides to be incorporated into viral DNA and a lack of the 3’-hydroxyl group results in the chain termination. HIV-1 reverse transcriptase can introduce mutations in viral genome that can result in resistance to reverse transcriptase inhibitors. Therefore, reverse transcriptase inhibitors are used in combination with other classes of drugs. Zidovudine was the first approved NRTI (Fischl et al., 1987; Mitsuya et al., 1985). The NRTIs currently used are abacavir, tenofovir disoproxil fumarate (TDF), emtricitabine, lamivudine and zidovudine. Although NRTIs are used widely due to its success in inhibiting viral reverse transcriptase, many NRTIs have also been associated with mitochondrial toxicity. Mitochondrial toxicity has been estimated to occur in approximately 15-20% individuals who are on NRTIs (Apostolova et al., 2011b). Although the exact mechanisms are not yet established, it is proposed that NRTIs inhibit DNA polymerase responsible for mitochondrial DNA (mtDNA) synthesis. Depletion of mtDNA, in turn, can result in impaired electron transport chain, mitochondrial dysfunction and reactive oxygen species (ROS) production (Apostolova et al., 2011a; Blas-Garcia et al., 2011). NRTI-associated mitochondrial toxicity can manifest into hepatic failure and lactic acidosis. NRTI-mediated mitochondrial toxicity has also been detected
in the brain of HIV-1 infected individuals on didanosine and/or zidovudine (Schweinsburg et al., 2005).

1.3.4 NNRTIs
NNRTIs are non-competitive inhibitors of viral reverse transcriptase that bind to a hydrophobic pocket near the substrate recognition site. They are administered in combination with NRTIs. Commonly prescribed NNRTIs are efavirenz, nevirapine and rilpivirine. Long-term NNRTI administration in HIV-1 infected individuals has been associated with a number of adverse effects including neuropsychiatric disorders, hepatotoxicity, gastrointestinal toxicity and metabolic disturbances. Some of the neuropsychiatric impairments include confusion, amnesia, impaired concentration, hallucinations, depression, anxiety and others. For example, common adverse effects associated with rilpivirine have been reported to be depression, insomnia, headache and rash (James et al., 2012).

1.3.5 Integrase inhibitors
Integrase inhibitor binds to the viral integrase competitively and prevents strand transfer which is essential for transfer of the proviral DNA into the host genome. Raltegravir, elvitegravir and dolutegravir are the three approved integrase inhibitors that are prescribed in combination with NRTIs.

1.3.6 PIs
PIs can bind to the active site of HIV-1 protease and prevent its catalytic activity. Saquinavir was the first approved PI. Since then other PIs have been identified such as atazanavir, darunavir, fosamprenavir, lopinavir, ritonavir, indinavir, nelfinavir and tipranavir. In the clinic, PIs are generally administered with two NRTIs or one NRTI. In order to improve PIs bioavailability, they are administered with low doses of ritonavir which is a potent inhibitor of cytochrome P450 enzymes (CYPs) and works as a booster.

PIs can be associated with toxicity and are prone to drug-drug interactions. In particular, they have been implicated in the development of hyperlipidemia, lipodystrophy as well as insulin resistance (Moyle, 2007;Pistell et al., 2010;Tsiodras et al., 2000). For example, saquinavir, indinavir and lopinavir administration have been shown to result in an increase in triglycerides and insulin resistance infected individuals (Calza et al., 2004;Galindo et al., 2008). A recent
study performed in mice also correlated PI-associated metabolic dysfunction with neurocognitive impairment (Gupta et al., 2012). In this study, mice receiving clinically relevant doses of lopinavir/ritonavir showed metabolic dysfunction as well as cognitive impairment. Decreased brain barrier integrity, decreased expression of synaptic markers and increased inflammatory markers were also observed in these animals indicating the potential detrimental effects associated with PIs (Gupta et al., 2012).
Table 1-1 Preferred and alternative antiretroviral regimens for antiretroviral therapy-naive patients (From Ashraf T, Robillard K, Chan G and Bendayan R. Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders. 2013. Current Pharmaceutical Design. 20: 1543-1563)

<table>
<thead>
<tr>
<th>Preferred Regimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>One of the following Antiretroviral Regimens</td>
</tr>
<tr>
<td>NNRTI</td>
</tr>
<tr>
<td>PIs</td>
</tr>
<tr>
<td>Integrase Inhibitor</td>
</tr>
<tr>
<td>Pregnant Women</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alternative Regimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>One of the following Antiretroviral Regimens</td>
</tr>
<tr>
<td>NNRTI</td>
</tr>
<tr>
<td>Rilpivirine</td>
</tr>
<tr>
<td>Tenofovir Disoproxil Fumarate and Emtricitabine</td>
</tr>
<tr>
<td>PIs</td>
</tr>
<tr>
<td>Fosamprenavir + Ritonavir or Lopinavir + Ritonavir</td>
</tr>
<tr>
<td>Abacavir and Lamivudine or Tenofovir Disoproxil Fumarate and Emtricitabine</td>
</tr>
<tr>
<td>Integrase Inhibitor</td>
</tr>
<tr>
<td>Abacavir and Lamivudine</td>
</tr>
</tbody>
</table>

Adapted from the 2012 Guidelines for the Use of Antiretroviral Agents in HIV-1 Infected Adults and Adolescents (http://aidsinfo.nih.gov/guidelines).
1.4 Brain barriers and parenchymal cellular compartments

1.4.1 Blood-brain barrier (BBB)

The BBB constitutes a remarkable physical and biochemical barrier between the brain and systemic circulation that regulates the traffic of molecules into/out of the brain parenchyma (Reese and Karnovsky, 1967). The discovery of the BBB is attributed to the German immunologist Paul Ehrlich who first reported in 1880 that injection of cationic dyes into the systemic circulation stained most of the organs with the exception of the brain and spinal cord (Ehrlich, 1904). Later work from Edwin E. Goldman demonstrated that if the dye is injected in the cerebrospinal fluid (CSF) directly, only the nervous tissue is stained whereas other tissues remain unstained confirming the existence of a physiological barrier between the systemic circulation and the brain (Goldmann, 1913). It was not until another 70 years when using electron microscopy several researchers observed lack of horseradish peroxidase (HRP) perfusion in mouse vascular endothelium suggesting the existence of a structural barrier at the blood-brain interface (Reese and Karnovsky, 1967). The identification of tight junction proteins that connect endothelial cells and restrict the paracellular trafficking of substrates further confirmed the concept of a tightly regulated physiological barrier surrounding the CNS (Reese and Karnovsky, 1967). To date, the BBB is known as a multi-cellular unit formed of a monolayer of non-fenestrated brain microvessel endothelial cells, surrounding pericytes, adjacent astrocytes and neurons. These cellular compartments and basal lamina are collectively referred to as the “neurovascular unit” (Neuwelt et al., 2011). Brain microvessel endothelial cells are joined by tight junctions that are maintained by trophic factors released from adjacent astrocytes and pericytes. The intercellular network of tight junctions regulates the traffic of immune surveillance cells and substances from the systemic circulation. Under physiological conditions, these tight junctions form a continuous, almost impermeable cellular barrier that limits paracellular flux and transport as well as the influx of endogenous and exogenous substances with the exception of very small, lipid soluble molecules. The high transendothelial electrical resistance (1500-2000 Ωcm²) of the BBB further restricts the free flow of water and solutes. Several receptors, ion channels, and influx/efflux transport proteins are prominently expressed at the BBB (Pardridge, 2012; Simionescu et al., 2002). In brain microvessel endothelial cells, specialized plasma membrane microdomains known as caveolae are believed to be involved in...
endocytosis of various macromolecules, including plasma proteins, immunoglobulins and metalloproteins. During neuropathological conditions such as stroke, trauma, bacterial or viral infections, multiple sclerosis, Alzheimer’s disease, Parkinson’s disease, epilepsy, brain tumors and pain, the integrity of the BBB has been reported to be compromised (Neuwelt et al., 2011; Ronaldson et al., 2008).

1.4.2 Pericytes

Pericytes are perivascular cells within the basal lamina that are important constituent of the BBB (Fisher, 2009). These cells surround the endothelial cells and maintain the structural integrity and stability of the BBB (Hori et al., 2004a). Pericytes are known to regulate many other brain functions such as angiogenesis, capillary flow, immune response as well as hemostatis (az-Flores et al., 1991; Bandopadhyay et al., 2001). Due to their multiple roles in maintaining a functional BBB, pericytes have been implicated in the pathogenesis of numerous cerebrovascular and neurodegenerative diseases (Dore-Duffy, 2008; Gonul et al., 2002).

1.4.3 Brain parenchymal cellular compartments

Neurons and the surrounding glial cells constitute the brain parenchyma. The term “glia” (derived from Greek, meaning glue) was first used by Rudolf Virchow in 1846 to describe a cell type that fastened the neuronal cells together. For decades, glial cells were viewed as structural support cells in the brain. However, emerging evidence suggest that glial cells in the brain perform a wide range of function that is critical to regulate and maintain brain homeostasis. Glial cells include astrocytes, oligodendrocytes and microglia.

1.4.3.1 Astrocytes

Astrocytes are the most abundant glial cells in the brain. They possess a stellate morphology and contain numerous cytoplasmic fibrils, of which glial acidic fibrillary protein (GFAP) is the main constituent. Astrocytes possess numerous functions that aid in maintaining the homeostatic environment of the CNS. Astrocyte-foot-processes surround more than 99% surface of the cerebral capillary basement membrane and this close interaction between astrocytes and brain microvessel endothelial cells has been implicated in the maintenance of tight junction integrity, proliferation and angiogenesis of endothelial cells (Abbott et al., 2006). Other functions include

Reproduced with permission from the copyright owner
regulation of immune and inflammatory events during injury and infection (i.e., production and secretion of cytokines), expression of adhesion molecules for neuronal development, buffering of excess K$^+$ during periods of neuronal hyperactivity and secretion of neurotropic factors (i.e., transforming growth factor-beta, glial-derived neurotropic factor, basic fibroblast growth factors) that are known to promote growth of neurons as well as differentiation and maturation of microglia. Astrocytes also express numerous transporters that mediate the trafficking of nutrients, solutes and xenobiotics across the cell membrane (Hirrlinger et al., 2005; Pardridge et al., 1997). Loss of astrocyte mediated neuroprotective effects as well as chronic activation of astrocytes have been reported in neurodegenerative diseases as well as in HIV-associated neuropathogenesis (Sidoryk-Wegrzynowicz et al., 2011).

1.4.3.2 Microglia

Microglia are the resident immunocompetent cells in the brain. These cells were first identified by the Spanish neuroanatomist del Rio-Hortega in 1932. Under normal physiological condition, these cells appear in a resting state, characterized by smaller cell body, ramified processes and low expression of surface antigens. In response to injury or inflammatory stimuli, microglia become activated and release inflammatory mediators such as pro-inflammatory cytokines, prostaglandins and NO. Activated microglia further recruit other microglia to the site of injury, trigger activation of astrocytes and signal recruitment of peripheral monocytes from the systemic circulation. Activated microglia have been implicated in many diseases such as Alzheimer’s, Parkinson’s, HIV-encephalitis (HIVE) and others (Garden and Moller, 2006). Many transport proteins are also expressed in these cells (Lee et al., 2001b). More than a decade ago, our group proposed that along with astrocytes, these cells constitute a secondary barrier to drug permeability in the brain and demonstrated functional expression of multiple drug efflux transporters in both microglia as well as astrocytes (Dallas et al., 2004a; Dallas et al., 2003; Lee et al., 2001b; Ronaldson et al., 2004b; Ronaldson et al., 2008).

1.4.3.3 Oligodendrocytes

The primary function of oligodendrocytes is to form the insulating myelin sheath that surrounds neuronal axons in the CNS. Myelin, an extension of the oligodendrocyte plasma membrane, is a lipid-rich biological membrane that forms multilamellar, spirally wrapped sheaths around

Reproduced with permission from the copyright owner
neuronal axons to increase the resistance for electrical impulses during an action potential. Injury and loss of oligodendrocytes are directly related to axonal damage (Benarroch, 2009). Several transporters have been detected at the gene or protein level in oligodendrocytes (Hirrlinger et al., 2002).

1.4.3.4 Neurons

Neurons form the basic structural and functional component of the CNS. The primary function of neurons is to transmit nerve signals through axonal processes. The interaction between blood vessels and neurons plays an essential role for the neurovascular network and maintenance of brain homeostasis. Irreversible neuronal injury or loss has been implicated in many diseases such as Alzheimer’s disease, Parkinson’s disease, infectious diseases, stroke and multiple sclerosis. Studies have demonstrated the expression of several drug transporters at this site (Lazarowski et al., 2004).

1.4.4 Blood-cerebrospinal fluid barrier (BCSFB)

The BCSFB composed of choroid plexus epithelial cells plays a major role in determining the permeability of nutrients and xenobiotics. The choroid plexus is a highly vascularised branched structure with numerous villi that project into all four cerebral ventricles (Spector and Johanson, 1989). The capillaries of the choroid plexus are fenestrated and provide little resistance to the movement of water and solutes. However, a barrier is formed by a monolayer of polarized epithelial cells surrounding the fenestrated capillaries that are joined together by tight junctional proteins (Groothuis and Levy, 1997; Segal, 2000). These tight junctions form a functional barrier that restricts the movement of molecules and ions. The main function of the choroid plexus epithelial cells is to secrete and maintain the homeostatic composition of the CSF. The CSF fills the ventricles of the brain, the spinal canal and subarachnoid space. In humans, the total volume of CSF is approximately 140 ml which is replaced four to five times daily (Enting et al., 1998). The CSF also provides a drainage system for the brain known as the sink effect into which products of metabolism and molecules penetrating into the CSF are diluted and subsequently removed (Davson et al., 1970; Saunders et al., 1999). The sink effect is greater for large molecular weight and hydrophilic compounds. At the level of choroid plexus epithelial cells,
numerous polarized expression of receptors, ion channels and transporters have been reported
(Davson and Segal, 1970; de Lange, 2004; Garner and Brown, 1992; Kusuhara and Sugiyama,

1.5 Brain HIV-1 infection and HIV-1-associated neurological disorders

1.5.1 HIV-1-associated neuropathogenesis

HIV-1 can penetrate the CNS early in the course of the infection either as a cell-free entity or
within infected monocytes that is also known as the “Trojan horse” hypothesis (Davis et al.,
1992). Infected monocytes can migrate into the brain either by transcellular or paracellular route.
The paracellular route involves migration in-between adjacent endothelial cells and the
transcellular route occurs directly through an individual endothelial cell likely through the
formation of a pore or channel (Wittchen, 2009). Studies have suggested that both transcellular
and paracellular migration of monocytes are associated with high intracellular adhesion molecule
(ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 enriched endothelial projections
(Carman and Springer, 2004).

Perivascular macrophages and brain resident microglial cells constitute the immunocompetent
cells in the CNS and are the primary targets of HIV-1 (Persidsky and Gendelman, 2003). These
cells express both CD4 and chemokine co-receptor for viral entry (Dalgleish et al., 1984; Deng et
al., 1996). In a healthy brain, microglia appear in a resting state. In response to HIV-1, microglia
become activated and release pro-inflammatory cytokines, prostaglandins, ROS and NO that
further trigger the activation of neighbouring microglia and astrocytes and signal recruitment of
peripheral monocytes from the systemic circulation (Garden, 2002; Persidsky et al., 1999).
Microglia are the main source of productive infection in the brain while infection of astrocytes
tends to be latent and noncytopathic. HIV-1 entry into astrocytes is independent of CD4 receptor
and mediated by galactosyl ceramide (Boutet et al., 2001; Gorry et al., 2003). It is proposed that
HIV-1 infection in astrocytes is controlled by different phases of restriction. A few potential
mechanisms could be low level of viral production which is controlled post-transcriptionally and
low level of basal promoter activity. Lower Rev function along with insufficient translation of
viral proteins have been implicated as potential mechanism of restrictive HIV-1 infection in
astrocytes. Sequestration of the virus in the astrocytes and prolonged viral viability in astrocytes
can lead to the formation of viral reservoir at this site. HIV-1 infection triggers activation of

Reproduced with permission from the copyright owner
astrocytes leading to inflammatory response and oxidative stress. A study by Churchill et al. has demonstrated that astrocyte infection was prominent in HIV-1 infected subjects with dementia (Churchill et al., 2009). In addition, astrocyte infection correlated with the severity of the neuropathological changes suggesting an important role of astrocytes in HIV-1 neuropathogenesis (Churchill et al., 2009).

A number of studies have demonstrated that virus-infected cells or induced glial cells are source of various neurotoxic factors such as arachidonic acid (AA) and its metabolites, platelet activating factor, quinolinic acid, ROS, NO and glutamate (Genis et al., 1992; Kong et al., 1996; Ronaldson and Bendayan, 2008). In addition, a number of pro-inflammatory cytokines (i.e., tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), IL-10, IL-18, interferonα (IFNα), IFNγ) are also known to be elevated during brain HIV-1 infection (Hult et al., 2008; Perrella et al., 1992; Torre et al., 1992; von Giesen et al., 2004). Several studies have reported detectable amount of these cytokines in the CSF of patients with HIV-associated neurocognitive disorder (HAND) and also found statistically significant correlations of these elevated cytokines with HAND pathogenesis (Sas et al., 2009). Heme oxygenase-1 (HO-1), a cytoprotective cellular enzyme, has been found to be significantly decreased due to HIV-1 replication in cultured macrophages and HO-1 deficiency was found to be associated with increased release of neurotoxic levels of glutamate (Ambegaokar and Kolson, 2014). Increased macrophage migration inhibitory factor (MIF) has also been reported in plasma of HIV-1 infected patients, in infected peripheral blood mononuclear cells (PBMCs) as well as in PBMCs treated with gp120 (Regis et al., 2010). MIF binds to different receptors (i.e., CD74, CD44, CXCR2, CXCR4) and promotes the release of pro-inflammatory cytokines (i.e., TNF-α, IL-6) (Calandra and Roger, 2003). In vitro studies in primary glial cell cultures suggest that shed viral proteins [gp120, transactivating protein (Tat), viral protein R (vpr)] can induce secretion of pro-inflammatory cytokines. For example, previous work from our laboratory has demonstrated that R5-tropic gp120 can mediate secretion of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) by interacting with CCR5 chemokine receptor in primary cultures of rodent astrocytes (Ronaldson and Bendayan, 2006).

HIV-1 infection may result in a neuropathological condition known as HIVE. HIVE is characterized by elevated monocyte/macrophage infiltration into the brain, myelin pallor, brain atrophy, multinucleated giant cells, activated microglia, reactive astrocytosis (proliferation and
activation of astrocytes) and presence of microglial nodules (Albright et al., 2003). Immune activation in the brain parenchyma during HIVE is associated with enhanced monocyte migration through the BBB (Kanmogne et al., 2007). Secretion of chemokines [i.e., monocyte chemoattractant protein-1 (MCP-1); macrophage inflammatory protein-1α/β (MIP-1α/β); regulated on activation, normal T cell expressed and secreted (RANTES)] from glial cells facilitate this migration (Asensio and Campbell, 1999). In addition, HIV-1 infection can disrupt the structural properties of the BBB by downregulating tight junction proteins [i.e., zona occluden-1 (ZO-1), occludin] in brain microvessel endothelial cells (Kanmogne et al., 2005; Nakamuta et al., 2008). A compromised BBB can facilitate entry of HIV-1 from the periphery and ultimately, can increase the spread of the virus in brain parenchymal cellular compartments. Several in vitro and in vivo studies have also demonstrated that HIV-1 proteins (i.e., gp120, Tat) can downregulate tight junction proteins expressed in brain microvessel endothelial cells (Kanmogne et al., 2005; Toborek et al., 2005) For example, Louboutin et al. demonstrated a significant decrease in the tight junction protein claudin-5 due to gp120 administration in rat brain (Louboutin et al., 2010b). Upregulation of adhesion molecules (i.e., ICAM, VCAM) in brain endothelial cells have also been implicated in facilitating viral infected monocyte infiltration (Andras et al., 2003; Pu et al., 2003). A recent study has identified mechanisms involved in HIV-1 transport across the BBB. Using in situ brain perfusion and in vitro BBB model, this study demonstrated that HIV-1 uses the mannose-6 phosphate receptor to cross the brain microvessel endothelial cells as a free virus (Dohgu et al., 2012).

Evidence also suggests a significant contribution of oxidative stress in the pathogenesis of HIV-1. Inflammatory cytokines and HIV-1 viral proteins (Tat, gp120) can induce oxidative stress in glial cells. In rat cortical neuroglial cultures, gp120 triggered an increased production of ROS and subsequent neuronal death (Wakabayashi et al., 2003). Upregulation of inducible nitric oxide synthase (iNOS) expression and associated increase in NO and peroxynitrite production have also been reported in astrocytes and microglia (Adamson et al., 1996; Hori et al., 1999; Zhao et al., 2001). High levels of iNOS expression were also reported to be localized to microglial nodules and reactive astrocytes in brain tissue isolated from patients with HIVE (Zhao et al., 2001). Increased oxidative stress markers have also been detected in the CSF of HIV-1 infected individuals with HIV-associated dementia implying their significance in the development of HIV-1 associated cognitive deficits (Sacktor et al., 2004).
Neurons do not appear to be susceptible to HIV-1 infection. However, chronic exposure to neurotoxic, inflammatory and oxidative stress markers during HIV-1 infection can cause irreversible neuronal damage (Kaul et al., 2001). Viral proteins (i.e., gp120, Tat, vpr) released from infected cells are known to be neurotoxic (Guha et al., 2012; Kanmogne et al., 2002). Studies have shown that mechanisms involved in neuronal apoptosis include interaction with viral proteins to neuronal chemokine receptors, caspase activation, calcium toxicity, excitotoxicity due to glutamate, loss of mitochondrial membrane potential and ultimately, DNA fragmentation (Lindl et al., 2010). For example, it has been demonstrated that gp120 mediates caspase-3-dependent apoptosis of neurons (Singh et al., 2004). Others demonstrated vpr associated neuronal apoptosis and neurodegeneration in vivo (Guha et al., 2012; Jones et al., 2007). Excessive K+ and excitatory neurotransmitter glutamate also lead to neuronal loss. Physiologically, astrocytes actively participate in removing excess K+ and excitatory neurotransmitter glutamate released from neurons. Functional glutamate uptake systems [i.e., excitatory amino acid transporter 1 (EAAT1), EAAT2] have been identified in astrocytes. Evidence suggests that these uptake systems may be compromised due to HIV-1 infection. Studies performed in cultured glial cells exposed to gp120 or Tat have reported reduced expression of EAAT2 expression as well as decreased glutamate uptake (Wang et al., 2003b; Zhou et al., 2004). Excess extracellular glutamate can further stimulate metabotropic glutamate receptors in astrocytes leading to more glutamate release (Volterra and Meldolesi, 2005). Figure 1-2 summarizes the different mechanisms involved in HIV-associated neuropathogenesis.

Reproduced with permission from the copyright owner
1.5.2 HIV-associated neurocognitive disorders (HAND)

Neurocognitive impairment remains highly prevalent in HIV-1 infected individuals due to persistence of viral replication and associated inflammation in the brain (Heaton et al., 2010; Heaton et al., 2011). Prior to the use of antiretroviral therapy, approximately 20-30% of HIV-1 infected patients developed HIV-associated dementia (HAD), the most severe form of cognitive impairment. Since the initiation of combined antiretroviral therapy, the development of HAD in HIV-1 infected patients has significantly decreased (Ances and Ellis, 2007). However, milder forms of HAND are becoming more predominant in the post-highly active antiretroviral therapy (HAART) era in part due to the longer life expectancy of infected individuals on treatment. HAND is characterized by cognitive, motor or behavioral abnormalities. Despite receiving treatment, HIV-1 infected patients may develop cognitive impairments (i.e., attention, learning, memory), psychiatric illness (i.e., depression, anxiety) and persisted fatigue interfering with their daily life functioning (i.e., employment, medication management, driving) and ultimately, resulting in a poor quality of life in these individuals (Warriner et al., 2010). Studies further suggest that cognitively impaired individuals with acute and early HIV-1 infection are at elevated risk of clinically significant changes in normal daily functioning (Doyle et al., 2013).

Several recently published studies have confirmed that despite the availability of combined antiretroviral therapy (cART), neurological disorders are still persistent in HIV-1 infected patients (Power et al., 2009; Vivithanaporn et al., 2010). In particular, Vivithanaporn et al. studied 1,651 patients with HIV-AIDS in Canada over a 10 year period and reported that more than one in four people with HIV-1 develops neurological disorders and more alarmingly, the mortality rate of these patients are significantly higher than those with no neurological disorder (Vivithanaporn et al., 2010). Vivithanaporn et al. further observed that patients suffering from neurological infection suffer from 53 brain-related conditions including HAND, pain, seizure, stroke and others (Vivithanaporn et al., 2010). Another study performed in 1,555 HIV-1 infected patients in the United States reported that 52% of the total subjects had neuropsychological impairment (Heaton et al., 2010).

One study performed by the CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) group reported that nadir CD4 cell count may be used as a predictor of neuropsychological impairment (Ellis et al., 2011). This study suggested that the risk of developing neurocognitive
deficit is lower in infected individuals whose CD4 levels were never allowed to fall to low levels (Ellis et al., 2011). McCombe et al. further reported that increased age, survival duration, low nadir CD4 cell count and high baseline viral load were predictors of the development of symptomatic HAND in patients with HIV-1 infection (McCombe et al., 2013). Brain HIV RNA and to a lesser extent HIV DNA in patients with HIVE and microglial nodule encephalitis were found to be correlated with worse neuropsychological performance (Gelman et al., 2013). HIV-1 infection and the development of dual-tropism may be associated with HAND in the relatively early stage of infection suggesting that viral interaction with cellular receptors may play an important role in the early manifestation of HAND (Morris et al., 2013).

Although CD4 count and viral load were important markers for the likelihood of developing HAND in the pre-cART era, there are currently no standard systemic or CSF biomarkers that can be used to diagnose HAND. Increased concentrations of CSF neopterin, a marker for macrophage activation, have been found in virally suppressed patients without any symptoms of HAND (Canestri et al., 2010). A correlation of cytokines present in the CSF of HIV-1 infected patients were found with their test results of neuropsychological functioning (Nolting et al., 2012). Increased IL-6 and MIP-1β were also found to be elevated in the CSF collected from advanced therapy-naive HIV-1 infected HAND patients (Airoldi et al., 2012). In this study, initiation and continuation of antiretroviral therapy for 12 weeks greatly reduced plasma RNA load, but did not affect CSF IL-6 and MIP-1β levels suggesting that CNS immune activation can persist despite administration of virologically effective therapy (Airoldi et al., 2012).

Different neuropsychological test batteries are being used to detect cognitive impairments in infected patients. Lack of reliable screening methods for early diagnosis and monitoring HAND is a key obstacle to evaluate HAND. Recently, a smartphone based screen test, neuroscreen, has been tested in HIV-1 infected individuals to detect neurocognitive deficits. Although it demonstrated similar ability to assess cognitive deficits as paper tests, it is yet to be determined if it can be useful for less cognitively impaired patients (Robbins et al., 2014). Neuroimaging techniques (i.e., morphometry, magnetic resonance spectroscopy, diffusion tensor imaging, positron emission tomography) have potential to detect neuropathological changes in HIV-1 infected patients and to evaluate the progression of disease and/or the efficacy of cART.
1.6 Treatment obstacles of brain HIV-1 infection: brain permeability of antiretroviral drugs

Treatment of brain HIV-1 infection remains challenging partly due to poor permeability of antiretroviral drugs across the BBB and into glial cells. Several classes of antiretrovirals have been reported to poorly permeate into the brain. In particular, a number of PIs and NRTIs exhibit low bioavailability following oral administration and reach subtherapeutic concentration in the brain, possibly permitting this site to become a sanctuary for HIV-1 (Best et al., 2009; Best et al., 2012; Gisolf et al., 2000). The inadequate concentration of PIs in the CNS could allow continuing production of HIV-1 and emergence of drug resistant viral strains despite adequate plasma concentrations and acceptable systemic antiviral efficacy indicators (Kravcik et al., 1999; Piacenti, 2006). Table 1-2 lists the CSF to plasma ratio of different antiretroviral drugs.

In order to determine the effectiveness of antiretroviral drug permeability in the brain, a CNS penetration effectiveness (CPE) score has been established by the CHARTER group. The ranking of individual antiretroviral drugs was based on their chemical properties, concentrations in CSF and/or effectiveness in the CNS reported in clinical trials (Letendre et al., 2008). In order to validate this ranking method, a study involving 833 HIV-1 infected individuals was designed to establish a correlation between CSF viral loads and penetration of antiretroviral drugs. As predicted, lower CPE ranks were found to be associated with higher CSF viral loads suggesting persistent viral replication due to poor penetration of antiretroviral drugs (Letendre et al., 2008). An updated CPE ranking has been proposed that incorporates data from recent studies and is more strongly associated with CSF viral loads than the older CPE method (Letendre et al., 2010). According to the most updated CPE ranking system, each drug has been given a score between 1 and 4 (Letendre et al., 2010). A higher CPE score indicates more CNS effectiveness. Based on this ranking zidovudine, nevirapine and indinavir/ritonavir are given a score of 4, while abacavir, emtricitabine, delavirdine, efavirenz, darunavir/ritonavir, fosamprenavir/ritonavir, indinavir, lopinavir/ritonavir, maraviroc and raltegravir have a score of 3. Drugs such as didanosine, lamivudine, stavudine, etravirine, atazanavir, atazanavir/ritonavir and fosamprenavir were given a score of 2. The lowest ranking was given to tenofovir, zalcitabine, nelfinavir, ritonavir, saquinavir, saquinavir/ritonavir, tipranavir/ritonavir and enfuvirtide indicating their poor permeability. Although this system has not been validated for clinical use yet, it suggests that
enhancing the penetration of antiretroviral drugs into the CNS can ultimately improve brain HIV-1 infection and associated cognitive impairments. Data from several clinical studies support that higher CPE ranks are associated with low CSF viral loads and/or improved cognitive performance in HIV-1 infected individuals (Marra et al., 2009). Besides increased permeability of antiretrovirals, early initiation of antiretroviral therapy may also reduce the risk of neurocognitive complications in HIV-1 infected individuals. In a study performed by the CHARTER group, nadir CD4 level was identified as a predictor of neuropsychological impairment suggesting that the risk of developing neurocognitive deficit is lower in infected individuals whose CD4 levels were never allowed to fall to low levels (Ellis et al., 2011). Therefore, other factors can also determine the effectiveness of antiretroviral therapy in improving neurocognitive impairment. In addition, as discussed in an earlier section, use of certain antiretroviral drugs can be associated with adverse side effects including neurotoxicity. More clinical evidence is required to understand penetration of antiretroviral drugs and improvement in neurocognitive outcome. Overall, these observations suggest that pharmacotherapy of brain HIV-1 infection is a complex process and further investigation is necessary to design treatment strategies that can benefit patients with neurocognitive deficits.
Table 1-2 Comparison of CSF to plasma ratios of antiretroviral drugs in HIV-infected patients. Values are reported as median and/or the range of CSF: plasma (from Ashraf T, Robillard K, Chan G and Bendayan R. Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders. 2013. Current Pharmaceutical Design. 20: 1543-1563)

<table>
<thead>
<tr>
<th>Class</th>
<th>Antiretroviral Drug</th>
<th>CSF : Plasma Ratio</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIs</td>
<td>Amprenavir</td>
<td>0.012 (0.008-0.018)</td>
<td>(Croteau et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Atazanavir</td>
<td>0.008 (0.02)</td>
<td>(Best et al., 2009b)</td>
</tr>
<tr>
<td></td>
<td>Darunavir</td>
<td>0.009 (0.003-0.078)</td>
<td>(Yilmaz et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Indinavir</td>
<td>0.11 (0-0.47)</td>
<td>(Antinori et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>0.002 (0.001-0.008)</td>
<td>(Capparelli et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Saquinavir</td>
<td>0.001-0.002</td>
<td>(Kravcik et al., 1999b)</td>
</tr>
<tr>
<td></td>
<td>Nelfinavir</td>
<td>Not detected</td>
<td>(Antinori et al., 2005; Solas et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>0.002 (0.001-0.005)</td>
<td>(Kravcik et al., 1999b)</td>
</tr>
<tr>
<td>NRTIs</td>
<td>Abacavir</td>
<td>0.35 (0.31-0.44)</td>
<td>(McDowell et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Didanosine</td>
<td>0.16 (0.03-0.24)</td>
<td>(Huang et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Emtricitabine</td>
<td>0.26 (0.05-0.41)</td>
<td>(Calcagno et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Lamivudine</td>
<td>0.23 (0-4.9)</td>
<td>(Antinori et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Stavudine</td>
<td>0-0.20</td>
<td>(Antinori et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Tenofovir</td>
<td>0.057 (0.004-0.84)</td>
<td>(Best et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Zidovudine</td>
<td>0.02 (0-6.74)</td>
<td>(Antinori et al., 2005)</td>
</tr>
<tr>
<td>NNRTIs</td>
<td>Efavirenz</td>
<td>0.007 (0.003-0.011)</td>
<td>(Tashima et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Enfuvirtide</td>
<td>Negligible</td>
<td>(Price et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Nevirapine</td>
<td>0.63 (0.41-0.77)</td>
<td>(Antinori et al., 2005)</td>
</tr>
<tr>
<td>Integrase Inhibitor</td>
<td>Raltegravir</td>
<td>0.06 (0.01-0.54)</td>
<td>(Croteau et al., 2010)</td>
</tr>
<tr>
<td>CCR5 Antagonist</td>
<td>Maraviroc</td>
<td>0.022 (0.004-0.17)</td>
<td>(Tiraboschi et al., 2010)</td>
</tr>
</tbody>
</table>

Reproduced with permission from the copyright owner
1.7 ATP-binding cassette (ABC) transporters

Subtherapeutic concentrations of antiretroviral drugs have been reported in the CNS. One potential mechanism that may lead to poor penetration of antiretrovirals into different brain cellular compartments is functional expression of efflux transporters that belong to the ABC superfamily. Along with ABC transporters, drug metabolizing CYPs may play a role in determining drug disposition into the CNS. In addition, drug-drug interactions can also lead to inadequate or toxic drug concentrations resulting in poor therapeutic outcome, treatment failure or adverse effects.

The ABC superfamily is one of the largest and ubiquitously expressed protein families containing numerous functionally diverse membrane-associated transporters. To date, 50 ABC proteins have been identified in humans that can be separated in seven subfamilies (A to G) based on their structural organization (Dean and Allikmets, 2001). These membrane-associated proteins transport numerous substrates across the lipid bilayer using ATP hydrolysis as the energy source. ABC transporters are characterized by the presence of a highly conserved Nucleotide Binding Domain (NBD) containing three distinct motifs, Walker A, Walker B and a signature motif (LSGG) (Leslie et al., 2001). Localization of different ABC transporters at the brain barriers and in brain cellular compartments is shown in figure 1-3 and figure 1-4, respectively.
**Figure 1-3** Localization of ABC transporters at the BBB and BCSFB. The arrows indicate the direction of substrate transport (Adapted from Ashraf T, Kao A and Bendayan R. 2014. Functional expression of drug transporters in glial cells: potential role on drug delivery to the CNS. In Thomas P. Davis, editor: pharmacology of the blood-brain barrier: targeting CNS disorders, vol 71, APHA, UK: Academic press; 45-111)
Figure 1-4 Localization of ABC transporters in astrocytes, microglia and neurons. The arrows indicate the direction of substrate transport (Adapted from Ashraf T, Kao A and Bendayan R. 2014. Functional expression of drug transporters in glial cells: potential role on drug delivery to the CNS. In Thomas P. Davis, editor: pharmacology of the blood-brain barrier: targeting CNS disorders, vol 71, APHA, UK: Academic press; 45-111)

Reproduced with permission from the copyright owner
1.7.1 P-glycoprotein (P-gp)

P-gp was the first identified ABC transporter. It was isolated and characterized by Victor Ling in Toronto, Canada, in a Chinese hamster ovary cell line where this transporter showed resistance to colchicine (Juliano and Ling, 1976). These cells also showed resistance to a number of structurally diverse anti-cancer agents, a phenomenon that was later termed as multidrug resistance (MDR). P-gp consists of 1276-1280 amino acids with a molecular mass of approximately 170 kDa. It is encoded by the MDR/mdr gene which has two isoforms in humans (MDR1 and MDR2) and three isoforms in rodents (mdr1a, mdr1b and mdr2) (Chen et al., 1986; Gottesman et al., 1995). P-gp is encoded by the MDR1 gene in humans, whereas, MDR2/mdr2 is exclusively expressed in the liver and involved in phosphatidylycholine translocation. In rodents, P-gp encoded by mdr1a and mdr1b demonstrate MDR phenotype.

The membrane topology of P-gp shows a tandemly duplicated structure with two homologous halves connected by a hydrophilic linker region (Loo and Clarke, 2005). Each half consists of a highly hydrophobic transmembrane domain (TMD) containing six transmembrane helices and an intracellular NBD with an ATP-binding site (Gottesman et al., 1995; Rosenberg et al., 2003). The hydrophilic linker region is phosphorylated at several sites by protein kinase C, although phosphorylation of this linker region does not appear to affect P-gp function. N-linked glycosylation sites have also been identified in P-gp within the first extracellular loop. Although glycosylation does not appear to be required for substrate transport, studies suggest that glycosylation may be required for proper protein folding. Recent structural studies have generated an X-ray crystal structure of mouse P-gp which shows an internal cavity with nucleotide-free inward facing conformation (Aller et al., 2009). This study also reported two cocrystal structures of P-gp bound to cyclic peptide inhibitor. In the drug-bound conformation, the drug-binding pocket of P-gp is open to the cytoplasm and lipid bilayer (Aller et al., 2009). This conformation likely represents a pre-transport state of P-gp where drug binding followed by ATP binding causes a dimerization in the NBDs, resulting in the outward facing conformation where the drug is released due to changes in conformation and/or accompanied by ATP hydrolysis.

Since its discovery, overexpression of P-gp has shown resistance to numerous chemotherapeutic drugs, immunosuppressants, cardiac glycosides, antibiotics and many more. A number of antiretroviral drugs have been reported to be substrates of P-gp. They include a number of HIV

Reproduced with permission from the copyright owner
PIs (i.e., darunavir, atazanavir, saquinavir, ritonavir, amprenavir), several NRTIs (i.e., tenofovir DF, nelfinavir), entry inhibitor (maraviroc) and integrase inhibitor (raltegravir) (Fujimoto et al., 2009; Janneh et al., 2007; Kassahun et al., 2007; Kim et al., 1998; Lee et al., 1998; Perloff et al., 2005; Shaik et al., 2007; Walker et al., 2005). Some of these antiretrovirals, in particular PIs, have also been identified as inhibitors of P-gp transport activity. Our laboratory has demonstrated that PIs such as indinavir, ritonavir and saquinavir can inhibit digoxin transport, an established P-gp substrate, in a rat brain endothelial cell line (RBE4), primary cultures of rat astrocytes and a rat microglial cell line (MLS-9) (Bendayan et al., 2002; Ronaldson et al., 2004a). P-gp has also been implicated in numerous drug-drug interactions and co-administration of drugs that are substrates, inducers or inhibitors of P-gp can result in altered pharmacokinetic and pharmacodynamic response (Kis et al., 2010).

![Figure 1-5](image)

**Figure 1-5** Membrane topology of P-gp (TMD= Transmembrane domain; NBD= Nucleotide binding domain) (From Sarkadi B, 2006. Physiol Review. 86:1179-236).

P-gp is expressed at several blood-tissue barriers (i.e., blood-intestinal, blood-testis, blood-placenta) including the BBB and the BCSFB where this transporter is directly involved in the elimination of pharmacological agents (Edwards et al., 2005; Su et al., 2009). Several studies have detected P-gp localization at the luminal membrane of brain microvessel endothelial cells and on the apical side of choroid plexus epithelia (Rao et al., 1999). Others including our group

Reproduced with permission from the copyright owner
have also reported P-gp localization at the abluminal surface of the endothelium (Bendayan et al., 2006; Pardridge et al., 1997). Pardridge et al. first demonstrated P-gp expression at the abluminal surface of the endothelium in human and primate astrocyte foot processes (Pardridge et al., 1997). Using immunogold cytochemistry, our laboratory has also detected P-gp localization at the luminal and abluminal surface of endothelial cells as well as in astrocyte foot processes (Bendayan et al., 2006). P-gp functional expression has been characterized in isolated brain capillaries as well as in several primary and immortalized brain cell culture systems including RBE4 and human brain microvessel endothelial cell line (Bendayan et al., 2002; Miller et al., 2000). Studies have also reported P-gp expression in parenchymal glial cells. Work from our laboratory has confirmed P-gp expression in a rat microglia cell line (MLS-9) as well as in cultured rat and human astrocytes (Lee et al., 2001b; Ronaldson et al., 2004a). Subcellular localization of P-gp in brain cellular compartments has also been investigated. Using immunogold immunocytochemistry, our laboratory has further detected P-gp expression in caveolae, nuclear envelope and in cytoplasmic vesicles in primary cultures of rat astrocytes (Ronaldson et al., 2004a). Additionally, we have shown P-gp expression in purified nuclear membranes prepared from isolated nuclei from RBE4 and MLS-9 cells (Babakhanian et al., 2007).

Studies performed in mdr1a/1b knockout mice models further support the role of P-gp in antiretroviral drug transport the brain. In mdr1a and mdr1b knockout mice, an enhanced accumulation of PIs in the brain has been reported suggesting the involvement of P-gp in limiting brain permeability of antiretrovirals. For example, brain permeation of indinavir, nelfinavir, ritonavir and saquinavir has been reported to be 4 to 36-fold higher in mdr1a knockout animals (Kim et al., 1998; Washington et al., 2000). In another study, administration of P-gp specific inhibitor zosuquidar in macaques resulted in significant brain accumulation of nelfinavir (Kaddoumi et al., 2007). A study by Choo et al. reported significant increases in [14C]-nelfinavir brain concentrations in mice pre-treated with several selective P-gp inhibitors including zosuquidar (LY335979) (25-fold increase), valspodar (PSC 833) (13-fold increase) and cyclosporin A (3-fold increase) (Choo et al., 2000). While these studies suggest that selective P-gp inhibitors have the potential to enhance accumulation of different drugs into the CNS in vivo, use of small molecule P-gp inhibitors to improve CNS drug delivery have been largely unsuccessful in the clinic. In fact, several clinical trials have attempted to incorporate...
pharmacological P-gp inhibitors into therapeutic regimens; however, these trials have largely failed due systemic toxicity associated with the high inhibitor doses necessary for effective transporter inhibitor (Kaye, 2008). Therefore, pharmacological approaches that involve inhibition of P-gp mediated transport should be employed with extreme caution to avoid an unwanted elevation in CNS drug concentrations and subsequent toxicity as well as unexpected adverse drug reactions.

Altered P-gp expression at the BBB and in brain parenchymal compartments can affect drug distribution during therapy. P-gp has been reported to be regulated by many physiological and pathological stimuli including pro-inflammatory cytokines, polypeptide hormone endothelin-1, viral proteins, bacterial lipopolysaccharide (LPS) and amyloid-β (Aβ) (Bauer et al., 2007; Lee and Piquette-Miller, 2001; Ronaldson and Bendayan, 2006). A number of signaling pathways have been reported to regulate P-gp such as mitogen-activated protein kinase (MAPK) pathway, nuclear factor-κB pathway (NF-κB), nuclear receptors [i.e., pregnane-X-receptor (PXR), peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR)], protein kinase C, protein kinase Akt and Rho pathways (Bauer et al., 2007; Miller, 2010; Miller et al., 2008; Ott et al., 2009; Pan et al., 2010; Wang et al., 2010b; Zhong et al., 2010). Recent studies in rodent brain capillaries also suggest that sphingolipid signaling through sphingosine-1-phosphate receptor 1 is involved in the regulation of P-gp (Cannon et al., 2012). Therefore, a more viable approach for controlling P-gp mediated drug transport in the CNS may be to target endogenous P-gp regulatory pathways.

**1.7.2 Multidrug resistance-associated proteins (MRPs)**

MRP family is a group of ABC transporters that are ubiquitously expressed in many tissues (i.e., brain, liver, kidney, intestine) and primarily confer resistance to a number of organic anions. The mammalian MRP family (humans, MRP; rodents, Mrp) has 13 members including nine proteins involved in drug transport, MRP1-9 (ABCC1-6 and ABCC 10-12), one ion channel (cystic fibrosis transmembrane regulator gene, CFTR), two receptors (sulfonylurea 1 and 2) and one truncated protein that does not mediate transport (ABCC13) (Dallas et al., 2006; Keppler, 2011). With regards to membrane topology, MRP transporters can be classified into two groups (MRP1-3, MRP6-7 and MRP4-5, MRP8-9). MRP1-3, MRP6 and MRP7 have predicted membrane topology of three TMDs showing a topology of 5 + 6 + 6 configuration of

Reproduced with permission from the copyright owner
transmembrane helices where the first TMD is linked to the core region by a shorter cytoplasmic loop. In the case of MRP1, the first TMD is not essential for transport activity, whereas, the linker region is required for function (Borst et al., 2000). In comparison, MRP4, MRP5, MRP8 and MRP9 contain two TMDs each having 6 transmembrane helices and a NBD, but do not possess the first TMD with five helices at their N-terminus (Bakos et al., 1998).

![Membrane topology of MRP1/Mrp1 (TMD= Transmembrane domain; NBD= Nucleotide binding domain) (From Dallas S, Miller DS, Bendayan R. 2006. Pharmacol Rev. 58:140-61).](image)

MRP1 was first identified in a human lung cancer cell line, H69AR, which displayed characteristics of the MDR phenotype in the absence of P-gp expression (Cole et al., 1992). MRP1 shows substrate selectivity towards organic anions or neutral organic drugs and unlike P-gp, MRP1 is also capable of transporting glutathione (GSH), glucuronide and sulphate conjugates (Hipfner et al., 1999;Leier et al., 1996). Substrates of MRP1 include anticancer drugs, inflammatory mediator leukotriene C4 (LTC4), PIs, oxyanions and several dietary constituents (i.e., bioflavanoids) (Dallas et al., 2006;Leslie et al., 2001). In the presence of GSH, Mrp1 has been shown to be involved in the transport of a few cationic drugs (i.e., vincristine, etoposide) (Loe et al., 1998;Rappa et al., 1997). MRP1 is also known to be involved in the transport of a number of HIV-1 PIs- atazanavir, saquinavir, ritonavir, indinavir and lopinavir (Dallas et al., 2004a;Janneh et al., 2009;Janneh et al., 2005;van, I et al., 2001). Interaction of emtricitabine, an NRTI and MRP1 has also been implicated (Bousquet et al., 2008). Using PBMCs, Bousquet et
al. have shown a decrease in MRP1 function after exposure to emtricitabine and increased intracellular calcein and \[^{3}\text{H}\]-vincristine accumulation. In the presence of an MRP-specific inhibitor, MK571, emtricitabine accumulation increased in PBMC suggesting an interaction with MRP1 in vitro (Bousquet et al., 2008).

MRP1 is known to be ubiquitously expressed with higher expression in lung, testes and PBMCs. In the CNS, MRP1 protein expression has been located in the luminal membrane of brain microvessel endothelial cells and basolateral membrane of choroid plexus epithelial cells (Gazzin et al., 2011; Rao et al., 1999). Studies have also detected MRP1 protein in pericytes, astrocytes, microglia, oligodendrocytes and neurons (Berezowski et al., 2004; Dallas et al., 2004a; Dallas et al., 2003). Functional expression of MRP1 has also been demonstrated in vivo. In Mrp1 knockout mice, vincristine permeation in the knockout animal brain was found to be significantly higher than the wild-type animal (Wang et al., 2010a). In addition, a few signaling pathways have been identified in MRP1 regulation such as MAPK, NF-κB and Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) (Ashraf et al., 2011; Hayashi et al., 2006; Ronaldson et al., 2010).

MRP1, along with other MRP isoforms (MRP2, MRP4 and MRP5), can also contribute towards cellular defence during oxidative stress by regulating intracellular GSH and glutathione disulfide (GSSG) concentrations. GSH levels within cells are tightly regulated and compromised levels lead to the progression of many disorders including cancer, inflammatory and neurodegenerative diseases. Elevated levels of GSH have been found in tissues from Mrp1 and Mrp2 knockout mice (Kruh et al., 2007). It is now known that both MRP1 and MRP2 can mediate transport of GSH as a substrate as well as cotransport of GSH in the presence of other compounds. MRP1 mediated transport of GSH has been well characterized in cultured astrocytes and this transporter has also been implicated in the transport of GSSG and other oxidized GSH derivatives (Hirrlinger et al., 2001). Other MRP isoforms are also known to be involved in GSH transport (MRP4 and MRP5), although their functional role in brain cellular compartments is not clear (Lai and Tan, 2002; Wijnholds et al., 2000b). For example, using astrocyte cultures derived from Mrp1 and Mrp5 knockout mice, Minich et al demonstrated that Mrp1, but not Mrp5 is involved in GSH and GSSG transport in cultured astrocytes (Minich et al., 2006). Another recent study observed depletion of cellular GSH and increase in the extracellular GSH content in primary rat astrocyte

Reproduced with permission from the copyright owner
cultures treated with PIs indinavir or nelfinavir. This process was attenuated in the presence of MRP-specific inhibitor, MK571 suggesting involvement of Mrp-mediated transport activity (Brandmann et al., 2012).

MRP1 and MRP2 share similar substrate specificities, although differences in kinetic properties exist between MRP1 and MRP2 mediated transport. Endogenous substrates of MRP2 include conjugated steroids, bile salts and LTC4. MRP2 also transports many exogenous compounds such as anticancer drugs, antiretrovirals, antibiotics, environmental toxins and metal complexes. MRP2 can also mediate transport of GSH independently or along with other compounds. With respect to antiretrovirals, studies have suggested that MRP2 can transport a number of PIs such as atazanavir, ritonavir, saquinavir, indinavir and lopinavir (Agarwal et al., 2007; Huisman et al., 2002; Janneh et al., 2005). MRP2-mediated transport of tenofovir has also been reported (Mallants et al., 2005). MRP2 has a more limited tissue distribution compared to MRP1 (Leslie et al., 2005). At the BBB, Mrp2 has been localized at the luminal side of brain microvessel endothelial cells and brain capillaries (Miller et al., 2000). At the BCSFB, the mRNA expression of Mrp2 has been reported to be negligible (Choudhuri et al., 2003). In brain parenchyma, Mrp2 mRNA, but not protein expression in astrocytes isolated from embryonic rats has been detected (Hirrlinger et al., 2002). Study by Potschka et al demonstrated an increased accumulation of phenytoin, an antiepileptic agent in Mrp2-transport deficient mice suggesting a functional role of this transporter at the level of the BBB (Potschka et al., 2003). Nuclear receptor CAR has been implicated in the regulation of Mrp2 in rodent capillaries (Wang et al., 2010b).

MRP3 has a narrower but similar substrate preference as MRP1 and MRP2. However, MRP3 cannot transport GSH and prefers glucuronide conjugates over glutathione conjugates (Zelcer et al., 2001). Weiss et al. demonstrated that NNRTIs and NRTIs enhance cellular accumulation of methylfluorescin glutathione complex, an MRP-specific substrate, in MRP1, MRP2 or MRP3 overexpressing cell lines indicating interaction between NRTIs/NNRTIs and MRP isoforms (Weiss et al., 2007). At the BBB, Mrp3 expression appears negligible and has not been detected in mouse brain microvessels; however, gene expression was reported in hCMEC/D3 cells (Dauchy et al., 2009). Low levels of Mrp3 have also been detected in bovine brain microvessel endothelial cells whereas expression was not observed in capillary enriched homogenate (Zhang et al., 2000). In choroid plexus epithelium, Mrp3 staining was observed at the inter-cellular junctions, although the function of this protein at this site is unknown (Sooontornmalai et al.,

Reproduced with permission from the copyright owner
In brain parenchyma, MRP3/Mrp3 gene expression has been detected in astrocytes, microglia, oligodendrocytes and neurons (Hirrlinger et al., 2002; Nies et al., 2004).

Unlike MRP1-3, MRP4 has the unique ability of transporting a range of endogenous molecules involved in cellular communication of signaling including cyclic nucleotides (cyclic adenosine monophosphate, cyclic guanosine monophosphate), eicosanoids, urate, conjugated steroid hormones, folate, bile acids, nucleotide and purine analogs (Chen et al., 2001). MRP4 substrates also include several NRTIs- abacavir, zidovudine monophosphate and tenofovir (Borst et al., 2004; Ray et al., 2006). In Mrp4 knockout mice model, increased brain penetration of adefovir has been reported suggesting a functional role of MRP4 in limiting drug penetration into the brain (Belinsky et al., 2007). Using immunocytochemical analysis, MRP4 expression has been detected both at the luminal and abluminal sides of brain capillary endothelial cells and at the basolateral side in the choroid plexus epithelia (Leggas et al., 2004; Nies et al., 2004; Roberts et al., 2008; Zhang et al., 2004). MRP4 expression has also been localized in astrocytes, microglia, oligodendrocytes and neurons (Ballerini et al., 2002). Our laboratory has also detected MRP4/Mrp4 expression in primary cultures of human and rat astrocytes (Ronaldson and Bendayan, 2008).

Similar to MRP4, MRP5 can function as a cyclic nucleotide export pump and confers resistance to nucleoside analogs. In addition to cyclic nucleotides, MRP5 substrates include a number of other organic anions such as nucleoside monophosphate analogs, glutathione S-conjugates and fluorescein diacetate. GSH is also a substrate for MRP4 and MRP5, although GSH cotransport is not necessary for all the substrates of these transporters. Among antiretroviral drugs, MRP5 has been reported to transport stavudine (Reid et al., 2003). MRP5 is also expressed in brain cellular compartments. In comparison to MRP4 expression, MRP5 is localized at the luminal membrane of brain microvessel endothelial cells, but is expressed at the basolateral membrane of the choroid plexus (Nies et al., 2004; Roberts et al., 2008; Zhang et al., 2004). MRP5 is also highly expressed in pyrimidal neurons and astrocytes (Nies et al., 2004). Others have reported Mrp5 gene expression in oligodendrocytes and microglia (Hirrlinger et al., 2002; Nies et al., 2004). Using 9-(2-phosphonomethoxyethyl)adamine (PMEA), a substrate for MRP4 and MRP5, our group has shown that these transporters are functional in a rat microglia cell line (MLS-9) (Dallas et al., 2004b).
Functional studies have shown that MRP6 exhibits a weak resistance to chemotherapeutic drugs such as etoposide, doxorubicin and daunorubicin. MRP6 can also transport GSH conjugates (i.e., LTC4, N-ethymaleimide S-glutathione). Transcripts of Mrp6 have been detected in primary cultures of bovine brain microvessel endothelial cells, in capillary enriched fraction of bovine brain homogenate and in human brain tissue (Zhang et al., 2000). Mrp6 expression at the gene and protein level has been detected in glial cells and neurons, but not in pericytes (Berezowski et al., 2004). Loss of MRP6 function causes an autosomal recessive multi-organ disorder known as pseudoxanthoma elasticum, resulting in calcification of elastic fibres (Varadi et al., 2011). To date, interaction of MRP6 and antiretroviral drugs has not been established.

A few in vitro studies have suggested that MRP7 is a lipophilic anion transporter that confers resistance to several natural product anticancer drugs (i.e., docetaxel, vincristine). High levels of MRP7 transcripts have been detected in various tumor specimens suggesting a potential role of this transporter in the intrinsic sensitivity of tumors (Takayanagi et al., 2004). Transcripts of Mrp7 have been detected in brain tissue (Kao et al., 2002). Although MRP7 showed resistance to lopinavir in an over-expressing cell line, functional expression of MRP7 in different brain cellular compartments is yet to be characterized (Bierman et al., 2010).

MRP8 has been characterized as a lipophilic anion efflux pump in vitro (Chen et al., 2005). MRP8, similar to MRP4 and MRP5, confers resistance to several purine and pyrimidine nucleotide derivatives (Oguri et al., 2007). Transcripts and protein expression of MRP8 have been detected in human brain samples and gliomas. MRP8 is considered an axonal protein since immunofluorescence microscopy studies have detected MRP8 protein to be colocalized with neurofilaments in white matter in human brain (Bortfeld et al., 2006). The axonal localization of MRP8 together with its substrate affinity towards steroids indicates that this transporter may potentially participate in regulating neurotransmitter receptors bymediating the efflux of neurosteroids from neurons. With respect to antiretrovirals, MRP8 has been reported not to be involved in the cellular efflux of zidovudine or lamivudine (Guo et al., 2003). Further studies are needed to characterize MRP8 involvement in transporting other antiretroviral drugs.

MRP9 overexpressing cells have been reported to show resistance to atazanavir, lopinavir and ritonavir (Bierman et al., 2010) suggesting the potential involvement of this transporter in antiretroviral therapy. Although MRP9 transcripts have been detected in brain, additional studies...
are required to clarify the functional expression of this transporter in the brain (Bera et al., 2002). Therefore, the role of MRP9 in antiretroviral drug transport in the brain is not yet clear.

1.7.3 Breast cancer resistance protein (BCRP)

This transporter was first identified in a P-gp and MRP1-negative breast cancer cell line that showed high resistance to mitoxantrone, an anthracycline anticancer drug (Doyle et al., 1998). ABCG2 (BCRP) consists of 665 amino acids with a molecular mass of approximately 72 kDa. Unlike most other ABC transporters, ABCG2 is a half-transporter and is predicted to function as a homodimer. Evidence also suggest that BCRP may also function as a monomer (Mitomo et al., 2003).

The substrate profile of ABCG2 overlaps with that of P-gp or MRP1 and includes many anticancer drugs (i.e., daunorubicin, doxorubicin, mitoxantrone, etoposide, topotecan, camptothecins, methotrexate), antibiotics (i.e., erythromycin, enrofloxacine, gepafloxacine), phototoxins (i.e., pheophorbide-A), calcium channel blockers (i.e., azidopine, dipyridamole), HMG-CoA reductase inhibitors (i.e., rosuvastatin, pitavastatin), fluorescent compounds (i.e., rhodamine123), carcinogens (i.e., aflatoxin B1) and many others (i.e., cimetidine, riboflavin) (Mao and Unadkat, 2005; Merino et al., 2006; Zhang et al., 2005). ABCG2 also confers resistance to zidovudine, lamivudine, abacavir and stavudine (Wang et al., 2003a). Experiments in bcrp knockout animal show enhanced brain penetration of abacavir suggesting BCRP involvement in brain antiretroviral drug transport (Giri et al., 2008).

![Membrane topology of BCRP](image)

**Figure 1-7** Membrane topology of BCRP (TMD= Transmembrane domain; NBD= Nucleotide binding domain) (from Sarkadi B, 2006. Physiol Review. 86:1179-236).

Reproduced with permission from the copyright owner.
ABCG2 is expressed in several blood-tissue barriers including the blood-brain, blood-placental and blood-testis barriers. Cooray et al. detected luminal expression of ABCG2 in rat capillaries. Several studies have also reported the expression of ABCG2/Abcg2 in primary cultures of human brain microvessel endothelial cells, mouse brain capillaries and immortalized rat brain endothelial cell line (Cooray et al., 2002; Eisenblatter and Galla, 2002; Lee et al., 2007; Zhang et al., 2003). ABCG2 expression has also been detected in pericytes and at the luminal membrane of choroid plexus epithelial cells (Eisenblatter et al., 2003). Our group has confirmed Abcg2 protein expression in primary cultures of rat astrocytes and in a microglia cell line, MLS-9 (Lee et al., 2007).

Several in vivo studies support that ABCG2 can restrict brain permeation of a number of compounds. Functionally active ABCG2 has been reported in intact brain capillaries (Hartz et al., 2010b). A few studies have also reported ABCG2 mediated transport in cultured human and rodent brain microvessel endothelial cells. However, several studies have suggested lack of ABCG2 mediated transport in endothelial cells (Hori et al., 2004b; Zhang et al., 2003). For example, accumulation of mitoxantrone, an established P-gp substrate, did not increase in the presence of ABCG2 inhibitors in primary cultures of human brain endothelial cells as well as in RBE4 cell line. In addition, a lack of ABCG2 mediated transport of mitoxantrone has been demonstrated in primary cultures of rat astrocytes and in a microglia cell line (Lee et al., 2007). A study by Cisternino et al. reported 700-fold higher Abcg2 mRNA in mouse brain capillaries compared to the cortex. In mdr1a/b deficient mice, they also observed 3-fold higher Abcg2 mRNA levels in the capillaries compared to wild-type mice. At the protein level, upregulated ABCG2 protein expression was observed in brain microvessels compared to wild type animals In mdr1a knockout mice model suggesting a compensatory role of ABCG2 (Cisternino et al., 2004). Recent studies also indicate that ABCG2, in conjunction with P-gp can limit the penetration of tyrosine kinase inhibitors into the brain (Agarwal et al., 2011; Breedveld et al., 2005; Gardner et al., 2009).
### Table 1-3 CNS expression/localization of ABC transporters implicated in antiretroviral drug transport (From Ashraf T, Robillard K, Chan G and Bendayan R. Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders. 2013. Current Pharmaceutical Design. 20: 1543-63)

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene name</th>
<th>CNS protein Expression/Localization</th>
<th>Known antiretroviral substrates</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp (MDR1)</td>
<td>ABCB1</td>
<td>Brain microvessel endothelial cells (LM/ALM), choroid plexus epithelium (AP), astrocytes, neurons</td>
<td>Darunavir, atazanavir, saquinavir, ritonavir, amprenavir, lopinavir, tipranavir, nelfinavir, tenofovir df, raltegravir, maraviroc</td>
<td>(Fujimoto et al., 2009; Janneh et al., 2007; Kassahun et al., 2007; Kim et al., 1998; Lee et al., 1998; Perloff et al., 2005; Walker et al., 2005)</td>
</tr>
<tr>
<td>BCRP (ABCG2)</td>
<td>ABCG2</td>
<td>Brain microvessel endothelial cells (LM), choroid plexus epithelium (AP)</td>
<td>Zidovudine, lamivudine, stavudine, abacavir, acyclovir</td>
<td>(Wang et al., 2003a)</td>
</tr>
<tr>
<td>MRP1</td>
<td>ABCC1</td>
<td>Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes, microglia, neurons</td>
<td>Atazanavir, saquinavir, ritonavir, indinavir, lopinavir, emtricitabine</td>
<td>(Bousquet et al., 2008; Janneh et al., 2009; Janneh et al., 2007; Janneh et al., 2005; van, I et al., 2001; Zastre et al., 2009)</td>
</tr>
<tr>
<td>MRP4</td>
<td>ABCC4</td>
<td>Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes</td>
<td>Tenofovir, abacavir, zidovudine</td>
<td>(Borst et al., 2004; Ray et al., 2006)</td>
</tr>
<tr>
<td>MRP5</td>
<td>ABCC5</td>
<td>Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes, neurons</td>
<td>Stavudine</td>
<td>(Reid et al., 2003)</td>
</tr>
</tbody>
</table>

(LM = luminal; ALM = abluminal; AP = apical; BL = basolateral)

Reproduced with permission from the copyright owner
1.7.4 Regulation of ABC transporters during HIV-1 infection

Evidence in literature suggests that functional expression of ABC transporters in the brain is altered during HIV-1 infection. Increased P-gp expression has been reported in brain autopsy tissues from patients with HIVE (Langford et al., 2004). Since antiretroviral drugs can modulate the expression of transporters in vitro and in vivo, it remains unclear if this increased P-gp immunoreactivity is due to therapy itself or due to HIV-associated pathogenesis. In contrast, decreased protein expression has been reported in brain autopsy samples from patients (not receiving pharmacotherapy) with HIVE and in brain tissue from severe combined immunodeficiency (SCID) mice model of HIVE (Persidsky et al., 2000). Using cell culture systems, many groups have demonstrated altered P-gp expression in human or rodent brain cellular compartments. Persidsky et al. reported decreased functional expression of P-gp in primary cultures of human brain microvessel endothelial cells exposed to TNF-α, IL-1β, IFN-γ and supernatants from HIV-1 infected macrophages (Persidsky et al., 2000). In both murine brain microvessel endothelial cells and astrocytes, HIV-1 tat exposure resulted in an upregulation of P-gp expression (Hayashi et al., 2005). Exposure to HIV-1 resulted in an increase in MDR1 gene and protein expression in primary cultures of brain microvessel endothelial cells. No significant change in MRP1 expression was observed (Roy et al., 2013). However, co-exposure of HIV-1 and PI saquinavir, showed induced functional expression of P-gp compared to HIV-1 exposure alone. Another group compared P-gp and MRP1 expression in PBMCs isolated from HIV-1 infected patients and healthy volunteers and observed significant decrease in P-gp expression, but no significant change in MRP1 (Meaden et al., 2001). In contrast, Turriziani et al. reported increased mRNA expression of MDR1, MRP1, MRP4 and MRP5 in PBMCs from infected individuals (Turriziani et al., 2008).

In our laboratory, we observed that HIV-1 gp120 can induce secretion of pro-inflammatory cytokines (IL-6, IL1-β and TNF-α) by interacting with CCR5 chemokine receptors in primary cultures of rat astrocytes and significantly decrease functional expression of P-gp (Ronaldson and Bendayan, 2006). Our group also examined the effect of different cytokines on P-gp expression and demonstrated that IL-6 could profoundly decrease P-gp expression, whereas, TNF-α or IL-1β exposure resulted in a modest enhancement in P-gp expression (Ronaldson and Bendayan, 2006). In regards to Mrp1 regulation, in primary cultures of rat astrocytes, we have

Reproduced with permission from the copyright owner
observed an increase in functional expression of this transporter in response to gp120 treatment which correlated with an enhanced efflux of GSH and GSSG, implying a potential role of MRP1 in regulating oxidative stress in glial cells (Ronaldson and Bendayan, 2008). In a transgenic rat model, significant changes in mRNA expression of several ABC transporters were observed in the brain, kidney, liver and testes (Robillard et al., 2014). In the brain, decreased mRNA expression of Mdr1a, Mdr1b, Mrp1, MRP4 and increase Mrp5 mRNA expression was observed (Robillard et al., 2014). These observations provide evidence that ABC transporters are regulated during HIV-associated brain inflammation which may result in altered brain permeability of antiretroviral drugs.

1.8 Potential adjuvant therapy

Antiretroviral drugs that are currently being used in HIV-1 therapy do not exhibit anti-inflammatory properties (with the exception of maraviroc) and majority of them demonstrate poor brain permeability. In addition, neurotoxicity has been associated with better permeable drugs (i.e., efavirenz) (Cavalcante et al., 2010). Therefore, alternative treatment options are being considered that can reduce HIV-associated brain inflammation.

A number of studies have reported the anti-inflammatory potential of different compounds against LPS-mediated inflammatory response. Various anti-psychotic compounds (i.e., spiperone, risperidone), nonsteroidal anti-inflammatory drugs (i.e., flurbiprofen) and natural extracts (i.e., tripchlorolide, xanthorrhizol, curcumin) are known to inhibit LPS induced cytokine and NO release in microglia (Ajmone-Cat et al., 2001; Lim et al., 2005; Pan et al., 2008; Zheng et al., 2008a; Zheng et al., 2008b). Administration of chloroquine, an anti-malarial drug, reduced gene expression of TNF-α in LPS treated PBMCs (Weber and Levitz, 2000). A plant derived compound, 2-cyano-3,12-dioxooleane-1,9(11)-dien-28-oic acid (CDDO)-methyl ester prevented induced transcription of TNF-α and IL-1β in primary cultures of mouse microglia and macrophages (Tran et al., 2008). Lipoic acid can be another potential suppressor of neuroinflammation due to its role against cognitive deficits in rodents (Holmquist et al., 2007). In a LPS injected rat model, minocycline, a tetracycline derivative, significantly reduced microglial activation in the hippocampus and rescued behavioral deficits (Zhu et al., 2014).
Several studies have also examined anti-inflammatory properties of different compounds in attenuating HIV-1-associated inflammatory response. For example, chloroquine, has been found to inhibit gp120 production and decrease mRNA synthesis of cytokines (Wozniacka et al., 2008; Wozniacka et al., 2006). Curcumin treatment prevented gp120-mediated release of ROS, TNF-α and MCP-1 in mouse microglial cells line N9 and protected primary rat cortical neurons from apoptosis (Guo et al., 2013). Simvastatin prevented Tat induced upregulation of inflammatory genes (Andras et al., 2008). Since neurological disorders are becoming more prevalent in HIV-1 infected patients and inflammation is a common immune response, identifying therapeutic compounds that can effectively permeate the BBB and exhibit anti-inflammatory properties may provide an additional option in preventing/treating HIV-associated neurological disorders.

1.8.1 Minocycline

Minocycline, a second generation tetracycline derivative, has been widely reported to have neuroprotective properties (Orsucci et al., 2009). Due to its small molecular weight (495 kDa) and highly lipophilic nature, minocycline can cross into BBB and has been known to penetrate into the CSF of humans better than other tetracyclines. Minocycline was originally developed to treat gram negative and gram positive bacterial infections. Currently, minocycline is recommended for treatment of anthrax, acne, gonorrhoea, acute intestinal amoebiasis, respiratory tract infection, chlamydial infections and a number of other infections.

Numerous published studies have documented that minocycline exhibits neuroprotective and anti-inflammatory effects in various animal models (Cai et al., 2010; Ryu and McLarnon, 2006). Administration of minocycline has been shown to successfully reverse the activation of glial cells and secretion of inflammatory cytokines in an Aβ injected rat model (Ryu et al., 2004). Minocycline administration resulted in beneficial effects in clinical trials for multiple sclerosis, rheumatoid arthritis and asthma (Daoud et al., 2008; Zabad et al., 2007). In a recent study, treatment with minocycline significantly attenuated the increase of immune activation markers (i.e., CD38, CD69, HLA-DR, CCR5, IL-10, soluble CD14, LPS) in a humanized NOD/LtsZ-scidIL-2Rγnull mice model (Singh et al., 2014).
Minocycline is also known to exhibit an anti-HIV-1 effect (Copeland and Brooks, 2010; Szeto et al., 2010). In vitro, minocycline reduced HIV-1 replication and decreased CD4 T-cell activation. Minocycline decreased the activation and proliferation markers (i.e., CD25, Ki-27) and decreased cytokine secretion (IL-2, IFN-γ, TNF-α) (Szeto et al., 2010). Minocycline also reduced CCR5 expression on CD4+ T-cells that may potentially reduce the spread of R5-tropic viruses (Szeto et al., 2010). In vivo, minocycline was able to decrease viral plasma load and viral RNA in the brain of Simian immunodeficiency virus (SIV) macaque model (Zink et al., 2005). However, in a clinical study, minocycline administration was unable to decrease HIV-1 viral load in the CSF (Ho et al., 2011). Another study has further reported that minocycline treatment was unsuccessful to improve the neurocognitive outcome in patients with cognitive impairment after 24 weeks of administration (Sacktor et al., 2011). Similarly, in a Ugandan population, minocycline treatment did not improve cognitive function in therapy naive, HIV-infected individuals (Nakasujja et al., 2013). Interestingly, in a SIV model of neuropathogenesis, early administration of minocycline was reported to be effective against striatal dopaminergic system dysfunction, suggesting that timely treatment initiation may contribute to minocycline efficacy (Meulendyke et al., 2012). Using proton magnetic resonance spectroscopy in SIV infected macaques and examining biomarkers in post-mortem tissues, Ratai et al. reported that minocycline could attenuate a progressive decline in neuronal integrity, decrease glial activation and CSF and plasma viral loads (Ratai et al., 2010). Following minocycline treatment, decreased plasma viral load and monocyte activation was also observed in this primate model (Campbell et al., 2011). It is still inconclusive if administration of minocycline in earlier stage of infection will have a better clinical outcome in HIV-infected patients. Therefore, further investigation is required to determine the potential anti-inflammatory effects of minocycline during HIV-1-associated inflammatory response.

1.8.2 Chloroquine

Chloroquine, an anti-malarial drug, has been used to treat malaria for several decades and it is recently being used to treat inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus. Studies have also demonstrated beneficial effects of chloroquine against HIV-1 infection. Chloroquine is a weak base that is known to affect acid vesicles leading to dysfunction of enzymes (i.e., acid hydrolases) necessary for post-translational modifications.
Tsai et al. demonstrated altered production of gp120 due to chloroquine (Tsai et al., 1990). Production of gp120 decreased in T-cells after chloroquine administration (Tsai et al., 1990). Chloroquine may also inhibit HIV-1 replication by restricting intracellular iron distribution which may lead to inhibition of ribonucleotide reductase necessary for viral replication. Chloroquine has been shown to interfere with toll-like receptor 7 downstream pathways to prevent activation of transcription factors to block the production of IFN-α (Martinson et al., 2010; Ries et al., 2012; Sun et al., 2007).

A number of studies have also investigated the anti-inflammatory properties of chloroquine in in vivo animal models. Chloroquine has been found to decrease mRNA synthesis of cytokines (Naarding et al., 2007; Savarino et al., 2001; Wozniacka et al., 2008; Wozniacka et al., 2006). In a LPS injected rat model, chloroquine significantly diminished LPS-mediated upregulation of serum cytokines (Hong et al., 2004). In another study, administration of chloroquine in rats up to four consecutive days completely prevented a bacterial toxin induced intracerebral toxicity (Hagihara et al., 2000). The administration of hydroxyl analogue of chloroquine (hydroxychloroquine) in HIV-1 infected patients resulted in a decrease in detectable viral RNA and IL-6 levels in plasma suggesting a therapeutic potential of this compound (Sperber et al., 1995). Another study reported reduced T-cell activation in chronic HIV-infected patients due to chloroquine administration (Murray et al., 2010). In contrast, chloroquine administration in HIV-1 infected patients on antiretroviral therapy did not improve T-cell activation or inflammatory markers (Routy et al., 2014). Therefore, it is inconclusive if chloroquine has the potential to reverse HIV-associated inflammatory response.

1.8.3 Simvastatin

Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, is known to have anti-inflammatory properties. Similar to other statins, simvastatin strongly inhibits endogenous cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase and also inhibits the synthesis of isoprenoid intermediates (i.e., geranylgeranylpyrophosphate) that act as important lipid attachments for post-translational modifications for a number of proteins. Simvastatin is known to have anti-inflammatory properties and demonstrates neuroprotective effects in various animal models (Adamson and Greenwood, 2003; Andras et al., 2008; Jiang et al., 2007). In dyslipidemic patients, simvastatin treatment decreased IL-8 production in
neutrophil leukocytes (Marino et al., 2014). In a rodent model of traumatic brain injury, administration of simvastatin in rats attenuated the activation of microglia and astrocytes (Li et al., 2009). In a study by Andras et al., simvastatin prevented Aβ and HIV-1 Tat induced upregulation of inflammatory genes in human brain microvessel endothelial cells (Andras et al., 2008). Besides anti-inflammatory properties, simvastatin also exhibits anti-HIV effects. A study by Navatob et al. demonstrated that simvastatin may limit the infection of R5 tropic virus in vitro by disrupting CC-chemokine receptor and CC-chemokine expression pattern (Nabatov et al., 2007). Amet et al. has demonstrated that simvastatin and lovastatin increased intracellular Gag and decreased viral release from chronically infected cells due to diminished geranylgeranylation (Amet et al., 2008). Simvastatin has also been reported to attenuate Aβ accumulation in brain endothelial cells by increasing low density lipoprotein related receptor protein-1 (LRP1) expression and decreasing HIV-1 induced receptor for advanced glycation end products (RAGE) expression in hCMEC/D3 cells (Andras et al., 2010). In addition, pre-treatment with simvastatin decreased HIV-1 induced accumulation of Aβ in these cells suggesting protective role of statins at the BBB against Aβ accumulation (Andras et al., 2010). Overall, these studies indicate that simvastatin has the potential to be used against the brain inflammatory responses observed during HIV-1 infection.

1.9 Signaling pathways

Experimental data from various in vitro and in vivo models suggest the involvement of different signal transduction pathways in the regulation of cytokine secretion and ABC transporters during HIV-associated brain inflammation (Ashraf et al., 2011;Hayashi et al., 2006;Ronaldson et al., 2010). Potential anti-inflammatory compounds are also known to target different signaling pathways to attenuate the inflammatory response. For example, simvastatin has been reported to reverse the production of TNF-α by inhibiting the MAPK and NF-κB pathways in cultured endothelial cells (Jiang et al., 2007). Minocycline is also known to attenuate LPS-induced increase in iNOS and nitric oxide expression by inhibiting MAPK and NF-κB in retinal microglia (Yang et al., 2007). These two pathways are discussed briefly below:
1.9.1 NF-κB

NF-κB is a transcription factor that was first discovered in the nuclei of mature B lymphocytes (Nabel and Baltimore, 1987). Since then, the activation of NF-κB has been associated with numerous immune and inflammatory responses. Unlike other transcription factors, NF-κB is a cytosolic protein bound to inhibitor of NF-κB (IκB) protein which is regulated by an upstream kinase known as IκB kinase complex (IKK). Upon activation by different stimuli, IκB is phosphorylated by IKK followed by degradation that in turn triggers the translocation of unbound NF-κB to the nucleus where it initiates transcription of specific target genes (Bonizzi and Karin, 2004; Schmitz et al., 2004). A number of signals can activate NF-κB such as inflammatory cytokines, phorbol esters, oxidative stress, UV light, bacterial and viral products and growth. NF-κB can activate and enhance transcription of genes involved in inflammation (i.e., TNF-α) (Bonizzi and Karin, 2004; Schmitz et al., 2004; Shah and Kumar, 2010).

Studies have demonstrated that NF-κB is activated in human brains of patients with Alzheimer’s disease (Boissiere et al., 1997). NF-κB activation was also found to be related with the pathophysiological responses associated with Parkinsons’s disease (Hunot et al., 1997). Since inflammation is a component of these neurodegenerative diseases, it has been suggested that this transcription factor may play a role in HIV-infection of the brain (Atwood et al., 1994). Studies have demonstrated that NF-κB plays an important role in HIV-1 transcription, thereby, making this transcription factor a potential pathway for anti-HIV therapy (Mingyan et al., 2009). NF-κB activation has been found to be associated with cytokine secretion in myeloid cells during HIV-1 infection suggesting a pivotal role of this pathway during inflammation in the CNS. In addition, NF-κB is known to be activated in different brain cells (i.e., endothelial cells, microglia and astrocytes) during HIV-1 infection (Hayashi et al., 2005; Sui et al., 2007). Interestingly, NF-κB is activated by inflammatory signals (i.e., cytokines) and in turn, can also activate and enhance transcription of genes involved in inflammation (i.e., TNF-α) (Bonizzi and Karin, 2004; Schmitz et al., 2004). Similar phenomenon has been observed with the MAPK pathway where various cytokines amplify their own signals by activating components of these pathways. Evidence also suggest that occasional cross-talk between NF-κB and MAPK occurs during regulation of several transcription factors and gene transcription (Liu and Lin, 2007). Therefore, the potential involvement of this pathway during HIV-associated inflammation needs further investigation.
1.9.2 MAPKs

The MAPK pathway consists of a large family of serine/threonine kinases that construct a highly regulated network of intracellular signaling pathways involved in generating various cellular responses in the presence of extracellular stimuli and in turn, regulate multiple critical cellular functions (i.e., gene expression, cytoskeletal organization, cell division) (Pearson et al., 2001). The three main subfamilies of the MAPK pathway are extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs) and P38 kinases (P38Ks). These kinases are known to be actively involved in regulating cytokine secretion as well as the expression of drug efflux transporters (Hayashi et al., 2006; Leghmari et al., 2008; Ronaldson et al., 2010). These pathways are also involved in various pathological processes associated with HIV-1 infection (i.e., neurotoxicity, macrophage activation, viral replication) (Furler and Uittenbogaart, 2010; Medders and Kaul, 2011). A recent study has further demonstrated that inhibition of these kinases can downregulate HIV-1 infection in vitro (Gong et al., 2011). Therefore, further examination of these pathways during HIV-associated brain inflammation is necessary.
1.9.2.1 ERK1/2

ERK1 and ERK2 were the first identified members of the ERK subfamily. These two kinases have been widely studied due to their pronounced role in cell growth and differentiation, cell-cycle regulation and cell survival (Whitmarsh and Davis, 2000). Although another five ERK isoforms have been identified in different cell systems, their function and physiological relevance is not yet fully understood. Previously, only growth factors were considered as activators of the ERK1/2 pathway and the regulatory role of these kinases in cancer or tumor cell growth have been examined extensively (Boulton et al., 1991; Cobb et al., 1991b). Besides growth factors, other factors such as pro-inflammatory cytokines, viral infection or carcinogens can also activate this pathway (Boulton et al., 1991; Cobb et al., 1991a). The upstream kinase of ERK1/2 is MEK1/2 (Alessi et al., 1995). PD98059 and U0126 are two well-established MEK inhibitors that can manipulate ERK1/2 regulated cellular activities (Dudley et al., 1995; Favata et
47

al., 1998). The downstream targets of ERK1/2 include transcription factors c-fos, ELK1 and CREB (Kyosseva, 2004).

Activation of the ERK1/2 has been observed in primary cultures of astrocytes and microglia in response to LPS (Pyo et al., 1998; Schumann et al., 1998). An increase in ERK1/2 phosphorylation was found to be associated with cognitive and motor deficits during brain injury in rats (Dash et al., 2002). In the context of HIV-1, gp120 or whole virus-mediated activation of Raf-1, an upstream kinase of ERK1/2, led to profound but transient upregulation of ERK1/2 (Popik and Pitha, 1996). The same group demonstrated that MEK/ERK pathway was activated by both CCR5 and CXCR4-tropic viruses. Other viral proteins have also been implicated in the activation of ERK1/2. This pathway was found to be involved in Tat stimulated TNF-α production in human macrophages (Leghmari et al., 2008). Using rat hippocampal slices, Tat-mediated release of TNF-α and MCP-1 was found to be mediated by ERK1/2 activation. Treatment with resveratrol inhibited ERK1/2 phosphorylation and attenuated Tat-induced release of TNF-α and MCP-1 (Lee et al., 2011). Another viral protein Nef-mediated upregulation of ICAM-1 was found to be ERK1/2 dependent and inhibition of ERK1/2 blocked ICAM-1 upregulation in endothelial cells (Fan et al., 2010).

1.9.2.2 JNKs

The three main subtypes of JNKs (JNK1, JNK2 and JNK3) are encoded by three different genes and a total of 10 isoforms result from alternative splicing (Kumagae et al., 1999; Mielke and Herdegen, 2000). JNKs respond to various cellular stress inducers (UV, heat shock, cycloheximide) as well as pro-inflammatory cytokines (i.e., TNF-α, IL-1β) (Sluss et al., 1994). As a result, these enzymes are also known as stress-activated proteins kinases (SAPKs). The full activation of JNKs requires the phosphorylation of both Thr183 and Tyr185 residues by specific upstream kinases, MKK4 and MKK7 (Johnson et al., 2007). Once activated, JNK phosphorylates and in turn activates a number of transcription factors and non-transcription factors. In addition to c-jun, the first identified downstream target of JNK, other JNK regulated transcription factors are ATF-2, ELK-1, p53 and c-myc (Mielke et al., 1999). The non-transcription factors that are also phosphorylated by JNK include members of the B-cell lymphoma 2 (Bcl-2) family and the result of their activation can lead to apoptosis, motility, immune response, metabolism or DNA repair (Jhonson et al., 2007).
JNK1 and JNK2 are ubiquitously expressed in the body whereas JNK3 is expressed primarily in the brain, and to a lesser extent in heart and testis (Kyriakis et al., 1994). However, JNKs may have an isoform specific role in different cell systems (Gupta et al., 1996; Pawate and Bhat, 2006). All three isoforms have been detected in glial cells (microglia and astrocytes), but their specific functional roles are not clearly understood (Hidding et al., 2002; Waetzig et al., 2005; Zhang et al., 1998; Zhang et al., 1996).

It is established that JNKs are involved in the pathogenesis of various CNS diseases, such as Alzheimer’s, Parkinson’s, ischemia, HIV-1 associated inflammation (Kumagae et al., 1999). Therefore, inhibition of JNKs may provide anti-inflammatory effects in the brain. Studies in cultured microglias and LPS injected mice showed that inhibition of JNKs attenuated LPS-induced TNF-α secretion (Ciallella et al., 2005). In the context of HIV-1 infection, JNKs are also known to be activated by viral proteins. Vpr induced apoptosis was found to be mediated by activation of JNKs and subsequent downregulation of anti-apoptotic genes (Mishra et al., 2007). HIV-1 or gp120-mediated activation of ERK1/2 and JNK pathways were also observed in primary culture of microglia, astrocytes and neurons where treatment with HIV-1 or gp120 resulted in apoptosis in microglias and neurons (Lannuzel et al., 1997).

Several inhibitors of the JNKs (i.e., CEP-11004, CEP-1347, SP600125) have been effectively used both in in vitro and in vivo models of brain inflammation (Bogoyevitch et al., 2004; Han et al., 2001; Saporito et al., 2002; Vincenti and Brinckerhoff, 2001) providing evidence that JNK inhibition might be a desirable target against neuroinflammation. CEP1347 has been shown to inhibit gp120-mediated apoptosis in hippocampal neurons (Bodner et al., 2002). In cultured rat embryonic neurons, the increased activity of JNKs and the subsequent neuronal death was blocked by CEP-1347 (Maroney et al., 1998). In another study, CEP-11004 inhibited LPS induced TNF-α secretion in cultured microglias and in LPS injected mice (Ciallella et al., 2005). These data provide evidence that JNK inhibition might be a desirable target against neuro-inflammation. A recently developed mixed lineage kinase 3 inhibitor, URMC-099, has shown increased BBB permeability and shown potential in in vitro and in vivo models (Goodfellow et al., 2013). URMC-099 inhibited LPS-induced TNF-α release in microglias, Tat- induced cytokine release in human monocytes and upregulation of phospho-JNKs in Tat-injected mouse brain.
This compound also exhibited neuroprotective and anti-inflammatory properties in both in vitro and in vivo models of HIV-1 associated brain inflammation (Marker et al., 2013).

1.9.2.3 P38Ks

To date, four isoforms of P38K have been identified in humans (α, β, γ and δ) and data suggest that P38αK and P38βK play a major role during inflammatory and immune responses. However, the cellular and physiological role of P38γ and P38δ in inflammation are not yet clearly understood. P38Ks are activated by dual phosphorylation on Thr180 and Tyr182 residues by upstream kinases, MKK3 and MKK6 (Kyriakis and Avruch, 2001). SB203580 is a known inhibitor of P38α and β isoforms. Some of the typical target transcription factors of P38Ks are ATF2, Elk1 or SAP1 (Cohen et al., 1997). Similar to the JNKs, P38Ks are activated by cellular stress, i.e., UV radiation, osmotic or heat shock, LPS and pro-inflammatory cytokines (Cuenda et al., 1995). These kinases also play a major role in mediating inflammation-related responses (Cuenda and Rousseau, 2007).

Evidence suggests that P38Ks are involved in the regulation of gene expression of several cytokines (i.e., TNF-α, IL1-β) during neuroinflammatory conditions. For example, inhibition of P38Ks prevented LPS-induced TNF-α production in rats (Zhu et al., 2005; Badger et al., 1996). Both Tat and Nef-induced TNF-α production in human macrophages was found to be P38K dependent (Kumawat et al., 2010; Leghmari et al., 2008). Viral protein Nef-mediated activation of JNK, P38K and NF-κB pathways have also been reported in murine macrophages (Mangino et al., 2012). Effect of Vpr on neuronal death has been found to be partly mediated through release of pro-inflammatory cytokines IL-1β and IL-10. JNK as well as P38K pathways were found to be involved in the release of these cytokines in monocyte derived macrophages (Guha et al., 2012). P38K was also found to be involved in LPS-enhanced transcytosis of HIV-1 across brain microvessel endothelial cells (Dohgu and Banks, 2008). This pathway is known to be involved in HIV-1 replication. In an in vitro study done in T-cell and monocyte cell lines, inhibition of P38K blocked viral replication (Muthumani et al., 2004). Therefore, the inhibition of this pathway leads to successful suppression of cytokine synthesis. Several P38K inhibitors that have shown successful cytokine suppression have been tested in clinical trials (Kaminska, 2005; Lee et al., 2000; Schindler et al., 2007). The protective role of P38K inhibitors in the CNS suggest a
therapeutic strategy against inflammatory conditions seen in HIV-1 infection and neurodegenerative diseases.

### 1.9.3 Regulation of ABC transporters by MAPK and NF-κB

Ample evidence suggest that MAPK and NF-κB are involved in the regulation of transporters. ERK1/2 are known to regulate the expression of both P-gp and Mrp1. In both human breast carcinoma and gastric carcinoma cell lines, ERK1/2 inhibition resulted in significant decrease in P-gp protein expression (Katayama et al., 2007). In cultured astrocytes, ERK1/2 inhibition resulted in the attenuation of Tat-mediated upregulation of Mrp1 protein expression (Hayashi et al., 2006). Activation of ERK pathway upregulated ABCG2 mRNA expression, whereas, activation of JNKs downregulated ABCG2 mRNA expression in human acute lymphoblastic leukemia cell lines (Tomiyasu et al., 2013).

Similar to ERK1/2, JNKs are also known to be involved in the regulation of ABC transporter expression. JNK activation led to a decrease in mRNA and protein expression of P-gp in several carcinoma cell lines (Zhou et al., 2006). C-jun, a downstream target of the JNK pathway, was also found to be involved in the downregulation of MDR1 gene in a human cell line (Miao and Ding, 2003). In the context of HIV-1 infection, Hayashi et al. has demonstrated that viral protein Tat-induced upregulation of Mrp1 is ERK1/2 and JNK pathway dependent in brain microvessel endothelial cells and in astrocytes (Hayashi et al., 2006). P38Ks are also involved in regulating transporter expression. In a mouse leukemic cell line, the reversal of MDR phenotype was observed after inhibiting the P38K pathway (Barancik et al., 2001). Other studies have demonstrated the role of the NF-κB pathway in the regulation of P-gp expression (Bentires-Alj et al., 2003; Yu et al., 2008). Regulation of ABC transporters has also been demonstrated in the mouse BV-2 microglia cell line where exposure to LPS significantly reduced functional expression of ABC transporters (e.g. Mdr1, Bcrp, Mrp4), presumably, through interactions with the NF-κB pathway (Gibson et al., 2012). Based on these observations, it is clear that regulation of these transporter is very complex, cell/tissue specific and dependent on disease related pathologies.
1.9.4 Effect of anti-inflammatory compounds on MAPK and NF-κB

Many compounds have demonstrated anti-inflammatory properties by interacting with MAPK or NF-κB pathway. For example, curcumin and CDDO-methyl ester have suppressed activation of NF-κB, whereas, catechols have been found to attenuate both NF-κB and P38K activation (Tran et al., 2008; Zheng et al., 2008b). Flurbiprofen derivative HCT1026 can inhibit cytokine induced activation of signaling pathways in macrophages (Idris et al., 2009). Simvastatin has been shown to reverse the production of TNF-α by inhibiting the MAPK and NF-κB pathways in cultured endothelial cells (Jiang et al., 2007). Simvastatin has also been reported to inhibit LPS-stimulated ERK1/2 activation (Sundararaj et al., 2008).

Chloroquine inhibited the phosphorylation of P38K in coronavirus infected human fetal lung cell line (Kono et al., 2008). SB203580, a P38K inhibitor, suppressed viral replication in this cell system suggesting that chloroquine may inhibit replication of coronavirus by suppressing P38K pathway (Kono et al., 2008). Both in a murine B-lymphoma and in a monocytic cell line, CpG-DNA induced phosphorylation of JNKs, P38Ks as well as their downstream targets c-Jun and ATF-1 were observed (Yi and Krieg, 1998). Administration of chloroquine inhibited CpG-DNA induced secretion of TNF-α and IL-6 by blocking phosphorylation of JNKs and P38Ks. NF-κB was also found to be activated in these cell lines due to CpG-DNA exposure. Inhibition of NF-κB as well as administration of a P38K inhibitor attenuated cytokine secretion suggesting involvement of signaling pathways in generating inflammatory response (Yi and Krieg, 1998). In a SIV model, minocycline administration has shown similar inhibitory effect on P38K and JNK phosphorylation in the brain (Follstaedt et al., 2008). In microglial and spinal cord culture system, minocycline attenuated glutamate-induced microglial activation, release of NO and IL-1β by preventing P38K phosphorylation (Tikka et al., 2001). These studies provide evidence that many compounds exert their anti-inflammatory properties by interacting with different signaling pathways.

Although the mechanisms of action for some of these compounds are unknown in astrocytes or microglia during HIV-1 infection, their ability to suppress inflammation indicates that signaling pathways associated with the regulation of pro-inflammatory genes are likely to be involved. Since these compounds have the potential to be used alongside antiretroviral drugs to mitigate
the sufferings of patients with neurocognitive disorders, there effect on HIV-associated brain inflammation should be investigated in greater detail.

1.10 *In vitro* and *in vivo* models to study drug transport and HIV-associated brain inflammation

1.10.1 *In vitro* systems

In order to understand the CNS-specific role of drug transporters, numerous brain microvessel endothelial cells and glial cell culture systems have been established (Nicolazzo et al., 2006; Poller et al., 2008; Tsuji et al., 1992). To date, many researchers have established primary cultures of brain microvessel endothelial cells from a number of mammalian species (i.e., bovine, porcine, murine, human). In addition, several immortalized rodent and human cell systems were also generated (i.e., rat RBE4, GPNT, b.End3, BB19, NKIM-6, HCMEC/D3) (Tsuji et al., 1992; Van Bree et al., 1992). Among these systems, the immortalized HCMEC/D3 cell line generated from human brain microvessel endothelial cells is one of the most established and extensively characterized system to study drug transport across the BBB (Poller et al., 2008). HCMEC/D3 cell line has been shown to retain several morphological and functional characteristics of the brain microvessel endothelial cells *in vivo*. Dauchy et al. compared the gene and functional expression of various ABC transporters between HCMEC/D3 and freshly isolated human brain microvessels and reported that P-gp and BCRP expression are lower in HCMEC/D3 than in the human microvessels, but functional expression remains comparable (Dauchy et al., 2009). Isolated brain capillaries from rodent brain tissues are also used to perform functional assay and understand regulation of drug transporters (Bauer et al., 2007; Miller et al., 2000).

In order to study drug transport in brain parenchymal cells, standard procedures have been established to generate primary cultures of astrocyte or microglia (Decleves et al., 2000; Hong et al., 2000; Tanaka and Maeda, 1996) (Dallas et al., 2003; Hong et al., 2001; Lee et al., 2001a; Ronaldson et al., 2004a; Schlichter et al., 1996). Mouse or rat neonatal brain tissues are generally used to establish astrocyte cultures since astrocytes are still immature and continue to divide at this stage. The process of astrocyte isolation involves mechanical and enzymatic methods. The isolation process and culture conditions (i.e., lack of flask coating) do not allow neuronal contamination. Microglia are the principal contaminating cells in astrocyte cultures. After confluency, the cultured astrocytes are shaken to remove microglia that can be used to
establish primary cultures of microglia. However, a major obstacle in working with microglia has been the difficulties associated with obtaining a large number of primary microglial cells to routinely perform studies. Therefore, immortalized cell systems, BV-2 and N9 are mostly utilized. However, microglial activation can be poorly reproduced in these systems and these cell lines also exhibit a limited cytokine and chemokine profile compared to primary cultures of microglia.

Since the BBB is considered a dynamic multi-compartment unit, advances have been made to establish co-culture systems where mixed cultures of cells are grown either together or in separate compartments using transwell systems. A co-culture system may allow cell-cell communication or generate specific signaling proteins or tropic factors necessary for the differentiation and proliferation of neighboring cells. Therefore, co-culture systems with mixed glia or pericytes have been considered a more physiological model of BBB (Meyer et al., 1991; Pardridge, 1999). Several studies have also described dynamic three-dimensional model of the BBB where drug transport properties are studies in brain microvessel endothelial cells co-cultured with other cellular components of the neurovascular unit under flow conditions (Cucullo et al., 2008).

Exposure to cultured brain microvessel endothelial cell or astrocytes or microglia to HIV-1/viral proteins/LPS can induce secretion of inflammatory cytokines, ROS and other neurotoxic factors. Therefore, these systems are often used to delineate regulation of transport activities during inflammation or oxidative stress (Ronaldson and Bendayan, 2008). For example, regulation of P-gp along with other ABC transporters has been demonstrated in the mouse BV-2 microglia cell line where exposure to LPS significantly reduced functional expression of ABC transporters (e.g., Mdr1, Bcrp, Mrp4) (Gibson et al., 2012).

1.10.2 In vivo models

Many animal models have also been generated to study HIV-associated brain inflammation. SIV infected rhesus macaques has been widely used as an established animal model to study HIV infection as well as HIV-associated brain inflammation (Rausch et al., 1999; Sasseville and Lackner, 1997). SIV infected primates develop a progressive immune dysfunction characterized by depletion of CD4+ T-cells. Similar to HIV-1 infected individuals, these non-human primates develop reservoirs in resting CD4+ T-cells as well in the brain. Microglial activation, astrogliosis
and multinucleated giant cells have been seen in SIV-infected macaque brains (Petry and Luke, 1997). Feline immunodeficiency virus (FIV) infection is also being used to study HIV-pathogenesis (Miller et al., 2011). FIV infects feline populations and causes pathology similar to HIV. Neuropathological changes as well as behavioral and psychological impairments have also been observed in this model (Fletcher et al., 2011; Maingat et al., 2009). HIV-1 transgenic rat model has been shown to be another model for examining HIV-associated immune response in the periphery as well as in the brain. This model is developed by expressing a modified HIV transgene with functional deletion of the gag and pol genes that makes the virus non-infectious (Reid et al., 2001). These transgenic rats also develop cognitive and motor deficits (Reid et al., 2001). Pro-inflammatory cytokines (IL-1β, IFN-γ, TNF-α) were found to be significantly elevated in brain lysates of transgenic rats compared to wildtype controls. Other transgenic mouse models are also available that express specific viral proteins (gp120 or Tat).

SCID mice injected with HIV-1 in the brain is a model that mimics several important pathological changes observed during HIVE (i.e., activation of microglia, reactive astrogliosis, neuronal apoptosis). This model is generated by inoculating cultured monocytes isolated from HIV-1 infected patients into the mice brain. Persidsky et al. demonstrated that inflammatory response was detectable on day 3 after inoculation and HIV-1 antigens were found in the mice brain tissue up to 5 weeks (Persidsky et al., 1996). Immunohistochemistry on fixed brain sections confirmed the inflammatory reactions such as activation of glial cells and presence of pro-inflammatory cytokines (i.e., TNF-α, IL-6, IL-1β). Pathological changes were also observed at sites distant from the inoculation area in the mice brain. In addition, neuronal cell death and behavioral abnormalities were also noticed in the SCID mice model of HIVE (Persidsky et al., 1996; Potula et al., 2005; Potula et al., 2008). A number of humanized mice models [i.e., hu-PBL-SCID, SCID-hu-thy/liv, NOD/SCID-γ(c)(−/−), NOD/SCID-Rag 2(-/-)γ(c)(−/−)] has also been used to investigate HIV-associated neuropathogenesis (Zhang et al., 2010).

Ample evidence suggests that intracerebral injection of gp120 or Tat leads to the activation of glial cells and neuronal loss in rodent brains (Bansal et al., 2000; Louboutin et al., 2010b; Nosheny et al., 2004). In different animal models, doses of gp120 ranging from 100 ng to 500 ng (administered intracerebrally) have been shown to induce an inflammatory response. One study demonstrated that when administered chronically (for 3 to 7 days) into the lateral ventricle,
gp120 can induce prominent neuronal cell-death in cerebral cortex by activating caspase-3 (Acquas et al., 2004). Louboutin et al. have shown that chronic expression of gp120 in rat can lead to ongoing brain inflammation (Louboutin et al., 2010b). In their system, an SV-40 derived vector expressing gp120 was injected in rat caudate-putamens and chronic apoptosis of microglia and neurons, secretion of MIP-1α and an increase in oxidative stress was observed. In this study, different doses of gp120 was also injected in the rat caudate-putamens for 1 h, 6 h, 1d, 2d, 4d, 7d, 14d and 28 days which resulted in increased glial population upto 14 days in a time and dose-dependent manner (Louboutin et al., 2010b). Therefore, intracerebral injection of viral proteins is another potential method to generate an in vivo model of HIV-1 associated brain inflammation.

2 Goal

The goals of this project are i) to understand the regulation of drug efflux transporters in astrocytes during HIV-1 gp120 associated inflammation and ii) to identify potential anti-inflammatory compounds that can successfully reverse HIV-1 gp120-associated brain inflammation in vivo.

3 Rationale

Inflammation is a common immune response associated with brain HIV-1 infection that can result in BBB disruption and neuronal loss (de Vries et al., 1997; Genis et al., 1992). Both microglia and astrocytes are known to play significant roles in HIV-associated chronic brain inflammation (Becher et al., 2000). Secretion of cytokines can further alter the expression of drug efflux transporters (Hayashi et al., 2005; Ronaldson and Bendayan, 2006). Our laboratory has extensively characterized the regulation of P-gp and Mrp1, two major transporters involved in antiretroviral drug permeation, by HIV-1 gp120 in primary cultures of rodent astrocytes in vitro (Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). Previous work from our laboratory suggests that HIV-1 gp120 mediated secretion of cytokines resulted in altered P-gp expression in primary cultures of rat astrocytes (Ronaldson and Bendayan, 2006). It is not known if P-gp is regulated in a similar manner in human astrocytes. The regulation of other ABC transporters by gp120-mediated inflammation has not been examined in astrocytes. It is yet to be established how different signaling pathways (MAPK and/or NF-κB) are involved in the
regulation of cytokine secretion and drug transporters in this cell system. The regulation of drug efflux transporters in astrocytes during HIV-1 gp120 associated inflammation is necessary to understand antiretroviral drug permeation into these cells during pathological conditions. In addition, identification of potential anti-inflammatory agents that can prevent cytokine secretion in both microglia and astrocytes can be an effective therapeutic approach against the severity of brain inflammation during brain HIV-1 infection. Therefore, development of an *in vivo* model of HIV-1 gp120 associated brain inflammation to assess the anti-inflammatory effect of different compounds is required. Understanding the mechanisms underlying CNS inflammatory response and drug resistance and identifying potential anti-inflammatory agents may benefit in reversing HIV-1 associated inflammatory responses in the brain.
4 Hypotheses

i) HIV-1 gp120 triggers secretion of cytokines (TNF-α, IL-6 and IL1-β) and regulates membrane drug efflux transporters (P-gp and Mrp1) through interacting with signaling pathways (i.e., MAPK) in astrocytes.

ii) HIV-1 gp120 induces an inflammatory response, alters drug efflux transporters and activates signaling pathways in rodent brain.

iii) Anti-inflammatory agents (i.e., simvastatin, chloroquine, minocycline) can reverse HIV-1 gp120-associated cytokine release in the brain.

5 Objectives

1) To investigate the role of MAPK and NF-κB pathways in the regulation of P-gp and Mrp1 in primary cultures of rat/human astrocytes exposed to pro-inflammatory cytokines (i.e., TNF-α, IL-6 and IL1-β) or gp120.

2) To implement and characterize an in vivo model of HIV-associated brain inflammation by intracerebroventricular (ICV) administration of gp120 in rodents

3) To evaluate the potential therapeutic effect of anti-inflammatory agents (i.e., minocycline, chloroquine) in reversing the cytokine secretion as well the disruption of BBB in our implemented rodent model of gp120-associated brain inflammation

4) To investigate, in vivo, the role of signaling pathways (i.e., MAPK) in the regulation of the inflammatory response in our implemented model of gp120-associated brain inflammation.
Chapter 2

6 Regulation of Mrp1 by TNF-α in cultured glial cells: involvement of NF-κB and JNK signaling pathways

This work is published and reproduced in this thesis with permission from ASPET:

Author contributions:

Research design: PT Ronaldson (first author), T Ashraf (second author) and R Bendayan (principle investigator).

Conducted experiments and data analysis: PT Ronaldson (Figures 6-1, 6-2, 6-3, 6-4, 6-6, 6-7,6-8, 6-11; table 1); T Ashraf (Figure 6-5, 6-6,6-7,6-8, 6-9,6-10)

Writing of the manuscript: PT Ronaldson (preparation of the manuscript and responses to reviewer’s comments); T Ashraf (revisions, submissions and responses to reviewer’s comments) and R Bendayan (overall conceptual and editorial review of several manuscript drafts and responses to reviewer’s comments)
6.1 Abstract

Pharmacotherapy of brain HIV-1 infection may be limited by ABC transporters [i.e., P-gp, Mrp1] that export antiretroviral drugs from HIV-1 brain cellular targets (i.e., astrocytes, microglia). Using an in vitro astrocyte model of an HIV-1 associated inflammatory response, our laboratory has shown that cytokines (i.e., TNF-α, IL-1β, IL-6), which are secreted in response to HIV-1 envelope gp120 exposure, can decrease P-gp functional expression; however, it is unknown if these same cytokines can alter expression and/or activity of other ABC transporters (i.e., Mrp1). In primary cultures of rat astrocytes, Mrp1 expression was increased by TNF-α (2.7-fold) but was not altered by IL-1β or IL-6. Cellular retention of 2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), an Mrp substrate, was reduced in TNF-α treated astrocytes, suggesting increased Mrp-mediated transport. Pharmacological inhibition of NF-κB signaling with SN50 prevented both TNF-α release and Mrp1 expression changes in astrocytes triggered with gp120; however, SN50 did not attenuate Mrp1 expression in cells triggered with TNF-α. In contrast, Mrp1 functional expression was not altered in the presence of gp120 or TNF-α when astrocyte cultures were pre-treated with SP600125, an established JNK inhibitor. SP600125 did not affect TNF-α release from cultured astrocytes triggered with gp120. Mrp1 mRNA expression was increased after treatment with gp120 (1.6-fold) or TNF-α (1.7-fold), suggesting altered Mrp1 gene transcription. These data suggest that gp120 and TNF-α can up-regulate Mrp1 expression in cultured astrocytes. Furthermore, our results imply that both NF-κB and JNK signaling are involved in Mrp1 regulation during an HIV-1 associated inflammatory response.
6.2 Introduction

Astrocytes, the most numerous cell type in the brain, perform multiple functions required for CNS homeostasis. During HIV-1 infection of the brain, astrocytes are known to participate in the immune response via release of proinflammatory cytokines (Speth et al., 2005). Increased cytokine secretion (i.e., TNF-α, IL-1β, IL-6) during brain HIV-1 infection is well established and may be triggered by soluble viral proteins (i.e., HIV-1 envelope gp120) (Kaul et al., 2005). Studies in cultured glial cells suggest that gp120 binding to chemokine receptors (i.e., CXCR4, CCR5) may mediate this inflammatory response (Ronaldson et al., 2008). In vitro, our laboratory has shown that proinflammatory cytokine release is increased in cultured rat astrocytes treated with gp120 via a CCR5-dependent mechanism (Ronaldson and Bendayan, 2006).

Although advances in HIV-1 pharmacotherapy have efficiently reduced systemic viral load, HAND remains a significant cause of morbidity and mortality in HIV-1 patients (McArthur et al., 2003). These neurological complications may be associated with poor CNS permeation of antiretroviral compounds, a phenomenon that may be attributed to expression of ABC efflux transporters [i.e., P-gp, MRPs/Mrps (MRPs in humans; Mrps in rodents)] at brain barrier sites (i.e., BBB, BCSFB) and in brain cellular targets of HIV-1 (i.e., microglia, astrocytes).

MRP1/Mrp1, a 190 kDa membrane protein, extrudes from cells many organic anions as well as their GSH, glucuronide, and sulfate conjugates (Ronaldson et al., 2008). Although MRP1/Mrp1 is primarily associated with efflux of anticancer drugs, antiretroviral agents (i.e., HIV-1 PIs) are also known substrates of this transporter (Dallas et al., 2004a; Williams et al., 2002). Mrp1 expression has been identified in several brain cellular compartments including brain capillary endothelial cells (Miller et al., 2000), choroid plexus epithelial cells (Wijnholds et al., 2000a) and glial cells (Dallas et al., 2003; Ronaldson and Bendayan, 2008). In the context of HIV-1 infection, expression levels of MRP1/Mrp1 remain controversial. While studies in PBMCs isolated from HIV-1 infected patients showed no difference in MRP1 expression as compared to healthy individuals (Meaden et al., 2001), another study has shown higher MRP1 expression levels in response to HIV-1 infection (Turriziani et al., 2008). The high variability in the data can be, in part, explained by differences in therapeutic regimens because some antiretroviral drugs are known to alter expression of membrane transporters (Ronaldson et al., 2008; Zastre et al., 2009).
Cytokine secretion (i.e., TNF-α, IL-1β, IL-6) in response to infection or cell stress may alter MRPI/Mrp1 functional activity. Using a human hepatoma cell line (HepG2), IL-1β and IL-6 treatment resulted in an increase in MRPI mRNA expression and transport activity (Lee and Piquette-Miller, 2003). Studies in Sprague-Dawley rats have demonstrated that treatment with LPS, a bacterial endotoxin that stimulates cytokine release, enhances hepatic Mrp1 mRNA expression, suggesting involvement of cytokines in regulating Mrp1 expression (Cherrington et al., 2004). In contrast, studies in human monocyte-derived macrophages reported that gp120-induced production and secretion of TNF-α and IL-6 were not correlated to altered expression of MRP1 (Jorajuria et al., 2004).

Cellular exposure to HIV-1 virions, HIV-1 viral proteins and/or cytokines is associated with activation of intracellular signaling systems such as NF-κB (Kim et al., 2005) and the MAPK pathway (Ghorpade et al., 2003; Hayashi et al., 2006; Hayashi et al., 2005). Additionally, both NF-κB and components of the MAPK pathway [i.e., JNKs] have been implicated in the regulation of ABC transporters such as P-gp (Bauer et al., 2007; Hartz et al., 2008; Zhou et al., 2006) and Mrp1 (Hayashi et al., 2006). Currently, there are no published reports demonstrating the involvement of either pathway in the regulation of Mrp1 in glial cells exposed to HIV-1 gp120 and/or cytokines.

Recently, our laboratory has reported increased functional expression of Mrp1 in response to oxidative stress in cultured rat astrocytes treated with gp120 (Ronaldson and Bendayan, 2008). Furthermore, we have also shown that gp120 treatment can induce secretion of TNF-α, IL-1β, and IL-6 from these astrocyte cultures (Ronaldson and Bendayan, 2006). It is unknown if cytokines can regulate Mrp1 expression in glial cells and, if cytokines are capable of altering Mrp1 expression, which intracellular signaling pathways may be involved. In the present study, we have i) evaluated Mrp1 functional expression in cultured rat astrocytes triggered with TNF-α, IL-1β, and IL-6 and ii) investigated the role of NF-κB and JNKs in the regulation of Mrp1 expression in cultured astrocytes exposed to HIV-196ZM651 gp120 or cytokines.
6.3 Materials and methods

6.3.1 Materials

HIV-1<sub>96ZM651</sub> gp120 full length protein (derived from subtype C, R5-tropic HIV-1) was obtained from the National Institute of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Bethesda, MD). PSC833 (i.e., vals podar) was a generous gift from Novartis Pharma (Basel, Switzerland). The rat monoclonal MRP1 antibody MRPr1 was obtained from Kamiya Biomedical Company (Seattle, WA). BCECF, acetoxymethyl ester and free acid, were purchased from Invitrogen (Mississauga, ON, Canada). MK571 was purchased from Biomol Inc. (Plymouth Meeting, PA). The cell-permeable NF-κB inhibitory peptide SN50 and the pharmacological NF-κB inhibitor (E)3-[((4-methylphenyl)sulfonyl]-2-propenenitrile (BAY 11-7082) were purchased from EMD Biosciences Inc. (La Jolla, CA). Rat recombinant TNF-α, the anthrapyrazolone JNK inhibitor SP600125 and the murine monoclonal actin antibody AC-40 were obtained from Sigma-Aldrich (Oakville, ON, Canada). Rat recombinant IL-1β, rat recombinant IL-6, the murine monoclonal TNF-α neutralizing antibody, and the murine monoclonal IL-1β neutralizing antibody were purchased from Chemicon Inc. (Temecula, CA). The rat monoclonal IL-6 neutralizing antibody was obtained from R&D Systems (Minneapolis, MN). The rabbit polyclonal total JNK/SAPK antibody and the rabbit polyclonal phosphorylated JNK/SAPK antibody were purchased from Cell Signaling Technology (Danvers, MA).

6.3.2 Cell culture

Primary cultures of rat astrocytes were prepared as previously described by our laboratory (Ronaldson et al., 2004a; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). All procedures were carried out in accordance with the University of Toronto Animal Care Committee and the Province of Ontario Animals for Research Act. Briefly, postnatal (1-3 day old) Wistar rats (Charles River Laboratories, St Constant, PQ, Canada) were killed by cervical dislocation and whole brains isolated. Cerebral cortices were dissected and subjected to enzymatic digestion for 30 min in serum-free minimum essential medium containing 2.0 mg/ml porcine pancreatic trypsin (Sigma-Aldrich) and 0.005% DNase I (Roche Applied Science, Laval, PQ, Canada). Tissue was mechanically disaggregated using a cell dissociation kit (Sigma-Aldrich) to yield a mixed glial cell suspension. The cell suspension was then centrifuged for 10
min at 100 g and resuspended in fresh culture medium consisting of minimum essential medium supplemented with 5% horse serum, 5% fetal bovine serum (FBS), and 50 μg/ml gentamicin. The cells were plated on 75 cm² polystyrene tissue culture flasks (Sarstedt, St. Leonard, PQ, Canada) and incubated in fresh medium at 37°C, 5% CO₂ and 95% air overnight for 7-10 days. The cells were then placed on an orbital shaker at 120 rpm for 6 h to remove contaminating oligodendrocytes, microglia, progenitor cells and neurons. The cells were harvested with 0.1% trypsin/ethylene diamine tetraacetic acid (EDTA) in Hank’s Balanced Salt Solution (HBSS) and plated at a density of 5 x 10⁴ cells/well on 48-well polystyrene plates (Becton-Dickinson, Franklin Lakes, NJ). The astrocytic nature of isolated cells and culture purity were previously assessed by morphological analysis and by immunostaining for standard biochemical markers (i.e., GFAP) (Ronaldson et al., 2004a).

The human cervical carcinoma cell line stably transfected with human MRP1 (MRP1-HeLa) was kindly provided by Dr. Susan Cole (Queen’s University, Kingston, ON, Canada). Cells were grown as monolayers on 75 cm² tissue culture flasks at 37°C in 5% CO₂ and 95% air. Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (4 mM L-glutamine; 25 mM D-glucose) supplemented with 400 μg/ml G418 and 10% FBS. Confluent cultures were subcultured with 0.25% trypsin-EDTA and were used as a positive control for western blotting experiments.

6.3.3 Gp120/cytokine Treatments

All treatments were performed on monolayers of primary cultures of rat astrocytes grown in 75 cm² tissue culture flasks. At the beginning of each experiment, culture medium was aspirated and fresh culture medium containing 1.0 nM HIV-196ZM651 gp120. HIV-196ZM651 gp120 is R5-tropic (also known as macrophage-tropic) and is derived from a subtype C viral isolate. R5-tropic viruses are the most prevalent strains of HIV-1 in the brain (Gabuzda and Wang, 2000). In HIV-1 infected patients, concentrations ranging between 12 ng/ml and 92 ng/ml have been reported to be released in serum (Oh et al., 1992). These serum concentrations correspond to a molar concentration range of 0.1 nM to approximately 1.0 nM. All experiments were conducted at 37°C in 5% CO₂ and 95% air. Control (i.e., untreated) cultures were comprised of untreated cells in fresh culture medium. For experiments examining the involvement of NF-κB or JNK on the
regulation of Mrp1 in gp120-treated cells, cultures were pre-treated with 1 µM SN50, 5 µM BAY 11-7082 or 20 µM SP600125 respectively for 30 min prior to HIV-196ZM651 gp120 exposure. At 6, 12, and 24 h, the cells were collected and prepared for immunoblot analysis as described below.

Cytokine exposure experiments were initiated by aspirating the culture medium and adding fresh medium containing 0.5 ng/ml or 10 ng/ml TNF-α, 0.4 ng/ml or 10 ng/ml IL-1β, or 0.3 ng/ml or 10 ng/ml IL-6. These proinflammatory cytokines were selected since their expression is increased during HIV-1 associated immunological responses in the brain (Kaul et al., 2005). The lower concentration of each cytokine was selected based on the maximum level of TNF-α, IL-1β, or IL-6 secreted from primary cultures of rat astrocytes triggered with HIV-196ZM651 gp120 as determined by ELISA (Ronaldson and Bendayan, 2006), while the higher cytokine concentration (i.e., 10 ng/ml) is widely reported in the literature to induce a profound inflammatory response in vitro. Untreated cells in 5% horse serum, 5% fetal bovine serum containing culture medium were used as control. For experiments examining the involvement of NF-κB or JNKs on the regulation of Mrp1 in cells exposed to TNF-α, cultures were pre-treated with 1 µM SN50 or 20 µM SP600125 respectively for 30 min prior to triggering with TNF-α. At 6, 12, and 24 h, the medium was aspirated and the cells were collected for immunoblot analysis.

Treatment of primary cultures of rat astrocytes with cytokine neutralizing antibodies and HIV-196ZM651 gp120 were conducted by aspirating culture medium and replacing it with fresh medium containing the cytokine neutralizing antibody and 1.0 nM HIV-196ZM651 gp120. At 6, 12, and 24 h, the medium was aspirated and the cells were collected for immunoblot analysis. Concentrations for the neutralizing antibodies were determined from cytokine activity curves provided by the manufacturer. For these experiments, the following concentrations were selected since they were shown to completely neutralize the biological activity of their respective cytokine: 0.2 μg/ml TNF-α neutralizing antibody, 0.5 μg/ml IL-1β neutralizing antibody and 0.5 μg/ml IL-6 neutralizing antibody.

6.3.4 Immunoblot Analysis

Whole cell lysates from primary cultures of rat astrocytes and HeLa-MRP1 cells were prepared by exposing the cells to 1.0 ml of modified radioimmunoprecipitation assay (RIPA) buffer [50
mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0 mM ethylene glycol tetraacetic acid (EGTA), 1% (v/v) Nonident P-40, 0.25% (m/v) sodium deoxycholate, 0.1% (m/v) Sodium dodecyl sulfate (SDS), 200 µM phenylmethylsulfonyl fluoride (PMSF), 0.1% PI cocktail (Sigma-Aldrich)]. The cells were then gently rocked for 15 min at 4°C to allow lysis to occur. Cell suspensions were collected and centrifuged at 3000 g for 15 min at 4°C to remove cellular debris. Supernatants were then collected for immunoblot analysis. Protein concentration of the cell lysates was determined using Bradford’s protein assay.

For immunoblotting, 1 µg or 25 µg or 50 µg aliquots of cell lysates were mixed in Laemmli buffer and resolved on a 10% SDS-polyacrylamide gel. The gel was then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. Protein transfer was verified by Ponceau S staining. The membranes were blocked overnight at 4°C in Tris-buffered saline (15 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.05% (v/v) Tween-20 (TBS-T) and 5% (m/v) dry skim milk powder. Following six washes (5 min each) with TBS-T, the membrane was incubated with the appropriate primary antibody for 4 h at room temperature. MRP1/Mrp1 protein expression was assessed using the monoclonal MRPr1 antibody, which was raised against a bacterial fusion protein containing amino acids 194-360 of human MRP1 and its epitope was subsequently localized to amino acids 238-247 (Hipfner et al., 1999). MRPr1 does not cross-react with P-gp or MRP2-6 (Hipfner et al., 1999; Scheffer et al., 2000). Total and phosphorylated JNK protein expression was determined using the polyclonal total JNK/SAPK antibody and the polyclonal phosphorylated JNK/SAPK antibody respectively. The polyclonal total JNK/SAPK antibody was produced by the immunization of rabbits with a GSH transferase/human JNK2 fusion protein (Product Data Sheet, Cell Signaling Technology, 2008). The polyclonal phosphorylated JNK/SAPK antibody was produced by immunizing the animals with a fusion protein corresponding to the amino acids surrounding threonine 183 and tyrosine 185 of human JNK and is specific for JNK isoforms that are phosphorylated at these residues (Product Data Sheet, Cell Signaling Technology, 2008). Actin expression was detected using the monoclonal AC-40 antibody, which recognizes a conserved C-terminal epitope on all actin isoforms (Product Data Sheet, Sigma-Aldrich Canada, 2005). Following a second wash, the membranes were incubated for 1.5 h in the presence of anti-mouse (Serotec Inc., Raleigh, NC), anti-rat (Sigma-Aldrich), or anti-rabbit (Sigma-Aldrich) HRP-conjugated secondary antibodies (1:5000 dilution) in 5% milk
at room temperature. Protein bands were detected by enhanced chemiluminescence and exposed to X-ray film for 1 min. The MRP1-HeLa cell line was used as a positive control for MRP1/Mrp1.

6.3.5 Quantitative PCR

Total RNA was extracted from confluent monolayers of primary cultures of rat astrocytes treated with either HIV-1_{96ZM651} gp120 (1.0 nM) or TNF-α (10 ng/ml) for 6 h, 12 h or 24 h using TRIZOL reagent (Invitrogen). Extracted RNA was treated with amplification grade DNase I (Invitrogen) to remove contaminating genomic DNA. The concentration of RNA in each sample was quantified spectrophotometrically by measuring UV absorbance at 260 nm. The High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) was used to synthesize first-strand cDNA. Primer pairs for the rat Mrp1 gene (5'-AGAAGGAATGTGTTAAGTCGAGGAA-3' and 5'-CCTTAGGCTTGGTGGGATCTT-3') and the rat Cyclophilin B gene (housekeeping gene; 5'-GGAGATGGCACAGGAGGAA-3' AND 5'-GCCCGTAGTGCTCGCTT-3') were designed using Primer Express 3 software (Applied Biosystems) and were validated for specificity and efficacy using BioTaq universal rat normal tissue cDNA (BioTaq Inc., Gaithersburg, MD). Quantitative PCR (qPCR) was performed using SYBR Green Master Mix (Applied Biosystems) on an ABI 7900HT Fast Real-time PCR System (Applied Biosystems). The quantity of the target gene (i.e., Mrp1) was normalized to Cyclophilin B using the comparative CT method (ΔΔCT). Results were expressed as mean ± standard deviation (SD) of at least three separate experiments.

6.3.6 Enzyme-linked immunoabsorbant assay (ELISA)

An ultrasensitive ELISA kit for detection of rat TNF-α (Pierce Biotechnology, Rockford, IL) was used to measure secretion of cytokines from primary cultures of rat astrocytes treated with HIV-1_{96ZM651} gp120 in the presence or absence of SN50. Standard curves for TNF-α (0-2500 pg/ml) were generated using purified recombinant rat TNF-α and the assay was performed according to manufacturer’s instructions. Absorbance was read at 450 nm using a SpectraMax Plus384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentration of secreted TNF-α was expressed as pg/ml. All experiments reflect eight separate measurements obtained from different cell cultures on different days.
6.3.7 Functional studies

These studies were performed on confluent monolayers of rat astrocytes triggered with HIV-1$_{96ZM651}$ gp120 or with TNF-α (0.5 or 10 ng/ml) and grown on 24-well polystyrene plates (Becton-Dickinson) at an approximate density of 8 x 10$^4$ cells/well. Cells were washed and incubated at 37°C for 30 min in HBSS, pH 7.4, containing 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) and 0.01% bovine serum albumin. The cells were then incubated for the desired time with the cell permeable ester BCECF-AM (5 μM) in the presence or absence of SP600125 (20 μM). Since BCECF-AM is a known P-gp substrate (Bachmeier et al., 2004), all incubations were performed in the presence of 1.0 μM PSC833, an established P-gp inhibitor. At the end of each time point, the incubation medium was aspirated and the reaction was terminated with 1000 μl ice-cold phosphate buffer saline (PBS). The cells were then solubilized with 200 μl 1% Triton-X-100 for 30 min. BCECF cellular retention was measured using a fluorescent assay plate reader at an excitation wavelength of 505 nm and an emission wavelength of 535 nm. All samples were corrected for background fluorescence. Cellular BCECF content was standardized to cellular protein content (mg/ml) determined by the Bradford colorimetric method using bovine serum albumin (Sigma-Aldrich) as the standard. Cellular retention of BCECF was expressed as nanomoles per milligram of protein (nmol/mg protein).

6.3.8 Data Analysis

Each set of experiments was repeated at least three times in cells pertaining to different isolations. In an individual experiment, each data point represents quadruplicate trials. Results are reported as a mean ± SD from at least three separate experiments. To determine significance of transport inhibition, Student’s $t$-test was used for unpaired experimental data. For multiple comparisons, the test of repeated measures Analysis of variance (ANOVA) and the post hoc multiple-comparison Bonferroni $t$-test were used. A value of $p < 0.05$ was considered to be statistically significant.
6.4 Results

6.4.1 Effect of cytokines on Mrp1 protein expression

Our laboratory has previously reported increased cytokine secretion (i.e., TNF-α, IL-1β, IL-6) in primary cultures of rat astrocytes exposed to HIV-196ZM651 gp120 (Ronaldson and Bendayan, 2006). In addition, we demonstrated that cellular exposure to these cytokines decreased functional expression of the ABC transporter P-gp (Ronaldson and Bendayan, 2006); however, it was unknown whether TNF-α, IL-1β or IL-6 were involved in the regulation of other ABC transporters that are expressed in astrocytes. Therefore, we explored the role of these cytokines in the regulation of Mrp1 protein expression (Fig 6-1A). Mrp1 protein was detected using the monoclonal MRPr1 antibody, which has been shown to react with both human MRP1 and rat Mrp1 (Dallas et al., 2003). As expected, in the MRP1-HeLa cell line (the positive control), a single band was observed at approximately 190 kDa, a size previously reported for MRP1/Mrp1 (Hipfner et al., 1999). Mrp1 protein expression was increased up to 2.7-fold by 24 h in primary cultures of rat astrocytes treated with TNF-α but was not altered in cultures treated with either IL-1β or IL-6 (Fig 6-1B). Appropriate loading of each sample was confirmed by detection of a single band at approximately 43 kDa, which corresponds to actin. We did not observe any change in actin protein expression in response to TNF-α, IL-1β, or IL-6 treatment at any of the time points examined (data not shown).
Figure 6-1 Effect of cytokines on Mrp1 protein expression in primary cultures of rat astrocytes.

A. Primary cultures of rat astrocytes were treated with TNF-α (0.5 ng/ml; 10 ng/ml), IL-1β (0.4 ng/ml; 10 ng/ml), and IL-6 (0.3 ng/ml; 10 ng/ml) for 6 h, 12 h, and 24 h and Mrp1 expression was assessed by immunoblot analysis. Crude membrane preparations of primary cultures of rat astrocytes (25 μg) and MRP1-HeLa cells (1 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The blots were incubated with the monoclonal MRPI/Mrp1 antibody MRPr1 (1:500 dilution). Equal sample loading was confirmed by the detection of actin using the monoclonal antibody AC40 (1:500 dilution). Primary cultures of rat astrocytes not exposed to cytokines were used as a control. B. Densitometric analysis of Mrp1 protein in cultured rat astrocytes treated with TNF-α, IL-1β, or IL-6. Results (% control) are expressed as mean ± SD of three separate experiments. Asterisks indicate data points that are significantly different from control.
In order to confirm the involvement of these cytokines in altering Mrp1 expression, we measured Mrp1 protein expression in cultured rat astrocytes treated with HIV-196ZM651 gp120 and various cytokine neutralizing antibodies. In the presence of cytokine neutralizing antibodies only, Mrp1 protein expression was not significantly altered in our rat astrocyte cultures (Fig 6-2). We observed no change in Mrp1 expression when cultures were treated with HIV-196ZM651 gp120 and the TNF-α neutralizing antibody (Fig 6-2A & 6-2B). In contrast, Mrp1 protein expression was significantly increased in primary cultures of rat astrocytes treated with HIV-196ZM651 gp120 and the IL-1β or the IL-6 neutralizing antibody. These data suggest that TNF-α, but not IL-1β or IL-6, is involved in upregulation of Mrp1 protein expression.
Figure 6-2 Effect of cytokine neutralizing antibodies on Mrp1 protein expression in primary cultures of rat astrocytes treated with HIV-196ZM651 gp120. A. Primary cultures of rat astrocytes were treated with neutralizing antibodies for TNF-α (0.2 ng/ml), IL-1β (0.5 ng/ml), and IL-6 (0.5 ng/ml) in the presence or absence of 1.0 nM HIV-196ZM651 gp120 for 6 h, 12 h, and 24 h and Mrp1 expression was assessed by immunoblot analysis. Crude membrane preparations of primary cultures of rat astrocytes (25 μg) and MRP1-HeLa cells (1 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The blots were incubated with the monoclonal MRP1/Mrp1 antibody MRPr1 (1:500 dilution). Equal sample loading was confirmed by the detection of actin using the monoclonal antibody AC40 (1:500 dilution). Primary cultures of rat astrocytes not exposed to HIV-196ZM651 gp120 or to the cytokine neutralizing antibodies were used as a control. B. Densitometric analysis of Mrp1 protein in cultured rat astrocytes treated with HIV-196ZM651 gp120 and various cytokine neutralizing antibodies. Results (% control) are expressed as mean ± SD of three separate experiments. Asterisks indicate data points that are significantly different from control. NAb = Neutralizing Antibody.
6.4.2 Functional studies

In order to investigate whether increased Mrp1 mRNA and protein expression in response to TNF-α exposure resulted in altered Mrp-mediated transport activity, we measured cellular retention of BCECF, a fluorescein derivative and established Mrp1, Mrp2, Mrp4, and ABCG2 substrate (Bachmeier et al., 2004). Mrp2 expression was not detected in our primary cultures of rat astrocytes and Mrp4 expression was not altered in response to either HIV-196ZM651 gp120 treatment (Ronaldson and Bendayan, 2008) or TNF-α exposure (data not shown). Additionally, a previous study by our laboratory demonstrated that ABCG2 was expressed but not capable of efflux transport in primary cultures of rat astrocytes (Lee et al., 2007). Therefore, we hypothesized that any change in BCECF cellular retention would most likely correspond to an alteration in Mrp1-mediated transport activity. For these experiments, cells were grown as monolayers and incubated in the presence or absence of 0.5 ng/ml TNF-α or 10 ng/ml TNF-α for 24 h. The time course of BCECF (5 μM) cellular retention at 37°C (Fig 6-3) showed increasing accumulation until approximately 15 min. At this point, BCECF cellular retention decreases for the duration of the experiment, suggesting the presence of an active efflux process for this fluorescent substrate. In cultured rat astrocytes treated with 0.5 ng/ml TNF-α for 24 h, BCECF cellular retention was significantly decreased up to 2.4-fold (Fig 6-3), suggesting an increase in Mrp1 functional activity. BCECF cellular retention was also decreased up to 4.4-fold in cultures treated with 10 ng/ml TNF-α, implying that TNF-α increases Mrp1 functional activity in a concentration-dependent manner.
Figure 6-3 Effect of 24 h TNF-α exposure on the cellular retention of BCECF, a fluorescent Mrp substrate, by cortical rat astrocyte monolayers. BCECF (5 μM) accumulation was measured at 37°C in the presence of 0.5 ng/ml or 10 ng/ml TNF-α. Results are expressed as mean ± SD of three separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control.
6.4.3 Role of NF-κB on cytokine release and Mrp1 protein expression

NF-κB is a redox-regulated transcription factor that is known to be activated in cultured cells triggered by gp120 (Saha and Pahan, 2007). In addition, NF-κB-mediated signaling has been implicated in the regulation of ABC transporters such as P-gp in rat brain capillaries (Bauer et al., 2007); however, it is currently unknown if NF-κB signaling can regulate Mrp1 expression. Therefore, we investigated the possible involvement of NF-κB in the regulation of Mrp1 protein expression in primary cultures of rat astrocytes triggered with gp120. Control experiments showed that Mrp1 protein expression was increased in cultured astrocytes triggered with 1.0 nM HIV-196ZM651 gp120 but was unchanged in cultures treated with denatured (i.e., heat-inactivated) HIV-196ZM651 gp120 (Fig 6-4A). These data imply that a cellular response specific for the native conformation of HIV-196ZM651 gp120 is required for enhancement of Mrp1 expression.

Furthermore, these results also indicate that altered Mrp1 expression was not associated with low level endotoxin contamination in recombinant HIV-196ZM651 gp120 samples. Immunoblot analysis of primary cultures of rat astrocytes triggered with 1.0 nM HIV-196ZM651 gp120 in the presence and absence of SN50, a cell-permeable NF-κB inhibitory peptide, was performed. SN50 has been previously shown to specifically inhibit NF-κB nuclear translocation at a concentration of 10 µM or less (Lin et al., 1995), thus rendering it a good pharmacologic inhibitor of NF-κB mediated signaling processes. Using trypan blue exclusion, we observed that cell viability was not compromised by exposure to SN50 at concentrations up to 10 µM (data not shown). In cells triggered with HIV-196ZM651 gp120, Mrp1 expression was increased by 2.5-fold; however, Mrp1 protein expression was unchanged in cultures treated with HIV-196ZM651 gp120 and 1.0 µM SN50 at the time points examined (6, 12, or 24 h) (Fig 6-4B).
A.
Figure 6-4 Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and SN50, a peptidic NF-κB inhibitor. A: Immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-196ZM651 gp120 and with denatured HIV-196ZM651 gp120. Whole cell lysates (25 μg) from primary cultures of rat astrocytes were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution) and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control. B: Immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-196ZM651 gp120 in the presence of 1.0 μM SN50, a cell permeable NF-κB inhibitory peptide. Whole cell lysates (25 μg) from primary cultures of rat astrocytes were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF.
membrane. MRP1/Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution) and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.

In order to confirm the involvement of NF-κB signaling in the regulation of Mrp1 protein expression, we also conducted experiments in the presence and absence of BAY 11-7082, an established pharmacological NF-κB inhibitor. Similar to our results with SN50, Mrp1 protein expression was not significantly different from control in rat astrocyte cultures triggered with HIV-1<sub>196ZM651</sub> gp120 (24 h) in the presence of 5.0 μM BAY 11-7082 (Fig 6-5). Appropriate loading of each sample was confirmed by the detection of a single band at approximately 43 kDa, which corresponds to actin. Overall, these data provide evidence for involvement of NF-κB signaling in the regulation of Mrp1 expression in primary cultures of rat astrocytes triggered with HIV-1 viral envelope proteins.
Figure 6-5 Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and BAY 11-7082, a pharmacological NF-κB inhibitor. A: Immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-1<sub>96ZM651</sub> gp120 in the presence and absence of BAY 11-7082 (5 μM). Whole cell lysates (50 μg) from primary cultures of rat astrocytes were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Whole cell lysate from HeLa-MRP1 cells (1 μg) was used as a positive control. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution). B: Densitometric analysis of Mrp1 expression in primary cultures of rat astrocytes triggered with HIV-1<sub>96ZM651</sub> gp120 in the presence and absence of 5 μM BAY 11-7082. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.
Since gp120 treatment is known to stimulate cytokine release (Ronaldson and Bendayan, 2006), we investigated the role of NF-κB in TNF-α secretion in primary cultures of rat astrocytes triggered with gp120. Secretion of TNF-α was measured in rat astrocyte cultures treated with HIV-196ZM651 gp120 and SN50. Ultrasensitive ELISA analysis demonstrated increased TNF-α protein expression (p < 0.01) in cell culture supernatants from primary cultures of rat astrocytes triggered with 1.0 nM HIV-196ZM651 gp120 for 6, 12, and 24 h (table 6-1). In contrast, TNF-α release in astrocyte cultures treated with 1.0 nM HIV-196ZM651 gp120 in the presence of 1.0 μM SN50 was below the detection limit of the assay (i.e., less than 15 pg/ml). Previous work by our laboratory has shown that basal levels of TNF-α in our primary cultures of rat astrocytes are below the detection limit of the assay (Ronaldson and Bendayan, 2006). These observations suggest that TNF-α secretion from primary cultures of rat astrocytes triggered with HIV-196ZM651 gp120 is mediated by an NF-κB dependent mechanism.
Table 6-1 ELISA analysis of TNF-α secretion in cultured astrocytes treated with HIV-196ZM651 gp120.

<table>
<thead>
<tr>
<th>Time of Exposure</th>
<th>HIV-196ZM651 gp120 (1.0 nM)</th>
<th>HIV-196ZM651 gp120 (1.0 nM) + SN50 (1.0 µM)</th>
<th>HIV-196ZM651 gp120 (1.0 nM) + SP600125 (20 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>583.08 ± 22.58</td>
<td>BDL **</td>
<td>570.43 ± 49.28</td>
</tr>
<tr>
<td>12 h</td>
<td>483.90 ± 32.81</td>
<td>BDL **</td>
<td>490.54 ± 36.43</td>
</tr>
<tr>
<td>24 h</td>
<td>384.71 ± 34.28</td>
<td>BDL **</td>
<td>369.56 ± 33.29</td>
</tr>
</tbody>
</table>

**p < 0.01; Results are expressed as mean ± SD of eight separate measurements obtained from different cultures on different days. Statistical comparisons are between cultures treated with HIV-196ZM651 gp120 alone and cultures triggered with HIV-196ZM651 gp120 plus inhibitor; Cytokine values are expressed as pg/ml; BDL= Values that are below detection limit of the assay.
The above results imply that NF-κB mediated signaling is involved in release of TNF-α in cultured astrocytes triggered with HIV-1<sub>96ZM651</sub> gp120; however, these data were unable to discern if NF-κB is directly involved in the regulation of Mrp1 itself. In order to address this question, we pre-treated our astrocyte cultures with 1.0 µM SN50 followed by exposure to 0.5 ng/ml or 10 ng/ml TNF-α. Immunoblot analysis showed increased expression of Mrp1 in astrocyte cultures treated with 1.0 µM SN50 and 0.5 ng/ml TNF-α (2.4-fold) or 10 ng/ml TNF-α (2.6-fold) (Fig 6-6), suggesting that NF-κB does not directly regulate Mrp1 expression. Appropriate loading of each sample was confirmed by detection of a single band at approximately 43 kDa, which corresponds to actin. Taken together, these data indicate that NF-κB signaling processes are indirectly involved in the regulation of Mrp1 protein expression by triggering release of cytokines (i.e., TNF-α) in glial cells following exposure to HIV-1<sub>96ZM651</sub> gp120.
**Figure 6-6** Expression of Mrp1 in primary cultures of rat astrocytes treated with TNF-α and SN50, a peptidic NF-κB inhibitor. Immunoblot analysis of primary cultures of rat astrocytes triggered with TNF-α (0.5 ng/ml or 10 ng/ml) in the presence of 1.0 µM SN50. Whole cell lysates from primary cultures of rat astrocytes (25 µg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution) and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.
6.4.4 Role of JNKs on Mrp1 functional expression

Components of the MAPK pathway such as the JNKs have also been shown to be activated in response to HIV-1 viral proteins and/or inflammation (Chen and Thorner, 2007; Hayashi et al., 2006). Additionally, JNK isoforms may be involved in regulation of ABC membrane transporters (Hartz et al., 2008; Hayashi et al., 2006). Therefore, we investigated the involvement of JNKs in regulation of Mrp1 protein expression in primary cultures of rat astrocytes triggered with HIV-1 gp120. Immunoblot analysis of cultured astrocytes triggered with 1.0 nM HIV-1 gp120 in the presence or absence of 20 μM SP600125, an established JNK inhibitor, was performed. SP600125 is known to reversibly inhibit JNK signaling with IC50 values in the range of 40-90 nM (Bennett et al., 2001). Furthermore, SP600125 displays greater than 300-fold selectivity for JNK over related MAPKs (i.e., ERK1 and p38 MAPK) and 10-100-fold greater selectivity over other intracellular kinases (Bennett et al., 2001). Using the trypan blue exclusion method, we observed that cell viability was not altered in the presence of 20 µM SP600125 (data not shown). In our hands, we demonstrated that 20 μM SP600125 decreased total JNK phosphorylation in primary cultures of rat astrocytes triggered with TNF-α to a level that was not significantly different from control untreated cells (data not shown). Mrp1 protein expression was unchanged in cultures treated with HIV-1 gp120 and SP600125 at the time points examined (6, 12, or 24 h) (Fig 6-7), suggesting that JNKs may be involved in the regulation of this ABC transporter. Appropriate loading of each sample was confirmed by the detection of a single band at approximately 43 kDa, which corresponds to actin.
Figure 6-7 Expression of Mrp1 in primary cultures of rat astrocytes treated with gp120 and SP600125, a pharmacological JNK inhibitor. Immunoblot analysis of primary cultures of rat astrocytes triggered with HIV-1<sub>96ZM651</sub> gp120 in the presence of 20 µM SP600125. Whole cell lysates from primary cultures of rat astrocytes (25 µg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution) and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.
To determine the role of JNKs in the regulation of cytokine release, we measured the secretion of TNF-α in primary cultures of rat astrocytes treated with HIV-1<sub>96ZM651</sub> gp120 in the presence of SP600125. TNF-α release in astrocyte cultures treated with 1.0 nM HIV-1<sub>96ZM651</sub> gp120 in the presence of 20 μM SP600125 was not statistically different (p > 0.05) from TNF-α secretion in astrocyte cultures treated with 1.0 nM HIV-1<sub>96ZM651</sub> gp120 alone (table 6-1). These observations imply that JNK signaling is likely not involved in the release of TNF-α from primary cultures of rat astrocytes triggered with HIV-1<sub>96ZM651</sub> gp120.

In order to determine if JNK signaling was directly involved in regulation of Mrp1 protein expression, we pretreated our rat astrocyte cultures with 20 μM SP600125 followed by exposure to 0.5 ng/ml or 10 ng/ml TNF-α. Control experiments in the absence of SP600125 demonstrated increased Mrp1 protein expression in primary cultures of rat astrocytes exposed to 0.5 ng/ml TNF-α (2.5-fold) or 10 ng/ml TNF-α (2.6-fold) (Fig 6-8A). In contrast, no change in protein expression of Mrp1 was observed in astrocyte cultures treated with 20 μM SP600125 and 0.5 ng/ml TNF-α or 10 ng/ml TNF-α (Fig 6-8B). Appropriate loading of each sample was confirmed by detection of a single band at approximately 43 kDa, which corresponds to actin.
Figure 6-8 Expression of Mrp1 in primary cultures of rat astrocytes treated with TNF-α and SP600125, a pharmacological JNK inhibitor. A: Immunoblot analysis of primary cultures of rat astrocytes triggered with TNF-α (0.5 ng/ml or 10 ng/ml). Whole cell lysates from primary cultures of rat astrocytes (25 µg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution) and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control. B: Immunoblot analysis of primary cultures of rat astrocytes triggered with TNF-α (0.5 ng/ml or 10 ng/ml) in the presence of 20 µM SP600125. Whole cell lysates from primary cultures of rat astrocytes (25 µg) were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Mrp1 was detected using the monoclonal antibody MRPr1 (1:500 dilution) and relative levels of Mrp1 expression were determined by densitometric analysis. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control.
In order to investigate if pharmacological inhibition of JNK isoforms resulted in altered Mrp-mediated transport activity, we measured cellular retention of BCECF in cortical astrocyte monolayers triggered with HIV-1\textsubscript{96ZM651} gp120 or TNF-\(\alpha\) in the presence or absence of SP600125. For these experiments, cells were grown as monolayers and incubated with 1.0 nM HIV-1\textsubscript{96ZM651} gp120 or 10 ng/ml TNF-\(\alpha\) for 24 h. In cultures treated with the JNK inhibitor, SP600125 (20 \(\mu\)M) was added 30 min prior to HIV-1\textsubscript{96ZM651} gp120 or TNF-\(\alpha\) exposure. In cultured rat astrocytes treated with 1.0 nM HIV-1\textsubscript{96ZM651} gp120 or 10 ng/ml TNF-\(\alpha\), BCECF cellular retention was significantly decreased up to 2.4-fold (Fig 6-9). In contrast, BCECF cellular retention was not altered in rat astrocyte cultures treated with SP600125 and HIV-1\textsubscript{96ZM651} gp120 or with SP600125 and TNF-\(\alpha\). Control experiments demonstrated that 20 \(\mu\)M SP600125 itself did not affect cellular retention of BCECF (data not shown). Taken together, these data indicate that inhibition of JNK signaling processes attenuates the increase in Mrp1 functional expression observed in glial cells following exposure to HIV-1 viral proteins or proinflammatory cytokines.
**Figure 6-9** Effect of 24 h gp120 or TNF-α exposure on the cellular retention of BCECF, a fluorescent Mrp substrate, by cortical rat astrocyte monolayers. BCECF (5 μM) accumulation was measured at 37°C in cultured astrocytes treated with 1.0 nM HIV-1<sub>196ZM651</sub> gp120 or 10 ng/ml TNF-α in the presence and absence of SP600125, a specific pharmacological JNK inhibitor. Results are expressed as mean ± SD of three separate experiments, with each data point in an individual experiment representing quadruplicate measurements. Asterisks represent data points that are significantly different from control.
6.4.5 Effect of HIV-196ZM651 gp120 and TNF-α on Mrp1 gene expression

Since we observed increased protein expression of Mrp1 in cultured rat astrocytes triggered with gp120 or TNF-α, we sought to evaluate Mrp1 mRNA expression in cultured astrocytes exposed to these same mediators. Quantitative PCR analysis was used to measure the expression of Mrp1 mRNA in primary cultures of rat astrocytes treated with HIV-196ZM651 gp120 or TNF-α. Mrp1 mRNA was significantly increased (1.6-fold) in cultured astrocytes triggered with 1.0 nM HIV-196ZM651 gp120 for 6 h; however, Mrp1 expression was not altered in primary cultures of rat astrocytes exposed to HIV-196ZM651 gp120 for 12 h or 24 h (Fig 6-10A). Similarly, Mrp1 mRNA expression was increased in cells treated with 10 ng/ml TNF-α for 6 h (1.7-fold) but no change in Mrp1 expression was observed in primary cultures of rat astrocytes triggered with TNF-α for 12 h or 24 h (Fig 6-10B). Taken together, these data suggest that increased Mrp1 protein expression in cultured rat astrocytes triggered with either gp120 or TNF-α may result, at least in part, from increased expression of Mrp1 mRNA.
Figure 6-10 Effect of gp120 or TNF-α exposure on Mrp1 mRNA expression in primary cultures of rat astrocytes. Primary cultures of rat astrocytes were triggered with 1.0 nM HIV-196ZM651 gp120 (A) or 10 ng/ml TNF-α (B) for 6 h, 12 h, or 24 h. Mrp1 mRNA expression was measured using quantitative PCR analysis. Results (% control) are expressed as mean ± SD of four separate experiments. Asterisks indicate data points that are significantly different from control.
6.5 Discussion

ABC transporters (i.e., P-gp, Mrp1) are important determinants of xenobiotic permeation across brain barriers and brain parenchyma cellular compartments (i.e., astrocytes, microglia) (Ronaldson et al., 2008). This is particularly significant for treatment of HIV-1 infection because antiretroviral agents (i.e., HIV-1 PIs) are known substrates for P-gp and/or Mrp1 (Dallas et al., 2004a; Ronaldson and Bendayan, 2006; Williams et al., 2002), a factor that may limit the ability of these drugs to attain efficacious CNS concentrations. Until recently, ABC transporter functional expression had only been characterized in non-pathological (i.e., healthy) astrocyte cultures (Ronaldson et al., 2004a). In order to elucidate the role of brain pathologies on ABC transporter expression and/or activity, we implemented an in vitro model of an HIV-1 associated inflammatory response by triggering cultured astrocytes with HIV-196ZM651 gp120 (Ronaldson and Bendayan, 2006). This model was characterized by increased production and secretion of proinflammatory cytokines (i.e., TNF-α, IL-1β, IL-6) as determined by semiquantitative RT-PCR and ELISA respectively (Ronaldson and Bendayan, 2006).

Previous in vitro and in vivo studies have shown that cytokines (i.e., TNF-α, IL-1β, IL-6) can alter Mrp1 expression (Cherrington et al., 2004; Lee and Piquette-Miller, 2003). In the context of HIV-1 associated inflammation, Jorajuria and colleagues reported increased TNF-α and IL-6 production and increased expression of MRP1 mRNA in human monocyte-derived macrophages infected with HIV-1 BaL, an R5-tropic viral strain (Jorajuria et al., 2004). Using Spearman’s rank correlation test, these researchers concluded that MRP1 mRNA expression was not directly correlated with TNF-α or IL-6 production (Jorajuria et al., 2004); however, a causal relationship between cytokine secretion and altered MRP1 mRNA levels was not established. In our study, we have directly triggered primary cultures of rat astrocytes with proinflammatory cytokines. While we observed no change in Mrp1 expression in cultured astrocytes triggered with IL-1β or IL-6, Mrp1 expression was increased in the presence of TNF-α (2.7-fold). We further examined the role of these cytokines on Mrp1 protein expression by treating primary cultures of rat astrocytes with HIV-196ZM651 gp120 in the presence of TNF-α, IL-1β or IL-6 neutralizing antibodies. Our results indicate that Mrp1 expression was not altered in the presence of TNF-α neutralizing antibody but was significantly increased when IL-1β or IL-6 neutralizing antibodies were utilized. These data confirm that TNF-α is prominently involved in upregulation of Mrp1.
expression in our astrocyte cultures. Taken together with our previous publication (Ronaldson and Bendayan, 2006), these results provide evidence for the complex manner by which cytokines regulate ABC transporter expression. With respect to P-gp, we observed decreased expression mediated by IL-6 but increased expression mediated by TNF-α and IL-1β (Ronaldson and Bendayan, 2006), suggesting that multiple cytokine signaling pathways are involved in regulation of P-gp expression. In the present study, we demonstrate that Mrp1 is increased by TNF-α, but not by IL-1β or IL-6, suggesting that Mrp1 expression is regulated by a TNF-α mediated pathway during an inflammatory response.

In order to determine if increased Mrp1 protein expression correlated with enhanced activity, we used BCECF, an established Mrp substrate (Bachmeier et al., 2004). An important consideration is that BCECF is also a substrate for Mrp2, Mrp4 and ABCG2. Since these transporters were either not expressed (i.e., Mrp2), nor affected by HIV-196ZM651 gp120 or TNF-α treatment (i.e., Mrp4) or not functional (i.e., ABCG2) in our primary cultures of rat astrocytes (Lee et al., 2007; Ronaldson and Bendayan, 2008), we are able to conclude that any difference in BCECF efflux is most likely attributed to changes in Mrp1 activity. Our studies showed that TNF-α treatment reduced BCECF cellular retention in a concentration-dependent manner, which implies an increase in Mrp-mediated transport. These data are particularly intriguing in light of our previous study, which showed a significant decrease in P-gp functional expression in the same in vitro model (Ronaldson and Bendayan, 2006). Therefore, we propose that Mrp1 may play an enhanced role in antiretroviral drug transport during HIV-1 associated inflammatory responses. Changes in Mrp1 functional expression may be particularly relevant for HIV-1 PIs, which are substrates for MRP1/Mrp1 (Dallas et al., 2004a; Williams et al., 2002).

Intracellular signaling mechanisms responsible for gp120 effects in glial cells have not been clearly identified. Previous studies have indicated that NF-κB mediated signaling pathways are activated in response to gp120 exposure in cultured rat astrocytes (Saha and Pahan, 2007). Since it has been shown that NF-κB activation is associated with changes in expression of other ABC transporters such as P-gp (Bauer et al., 2007; Hayashi et al., 2005), we hypothesized that NF-κB may also be involved in Mrp1 regulation. In the present study, we show that increased Mrp1 expression induced by HIV-196ZM651 gp120 was attenuated by SN50, an NF-κB inhibitory peptide. Although SN50 was used primarily as an inhibitor of NF-κB nuclear import, it may also
affect nuclear translocation of other transcription factors. Using an immortalized human T-lymphocyte cell line, SN50 (210 µg/ml; 75 µM) was shown to inhibit nuclear import of multiple transcription factors including AP-1, NFAT, STAT1, and NF-κB (Torgerson et al., 1998). In contrast, studies in primary cultures of human peripheral blood-derived T-lymphocytes demonstrated that SN50 had no effect on nuclear translocation of AP-1 or NFAT at a concentration that was 5.6-fold lower than used by Torgerson and colleagues (Kolenko et al., 1999), suggesting that cross-talk with other signaling pathways occurs only at high concentrations of SN50. This corroborates data obtained in a murine fibroblast cell line (3T3), which showed that SN50 specifically inhibited NF-κB nuclear translocation at concentrations less than 10 µM (Lin et al., 1995). We used a much lower concentration of SN50 than any of these studies (i.e., 2.8 µg/ml; 1 µM), suggesting that our results reflect an inhibition of NF-κB with little contribution from other signaling pathways.

NF-κB activation is associated with production/secretion of cytokines such as TNF-α (Filipov et al., 2005). Therefore, we examined TNF-α release from primary cultures of rat astrocytes exposed to HIV-1<sub>96ZM651</sub> gp120 in the presence and absence of SN50. Indeed, pre-treatment with SN50 reduced TNF-α secretion to levels that were below ELISA detection limits (i.e., less than 15 ng/ml), suggesting involvement of NF-κB. When we treated our cultures with TNF-α in the presence of SN50, we observed a significant increase in Mrp1 protein expression, implying that NF-κB does not directly regulate Mrp1 expression. A recent study in LPS-treated mice deficient in IκB demonstrated a similar increase in hepatic Mrp1 mRNA levels as compared to LPS-treated wild-type mice (Lickteig et al., 2007). LPS treatment has been shown to induce the cellular release of proinflammatory cytokines that can alter the expression of ABC transporters including Mrp1 (Cherrington et al., 2004). Taken together with our present study, these data suggest that NF-κB activity is involved in the regulation of Mrp1 expression only by enhancing the release of TNF-α.

The cellular response to gp120 and/or cytokines involves a multiplicity of signaling pathways in addition to NF-κB. It has been previously shown that both gp120 and TNF-α can activate the MAPK pathway, in particular the JNKs (Barbin et al., 2001;Bodner et al., 2004). Other HIV-1 proteins (i.e., Tat) have been shown to up-regulate Mrp1 expression in primary cultures of murine astrocytes via a JNK-dependent mechanism (Hayashi et al., 2006). Therefore, we
investigated the role of the JNK pathway on regulation of Mrp1 expression in cultured astrocytes exposed to gp120 and/or TNF-α. Pharmacological inhibition of JNK signaling with SP600125 prevented upregulation of Mrp1 expression in HIV-196ZM651 gp120 triggered astrocyte cultures. Pre-treatment with SP600125 attenuated upregulation of Mrp1 in response to TNF-α exposure; however, SP600125 had no effect on HIV-196ZM651 gp120 induced release of TNF-α from rat astrocyte cultures. Furthermore, SP600125 prevented the increase in cellular BCECF efflux in cultures treated with HIV-196ZM651 gp120 or TNF-α. Our data corroborates the work of Hayashi and colleagues (2006) and implies that JNKs are involved, in part, in regulation of Mrp1 functional expression in glial cells exposed to HIV-1 viral proteins and/or inflammatory mediators (Fig. 6-11). Specifically, our results indicate that JNK phosphorylation and upregulation of Mrp1 occurs subsequent to NF-κB-mediated TNF-α release. Studies in human macrophages and microglia have demonstrated that JNK phosphorylation may also occur in response to gp120 binding to CCR5 (Yi et al., 2004). Since we did not observe a change in Mrp1 protein expression in cultured astrocytes treated with HIV-196ZM651 gp120 and the TNF-α neutralizing antibody, we can conclude that JNK phosphorylation resulting from the gp120-CCR5 interaction was not a confounding factor in our study.

Our data shows increased Mrp1 mRNA expression in cultured astrocytes triggered with HIV-196ZM651 gp120 or with TNF-α, suggesting that an HIV-1 inflammatory response may, in part, alter transcription of the Mrp1 gene. MAPK signaling cascades (i.e., JNK) are complex and may affect the expression of ABC transporter genes by the recruitment of transcription factors (i.e., AP-1, c-Jun) (Hartz et al., 2008; Shinoda et al., 2005; Zhou et al., 2006). Using chromatin immunoprecipitation, increased c-Jun binding to the MRP1 promoter was observed in human small cell lung cancer cell lines treated with the anticancer drug doxorubicin (Shinoda et al., 2005). Additionally, this study showed that SP600125 inhibited c-Jun binding, confirming the involvement of JNK signaling in MRP1 regulation. Although conducted in cancerous human cell culture systems, the hypotheses proposed in this study can be tested in healthy rodent cell culture systems because similar signaling pathways and/or transcription factors are expressed in both models. Some of these similarities include expression of JNK/AP-1 (Nair et al., 2008), JNK/c-Jun (Shinoda et al., 2005), and Nrf2 (Song et al., 2009). Furthermore, human MRP1 and rat Mrp1 are both regulated by a highly-conserved 100 nucleotide sequence in the promoter region, suggesting that regulatory mechanisms for both genes may be structurally and functionally
similar (Muredda et al., 2003). Nonetheless, studies are required to determine specific JNK-associated transcription factors involved in regulation of Mrp1 during cellular exposure to HIV-1\textsubscript{96ZM651} gp120 and/or proinflammatory cytokines.

In addition to inflammatory processes, oxidative stress is also involved in HIV-1 associated pathologies in the CNS. Our group has recently shown that gp120 treatment can lead to an oxidative stress response in primary cultures of rat astrocytes characterized by increased free radical production and increased oxidation of intracellular glutathione (Ronaldson and Bendayan, 2008). Our study demonstrated, for the first time, that gp120-induced oxidative stress increases Mrp1 functional expression in cultured glial cells (Ronaldson and Bendayan, 2008). Oxidative stress responses in astrocytes are associated with activation of several intracellular signaling mechanisms including NF-κB (Caccamo et al., 2005) and JNK MAPK (Chen et al., 2008). Additionally, Nrf2 signaling is also known to be activated in response to oxidative stress and may be involved in the regulation of ABC transporters such as MRP1/Mrp1 (Hayashi et al., 2003; Song et al., 2009). Clearly, the cellular response to HIV-1 viral proteins such as gp120 is complex and involves multiple pathophysiological responses (i.e., inflammation, oxidative stress). Future studies will delineate those cellular signaling processes that are activated by proinflammatory cytokines and those that are induced by oxidative stress in an effort to clarify mechanisms of HIV-associated pathophysiological processes as well as novel strategies for the treatment of brain HIV-1 infection.
Figure 6-11 Proposed mechanism of NF-κB and JNK signaling in glial cells during an HIV-1 associated inflammatory response. Brain HIV-1 infection is characterized by the presence of HIV-1 viral proteins such as gp120 within the brain parenchyma. In our model, R5-tropic gp120 (i.e., HIV-1_{96ZM651} gp120) directly binds to specific chemokine receptors (i.e., CCR5) expressed at the astrocyte cell surface (1). In turn, this activates NF-κB mediated signaling (2), leading to increased production and secretion of proinflammatory cytokines including TNF-α (3). Once secreted, TNF-α may bind to its receptor (TNFRs) that are expressed at the plasma membrane of astrocytes (4). The activation of TNFRs leads to increased phosphorylation (i.e., activation) of JNK isoforms (5). Our data show that these signaling events can lead to increased mRNA and protein expression of ABC membrane transporters such as Mrp1 (6). The end result of this signaling mechanism is an increase in Mrp1 transport activity. Overall, these data may point to a greater role for Mrp1 in antiretroviral drug resistance during an HIV-1 associated inflammatory response.
6.6 Acknowledgements

The authors thank Dr. Carolyn Cummins (Leslie Dan Faculty of Pharmacy) for providing the equipment and facility for the qPCR analysis. The authors also thank Ms. Manisha Ramaswamy and Mr. Vijay Rasaiah for excellent technical assistance.
Chapter 3

7 Regulation of P-gp by HIV-1 in primary cultures of human fetal astrocytes (HFAs)

This work is published and reproduced in this thesis with permission from John Wiley and Sons: T Ashraf, PT Ronaldson, Y Persidsky and R Bendayan. Regulation of P-glycoprotein by HIV-1 in primary cultures of human fetal astrocytes. 2011. Journal of Neuroscience Research. 89: 1773-1782.

Author contributions:

Research design: T Ashraf (first author), PT Ronaldson, Y Persidsky and R Bendayan (principle investigator).

Conducted experiments and data analysis: T Ashraf (figures 7-2C, 7-4, 7-5; table 1); PT Ronaldson (figures 7-1, 7-2B, 7-3; table 1) and Y Persidsky (Figure 7-2A)

Writing of the manuscript: T Ashraf (preparation of the manuscript drafts and responses to reviewer’s comments); PT Ronaldson (conceptual and editorial review of several manuscript drafts and responses to reviewer’s comments), Y Persidsky (editorial review) and R Bendayan (overall conceptual and editorial review of several manuscript drafts and responses to reviewer’s comments)
7.1 Abstract

P-gp, a drug efflux pump, is known to alter the bioavailability of antiretroviral drugs at several sites including the brain. We have previously shown that HIV-1 gp120 induces pro-inflammatory cytokine secretion and decreases P-gp functional expression in rat astrocytes, a cellular reservoir of HIV-1. However, whether P-gp is regulated in a similar way in human astrocytes is unknown. This study investigates the regulation of P-gp in an *in vitro* model of gp120-triggered human fetal astrocytes (HFAs). In this sytem, elevated levels of IL-6, IL-1β and TNF-α were detected in culture supernatants. Pretreatment with CCR5 neutralizing antibody attenuated cytokine secretion suggesting that gp120-CCR5 interaction mediated cytokine production. Treatment with gp120 (R5-tropic) resulted in reduced P-gp expression (64%) and function as determined by increased (1.6-fold) cellular accumulation of [3H]digoxin, a P-gp substrate. Exposure to R5 or R5/X4-tropic viral isolates also resulted in a downregulation in P-gp expression (75% and 90% respectively) and treatment with IL-6 also showed lower P-gp expression (50%). Moreover, IL-6 neutralizing antibody blocked gp120 mediated P-gp downregulation, suggesting that IL-6 is a key modulator of P-gp. Gp120 or IL-6 mediated downregulation of P-gp was attenuated by SN50 (an NF-κB inhibitor), suggesting involvement of NF-κB signaling in P-gp regulation. Our results suggest that, similarly to the case with rodent astrocytes, pathophysiological stressors associated with brain HIV-1 infection have a down-regulatory effect on P-gp functional expression in human astrocytes, which may ultimately result in altered antiretroviral drug accumulation within brain parenchyma during HIV-1 infection.
7.2 Introduction

Neuroinflammation is a common immune response associated with HIV-1 infection. The sequestration of HIV-1 in brain cellular reservoirs (i.e., astrocytes, microglia) may allow the virus to evade antiretroviral therapy, thereby prolonging HIV-1 associated inflammatory responses (i.e., production of pro-inflammatory cytokines such as TNF-α and IL-6, chemokines and other neurotoxins) (Alexaki et al., 2008; Persidsky and Gendelman, 2003). Brain autopsy samples from HIV-1 infected individuals and tissue samples from HIV-1 inoculated animal models confirm elevation of inflammatory mediators during progression of HIV-1 associated neuroinflammation (Persidsky et al., 1997; Persidsky et al., 1999). The release of circulatory viral proteins and cytokines in brain parenchyma often results in neurologic impairment and neuronal cell loss that are associated with cognitive and motor disorders in patients with prolonged HIV-1 infection (Ances and Ellis, 2007; Kaul, 2009).

Poor penetration of antiretroviral drugs across the BBB and subsequently into parenchymal cells remains a major obstacle in efficient suppression of HIV-1 infection in the brain (Ronaldson et al., 2008). Inadequate permeability of different antiretroviral drugs, in particular HIV-1 PIs and NRTIs, into different brain cellular compartments is primarily due to functional expression of ABC efflux transporters (P-gp, MRPs and BCRP). P-gp is encoded by the MDR gene, which has two isoforms in humans (i.e., MDR1, MDR2) and three isoforms in rodents (i.e., mdr1a, mdr1b, mdr2). P-gp substrates include various classes of antiretroviral drugs such as PIs (i.e., darunavir, ritonavir) and NRTIs (i.e., abacavir) (Kis et al., 2010). Mdr1a/1b knockout animal models show increased CNS accumulation of several PIs, which confirms a role for P-gp in antiretroviral drug permeability into the brain (Salama et al., 2005; Spitzenerberger et al., 2007). Localization of P-gp has been reported at the BBB in brain microvessel endothelial cells, adhesive pericytes and astrocytes (Bendayan et al., 2006). Furthermore, we have also confirmed localization of P-gp in a rat microglia cell line, in cultured rat astrocytes, in a rat brain endothelial cell line and in a human brain microvessel endothelial cell line (Bendayan et al., 2002; Lee et al., 2001b; Ronaldson et al., 2004a; Zastre et al., 2009).

Evidence in the literature suggest that P-gp functional expression in the brain is altered during HIV-1 infection. Increased P-gp immunoreactivity in glial cells has been reported in brain autopsy tissues from patients with HIVE (Langford et al., 2004; Persidsky et al., 2006b). It is not
yet conclusive if the induced P-gp expression observed in post-mortem brain tissue is due to the
disease manifestation itself or the therapeutic regimen. It is known that antiretrovirals, in
particular, HIV-PIs, through their interaction with orphan nuclear receptors (i.e., PXR) may play
an important role in the upregulation of P-gp expression (Chan et al., 2011; Chandler et al.,
2003; Zastre et al., 2009). Therefore, in vitro systems have been used to delineate the cell-specific
effect of viral proteins and cytokines. Hayashi et al. have reported HIV-1 viral protein Tat
induced upregulation of P-gp expression in both murine brain microvessel endothelial cells and
astrocytes (Hayashi et al., 2005). Our group has demonstrated that both gp120 and IL-6 can
decrease P-gp expression in primary cultures of rat astrocytes, whereas, TNF-α or IL-1β
exposure results in an enhancement in P-gp expression. Due to the expression of ABC drug
transporters, astrocytes may act as a secondary barrier to drug permeability within brain
parenchyma, thereby, resulting in altered drug distribution in the brain extracellular fluid (Lee et
al., 2001a). Since drug efflux transporters such as P-gp are known to limit the uptake of
antiretroviral drugs in astrocytes, the upregulation or downregulation of P-gp along with other
ABC transporters can significantly alter the availability of these drugs within brain parenchyma.

Currently, much interest has focused on studying intracellular signaling systems/ transcription
factors that regulate P-gp. For example, a recent study showed that the transcription factor NF-
κB regulates TNF-α-induced upregulation of mdr1b promoter activity in a rat brain endothelial
cell line (Yu et al., 2008). Although these studies provide insight on P-gp regulation in rodent or
murine astrocytes, regulation of P-gp in human astrocytes in the context of HIV-1 infection has
not yet been characterized. Therefore, this study aims to determine if gp120 can induce an
immune response in primary cultures of human fetal astrocytes and examines how P-gp protein
expression is regulated by viral isolates, gp120 and IL-6.
7.3 Materials and Methods

7.3.1 Reagents

HIV-1\textsuperscript{96ZM651} gp120 full-length protein (subtype C; R5-tropic), rabbit polyclonal anti-CCR5 and anti-CXCR4 were obtained from the NIH AIDS Research and Reference Reagent program (Bethesda, MD). HIV-1\textsubscript{ADA} gp120 (subtype B; R5-tropic) full-length protein was purchased from Immunodiagnostics (Woburn, Ma). Murine monoclonal C219 antibody against P-gp was purchased from ID labs (London, ON, Canada). Murine monoclonal AC-40 antibody against actin, horseradish peroxidase conjugated anti-mouse, human IL-6, CXCR4 and CCR5 neutralizing antibody were obtained from Sigma-Aldrich (Oakville, ON, Canada). IL-6-neutralizing antibody was purchased from R&D systems (Minneapolis, MN). NF-κB inhibitory peptide, SN50, was purchased from EMD Biosciences (La Jolla, CA). [\textsuperscript{3}H]Digoxin (Specific activity 23.5 Ci/mmol), a substrate for P-gp was purchased from PerkinElmer Life and analytical Sciences (Boston, MA).

7.3.2 Primary Cultures of HFAs

Human fetal brain tissue samples were collected from consenting patients undergoing elective pregnancy termination (between 12 to 14 weeks of gestation age). Ethics approval was obtained from University Health Network (Toronto, ON, Canada). Primary cultures of HFAs were established according to previously published protocol (Hammond et al., 2002) with few modifications. Briefly, tissue was collected in ice-cold medium (DMEM supplemented with 5% FBS and 5% penicillin/streptomycin). After removal of meninges, tissue was triturated until disaggregated. The cell suspension was then centrifuged and re-suspended in initial growth medium containing DMEM supplemented with 20% FBS and 0.025% penicillin/streptomycin. Cells were distributed in 75 cm\textsuperscript{2} tissue culture flasks and incubated overnight at 37°C under 5% CO\textsubscript{2} and 95% air so that viable cells can adhere to culture flasks. The medium was then replaced with feeding medium (DMEM with 10% FBS and 0.025% penicillin/streptomycin) and cells were grown as monolayer until confluence. HFAs were characterized by the detection of vimentin, a biochemical marker for fetal astrocytes.
7.3.3 Isolation of CCR5 HIV-1 ADA and CCR5/CXCR4 HIV-1 89.6 viral isolates

HIV-1 89.6, a macrophage/T-lymphocyte dual-tropic (CCR5 and CXCR4-tropic) viral isolate, was obtained from the AIDS Research and Reference Reagent program, National institute of Allergy and infectious Diseases. HIV-1 ADA, a macrophage-tropic (CCR5-tropic) strain, was isolated from PBMCs from an HIV-1 infected patient. Infected monocytes were cultured and maintained following previously published protocols (Ricardo-Dukelow et al., 2007).

7.3.4 Treatment with HIV-1, HIV-1 gp120 and IL-6

Confluent HFA monolayers were treated with either CCR5 HIV-1 ADA or CCR5/CXCR4 HIV-1 89.6 virus (100x concentrated stocks; reverse transcriptase activity 38.5 and 29.1 cpm/mlx105 day) for 24h. Similarly, HFAs were treated with HIV-1\textsubscript{96ZM651} gp120 (1.0 nM) in the presence or absence of CXCR4 or CCR5 neutralizing antibodies (1mg/ml) for 6h and 24h. Additionally, cells were treated with IL-6 (0.5 ng/ml or 10 ng/ml) or HIV-1\textsubscript{96ZM651} gp120 (1.0 nM) for desired time (6h, 12h and 24h) in the presence or absence of SN50 (1.0 µM), an NF-κB inhibitory peptide.

7.3.5 ELISA analysis

IL6, IL-1β and TNF-α secretion from cultured HFAs in response to HIV-1\textsubscript{96ZM651} gp120 (1.0 nM) were measured using commercially available ultrasensitive ELISA kits according to the manufacturers protocol (Pierce Biotechnology, IL). Standard curves for all three cytokines (0-1000 pg/ml) were generated using the appropriate recombinant human cytokines. For the IL-6 and IL-1β kits, the detection level ranged from 10.2 to 400 pg/ml and for the TNF-α kit, it ranged from 15.6 to 1,000 pg/ml.

7.3.6 Immunoblot analysis

Protein expression of P-gp, CXCR4 and CCR5 were determined by SDS-PAGE according to previously published protocols (Ronaldson et al., 2004b). Briefly, whole cell lysates were isolated using the RIPA buffer. Proteins (50µg) were resolved by SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were blocked overnight in TBS containing 0.1% twin and 5% skim milk. Then blots were incubated with appropriate primary
antibody followed by HRP-conjugated secondary antibody. C219 (1:500), AC40 (1:300), anti-CXCR4 (1:1000) and anti-CCR5 (1:1000) antibodies were used to detect P-gp, actin, CXCR4 and CCR5, respectively. Finally, proteins bands were detected using enhanced chemiluminescence kit. Densitometric analysis was performed using the software AlphaDigiDoc RT2 to quantify relative protein expression.

7.3.7 Transport assay

Primary cultures of HFAs were seeded on 48-well plates and accumulation of $[^3]H$ digoxin was measured following previously published protocols (Ronaldson et al., 2006). Briefly, cells were washed and incubated at 37°C for 30 min in HBSS, pH 7.4, containing 10 mM HEPES and 0.01% bovine serum albumin. The cells were then incubated for the desired time with $[^3]H$ digoxin (100nM; Specific activity 23.5 Ci/mmol). At the end of each time point, the incubation medium was aspirated, and the reaction was terminated with 1000 μl of ice-cold PBS. The cells were then solubilised with 200 μl of 1% Triton-X-100 for 30 min. Radioactivity was measured by standard liquid scintillation counting. Cellular protein content was determined by the Bradford colorimetric method using bovine serum albumin as a standard.

7.3.8 Data analysis

Each experiment was repeated at least three times on cultured astrocytes isolated from different tissue. Student’s $t$-test was used to determine statistical significance between two groups. Multiple comparisons were performed using ANOVA and Bonferroni’s post-hoc analysis. A $p$ value less than 0.05 was considered as statistically significant.
7.4 Results

7.4.1 Interaction of viral protein gp120 (R5-tropic) with chemokine receptor and pro-inflammatory cytokine secretion

To characterize the inflammatory response mediated by gp120 in human astrocytes, we exposed HFAs to HIV-1 gp120 (R5-tropic strain) and observed a significant increase in the secretion of various pro-inflammatory cytokines (i.e., IL-6, IL-1β, TNF-α) (Table 7-1). When primary HFA cultures were exposed to gp120 in the presence of neutralizing antibodies directed against CXCR4 and CCR5, the CCR5 neutralizing antibody significantly decreased gp120 induced secretion of all three cytokines examined whether administered alone or in conjunction with CXCR4 neutralizing antibody. In contrast, administration of HIV-1 gp120 and CXCR4 neutralizing antibody failed to affect gp120 mediated cytokine secretion.
Table 7-1 Pro-inflammatory cytokine secretion in primary cultures of HFAs treated with HIV-1<sub>96ZM651</sub> gp120

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-6 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6h</td>
<td>24h</td>
<td>6h</td>
</tr>
<tr>
<td>Untreated</td>
<td>_</td>
<td>182.36 ± 26.83</td>
<td>_</td>
</tr>
<tr>
<td>gp120</td>
<td>431.57 ± 73.09***</td>
<td>336.33 ± 54.32***</td>
<td>679.50 ± 31.80***</td>
</tr>
<tr>
<td>CXCR4 Nab ± gp120</td>
<td>452.40 ± 69.99***</td>
<td>347.52 ± 65.75***</td>
<td>735.85 ± 108.93***</td>
</tr>
<tr>
<td>CCR5 Nab ± gp120</td>
<td>204.90 ± 18.55***</td>
<td>164.20 ± 10.59</td>
<td>12.09 ± 4.84</td>
</tr>
<tr>
<td>CXCR4 &amp; CCR5 Nab ± gp120</td>
<td>189.65 ± 19.29***</td>
<td>167.59 ± 17.32</td>
<td>19.91 ± 13.04</td>
</tr>
</tbody>
</table>

*** p<0.001; Nab = neutralizing antibody; Data points are expressed as mean ± SD of 8 separate measurements obtained from different cultures. For the 24h treatments, statistical comparisons are made between untreated controls and treated groups. For the 6h treatments, all the values are compared to the minimal detection limit of the respective kit. For the IL-6 and IL-1β kits, the detection level ranged from 10.2 to 400 pg/ml and for the TNF-α kit, it ranged from 15.6 to 1,000 pg/ml.
7.4.2 Effect of R5/X4 and R5-tropic viral isolates on P-gp protein expression

It is currently unknown whether interaction of intact HIV-1 virus with chemokine receptors in astrocytes can modify functional expression of drug transporters such as P-gp. In intact HIV-1 viral isolates, gp120 is expressed at the viral envelope and mediates attachment to chemokine receptors expressed at the surface of target cells. We detected protein expression of both CXCR4 and CCR5 in our HFA cultures (Figure 7-1).

![Immunoblot analysis of CXCR4 and CCR5 in primary cultures of HFAs.](image)

**Figure 7-1** Immunoblot analysis of CXCR4 and CCR5 in primary cultures of HFAs. Whole cell lysates (50 µg) from primary cultures of HFAs, 3T3-CXCR4 and 3T3-CCR5 cells were resolved on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane. Cell lysates prepared from 3T3-CXCR4 and 3T3-CCR5 cells were used as positive controls. CXCR4 and CCR5 were detected using the appropriate polyclonal antibody (anti-CXCR4, 1:1000 dilution; anti-CCR5, 1:1000 dilution).
In order to test if HIV-1 can alter P-p expression, primary cultures of HFAs were exposed to R5-tropic and X4/R5-tropic viral isolates. Since R5-tropic viruses are known to predominate in the brain, we used HIV-1 ADA isolates. We also utilized HIV-1 89.6, an X4/R5 viral isolate, to test the effect of dual tropism on P-gp expression. Exposure to either X5-tropic or X4/R5-dual-tropic viral isolates for 24h resulted in decreased \((p<0.001)\) P-gp expression by up to 75% and 90% respectively (Figure 7-2A).

7.4.3 Effect of gp120 and IL-6 on P-gp protein expression

We have previously shown that gp120 exposure can significantly decrease P-gp protein expression in primary cultures of rat astrocytes. However, it was unknown if gp120 had a similar effect on P-gp expression in human astrocytes. Immunoblot analysis showed that HIV-1 gp120 treatment resulted in a time dependent decrease in P-gp protein expression (approximately 64% or 2.8-fold after 24h) compared to untreated cells (Figure 7-2B). No significant change in protein expression was observed after 6h or 12h of treatment.
Figure 7-2 Immunoblot and densitometric analysis of P-gp in primary cultures of HFAs after exposure to (A) either CCR5-tropic HIV-1 ADA or CCR5/CXCR4 dual-tropic HIV-1 89.6 viral isolates, (B) 1.0 nM gp120 or (C) IL-6 (0.5 ng/ml or 10 ng/ml). Whole cell lysates (50 µg) from primary cultures of HFAs were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. MDR1 overexpressing cell lines (MDCK-MDR1 and MDA-MDR1) were used as positive controls. P-gp was detected using the monoclonal antibody C219 (1:500 or 1:300 dilution) and actin was detected using AC40 antibody (1:500 dilution). Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control (***p<0.001; *p<0.05).
Decreased P-gp protein expression correlated with a reduction in P-gp mediated drug transport activity. This was demonstrated by an increase in cellular [\(^3\)H]digoxin accumulation, an established substrate for P-gp, in gp120-treated HFA cultures as compared to untreated controls (Figure 7-3).

**Figure 7-3** Accumulation of [\(^3\)H]digoxin by HFAs in the presence or absence of gp120. [\(^3\)H]digoxin (100 nM) accumulation was measured at 37°C in cells treated with 1.0 nM gp120. Results are expressed as mean ± SD of three separate experiments and each data point represents quadruplicate measurements. Asterisks (*) represent data points that are significantly different from control (p<0.05).
Previously, we have demonstrated that IL-6 was a key regulator of P-gp expression in rat astrocytes. Therefore, we investigated the effect of this cytokine in human astrocytes. IL-6 (0.5 ng/ml and 10 ng/ml) decreased P-gp expression up to 50% after 12h exposure (Figure 7-2C). However, P-gp protein expression was not altered by longer exposure up to 48h (Data not shown). In the presence of IL-6-neutralizing antibody, P-gp protein expression was not significantly decreased by HIV-1 gp120 as compared to HFAs treated with only HIV-1 gp120 (Figure 7-4).
Figure 7-4 Immunoblot and densitometric analysis of P-gp in primary cultures of HFAs treated with 1.0nM gp120 in the presence of 0.5µg/ml IL-6 neutralizing antibody (NAB). Whole cell lysates (50 µg) from primary cultures of HFAs were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. An MDR1 overexpressing cell line (MDA-MDR1) was used as positive control. P-gp was detected using the monoclonal antibody C219 (1:300 dilution) and actin was detected using AC40 antibody (1:500 dilution). Results are expressed as mean ± SD of three separate experiments. Asterisks (*) represent data points that are significantly different from control (p<0.05).
7.4.4 Involvement of NF-κB in the regulation of P-gp expression

NF-κB signaling has been reported to play a role in inflammation-mediated regulation of P-gp. However, the role of this pathway in regulating P-gp expression in human astrocytes has not been demonstrated. Using immunoblot analysis, we investigated whether NF-κB can regulate P-gp protein expression in HFAs exposed to gp120 or IL-6. Treatment with gp120 or IL-6 resulted in a significant decrease in P-gp expression (64% and 50%, respectively). However, in the presence of SN50, a peptidic inhibitor of NF-κB nuclear translocation, P-gp protein expression remained unchanged when exposed to gp120 or IL-6 in HFAs, suggesting a role for this transcription factor in P-gp downregulation (Figure 7-5).
Figure 7-5 Immunoblot and densitometric analysis of P-gp in primary cultures of HFAs treated with (A) 1.0nM gp120 or (B) IL-6 (0.5 or 10ng/ml) in the presence of NF-κB inhibitory peptide, SN50 (1.0 μM). Whole cell lysates (50 µg) from primary cultures of HFAs were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. An MDR1 overexpressing cell line (MDCK-MDR1) was used as positive control. P-gp was detected using the monoclonal antibody C219 (1:500 dilution) and actin was detected using AC40 antibody (1:500 dilution). Results are expressed as mean ± SD of three separate experiments. Asterisks (*) represent data points that are significantly different from control (p<0.05).
7.5 Discussion

Pharmacotherapy of HIV-1 brain infection is limited. This, in part, is attributed to functional expression of ABC drug transporters such as P-gp actively pumping several xenobiotics including antiretroviral drugs out of several cellular compartments. Since astrocytes in brain parenchyma are able to harbour latent HIV-1 infection (Gorry et al., 2003), expression of P-gp in these cells is a major determinant of successful antiretroviral drug cellular permeability and suppression of viral infection. Since the regulation of P-gp expression by HIV-1 in human astrocytes had not been investigated prior to the present study, we investigated the effect of HIV-1 isolates on P-gp protein expression in an in vitro model of HFAs. In our primary cultures of HFAs, we observed a significant decrease in P-gp protein expression in the presence of R5-tropic and R5/X4-tropic HIV-1 viral isolates suggesting a downregulatory effect of HIV-1 on P-gp expression. Previously, Gollapudi and Gupta reported altered expression of P-gp in a HIV-1 infected T-cell line and in a monocytic cell line. In contrast to our findings, they reported upregulation of P-gp expression in their leukocytic cell systems when subjected to HIV-1 infection (Gollapudi and Gupta, 1990). Compared with T-cells or monocytes, astrocytes do not harbour productive HIV-1 infection and utilize different mechanisms of HIV-1 viral entry and sequestration (Boutet et al., 2001; Liu et al., 2004; Sabri et al., 1999). Therefore, it is possible that regulation of P-gp expression by HIV-1 is also cell-type specific, which may account for the apparent discrepancies between our present study and the work of Gollapudi and Gupta.

Shed viral proteins are also known to alter expression of ABC transporters. For example, viral protein Tat-induced P-gp expression has been reported both in cultured brain microvessel endothelial cells and in astrocytes (Hayashi et al., 2005), whereas, we have previously demonstrated that gp120 exposure can significantly downregulate P-gp protein expression (4.7-fold) resulting in increased accumulation of saquinavir, an antiretroviral drug, in primary cultures of rat astrocytes (Ronaldson et a., 2006). HIV-1 Tat is an accessory protein that is critical for viral replication, whereas, gp120 is a structural protein that is part of the viral envelope and is essential for the viral binding to host cells that initiates HIV-1 cellular entry process. The mechanism of action of both proteins are different. Tat is known to cross the cell membrane and interact with transcription factors within a cell (Mahlknecht et al., 2008; Sune and Garcia-Blanco, 1995), whereas, gp120 is known to interact with chemokine receptors on the cell surface.
Therefore, it is anticipated that these proteins may activate signaling pathways differently and in turn, have different effects on P-gp expression. In our present study, we further demonstrate that gp120 exposure results in a 2.8-fold decrease in P-gp protein expression in human astrocytes. As expected, the magnitude of P-gp downregulation is less profound in HFAs exposed to viral protein as compared to cells exposed to R5-tropic and dual-tropic HIV-1 viral isolates. In our hands, exposure to gp120 also results in a significant reduction in P-gp function as shown by increased accumulation of digoxin (approximately 1.6-fold in gp120 treated cells compared to the control). Although the decrease in P-gp protein expression is less profound in HFAs than in cultured rat astrocytes (2.8-fold versus 4.7-fold), the functional activity of P-gp was downregulated to a similar degree in both models (1.5-fold increase in digoxin accumulation in rat astrocytes versus 1.6-fold increase in HFAs) (Ronaldson and Bendayan, 2006). The comparable functional loss of P-gp in both human and rat astrocytes in the presence of gp120 suggests that although the effect of this viral protein on the P-gp expression is different between two models, the effect on drug transport activity remains similar.

In this study, we have further characterized gp120 mediated cytokine secretion in HFAs. It is known that during HIV-1 infection, secreted gp120 can interact with microglia/macrophages as well as astrocytes in the CNS and induce secretion of neurotoxic cytokines (Kaul and Lipton, 2006). Transgenic mice expressing gp120 show activation of glial cells and neuronal damage suggesting a significant role for this viral protein in HIV-1 mediated neuroinflammation (Toggas et al., 1994). Previously, gp120 triggered cytokine secretion (IL-6, IL-1β and TNF-α) has been characterized in primary cultures of rat astrocytes. Here, we have characterized cytokine secretion by gp120 in human astrocytes. Our findings show that R5-tropic gp120 induced secretion of IL-6, IL-1β and TNF-α in HFAs indicating that an inflammatory response can be triggered by gp120 in these cells. Neutralization of cell surface CCR5 chemokine receptor significantly diminished gp120-mediated cytokine production suggesting that interaction of gp120 with CCR5 is responsible for cytokine release in these cells. Our findings in HFAs are in agreement with previously published findings in a rodent model and emphasize that the gp120-CCR5 interaction is a critical event in neuroinflammatory signaling within human brain parenchyma.
Ample literature evidence suggests alteration of P-gp expression by pro-inflammatory cytokines. In human hepatocytes, IL-6 exposure resulted in a reduced MDR1 gene expression and P-gp activity (Lee and Piquette-Miller, 2001). Recently, an IL-6 mediated decrease in P-gp expression has been reported in cultured human brain microvessel endothelial cells further supporting the fact that P-gp expression can be altered by this cytokine (Poller et al., 2010). In rodent astrocytes, IL-6 treatment resulted in a continuous and profound decrease in P-gp expression from 6h to 24h (8.9-fold after 24h). Comparison of P-gp expression following IL-6 exposure in different cells suggests that this cytokine may have a down-regulatory effect on P-gp expression. Accordingly, treatment with IL-6 also results in a time-dependent decrease in P-gp protein expression in cultured HFAs. P-gp protein expression is 50% (2-fold) downregulated after 12h of treatment with either low or high concentration of IL-6. However, the downregulatory effect of IL-6 is lost during longer exposure (24h) and P-gp protein expression returned to the control levels. Time dependent regulation of P-gp by other cytokines has been reported previously. Bauer et al. have shown that TNF-α induced regulation of P-gp is time-dependent where a transient exposure to TNF-α caused a decrease in P-gp expression and a longer exposure increased the expression of this transporter in rat brain capillaries (Bauer et al., 2007). Compared to the profound downregulation (8.9-fold) previously reported in rodent astrocytes (Ronaldson and Bendayan, 2006), the IL-6 mediated decrease in P-gp expression in HFAs is modest (2-fold). This finding may be explained by the difference in endogenous levels of IL-6 between the culture supernatants of rat and human astrocytes. IL-6 was undetectable in primary cultures of rat astrocytes. Although previous studies reported no evidence of IL-6 presence in untreated cultured HFAs (Lee et al., 1993), we detect IL-6 in HFA culture supernatant (approximately 182.36 pg/ml). Astrocytes are known to be the primary source of IL-6 cytokine during inflammation; however, elevated endogenous levels of IL-6 in HFAs may be related to the prenatal phenotype of these cells. Previously, a beneficial role of IL-6 has been reported in neuronal protection implying that endogenous IL-6 secreted from HFAs may have a neuroprotective role during fetal brain development (Gadient and Otten, 1997). Since the HFAs are continuously exposed to endogenous IL-6 levels, it is likely that the IL-6 mediated decrease in P-gp expression may substantially differ between human and rat astrocytes.
We have further examined the role of IL-6 in regulating P-gp expression by treating cultured HFAs with HIV1 gp120, in the presence of IL-6-neutralizing antibody. Our results show that P-gp protein expression was not significantly altered in these conditions confirming that IL-6 secretion is primarily responsible for the gp120-mediated downregulation observed in HFAs. These findings further support our findings in rodent astrocytes where IL-6 was also the principal cytokine responsible for P-gp downregulation (Ronaldson and Bendayan, 2006).

Viral proteins and/or cytokines are known to activate intracellular signaling pathways (Kaul et al., 2005; Sluss et al., 1994). Few studies have reported the activation of NF-κB in response to gp120 in various cell systems including astrocytes (Saha and Pahan, 2007). Furthermore, NF-κB signaling is known to be involved in ABC drug transporters regulation. Using rat brain capillaries, Bauer et al. demonstrated that alteration of P-gp expression by TNF-α is NF-κB dependent. Previous work by our group showed that NF-κB is involved in the regulation of Mrp1, another drug efflux transporter, in gp120 treated cultured rat astrocytes, by inducing TNF-α secretion (Ronaldson et al., 2010). However, the role of NF-κB in P-gp regulation in HFAs triggered with gp120 or IL-6 has not been elucidated. Therefore, we investigated the involvement of NF-κB in our HFA model by inhibiting this transcription factor using the inhibitory peptide, SN50. In HFAs, inhibition of NF-κB attenuated both gp120 and IL-6 mediated decreases in P-gp expression whereas inhibition of this transcription factor did not affect endogenous P-gp expression. These findings demonstrate that NF-κB is involved in the downregulation of P-gp by gp120 or IL-6 in HFAs.

To date, a limited number of studies have examined the expression of ABC transporters in adult human brain and none of these studies have investigated P-gp expression in HIV-1 infected treatment-naive patients. Due to the limitation in obtaining appropriate controls (i.e., treatment naive, age-matched), it is still unclear how HIV-1 associated neuroinflammation affects the functional expression of these transporters in adult human brain. Therefore, we have chosen to use an in vitro system to determine the effect of viral proteins and cytokines in HFAs. Our findings demonstrate for the first time that HIV-1 isolates, gp120 and/or IL-6 exposure can downregulate P-gp protein expression in primary cultures of HFAs and that NF-κB signaling pathway is involved in this regulation. The present study performed in human astrocytes validates our previous data obtained in primary cultures of rat astrocytes by showing that gp120
induced inflammatory response and loss of P-gp function exist and remain comparable in both models. Furthermore, our results suggest that astrocytes may be involved in antiretroviral drug sequestration within brain parenchyma during HIV-1 infection. The availability of antiretroviral drugs in astrocytes, in turn, may aid in limiting brain viral infection by preventing the latent virus from becoming infectious. However, in addition to P-gp, other transporters of the ABC family are also known to be involved in antiretroviral drug transport. For example, MRPl can transport several HIV-PIs used in antiretroviral therapy (Dallas et al., 2004a; Ronaldson et al., 2008). Using rodent astrocytes, we have previously demonstrated that gp120 exposure results in an upregulation of Mrpl expression in primary cultures of rat astrocytes (Ronaldson et al., 2010). Although we have not yet investigated the regulation of MRPl or other MRP isoforms involved in antiretroviral drug transport in human astrocytes, the regulation of these transporters during HIV-associated neuroinflammatory response will also contribute towards the overall antiretroviral drug permeability in astrocytes. Further work is needed to clarify the effect of downregulated P-gp in astrocytes on antiretroviral therapy efficacy. Overall, the results of our work emphasize that rodent astrocytes can be used as an effective glial culture model for understanding and elucidating mechanisms involved in inflammation and drug resistance in HIV-1 infection of human astrocytes.

7.6 Acknowledgements

The authors thank Dr. Michelle Farrugia (Mount Sinai Hospital, Toronto, ON) for her remarkable assistance in obtaining human fetal brain tissue.

This work is supported by the Canadian Institutes of Health Research (CIHR, MOP-56976 to RB), the Ontario HIV Treatment Network (OHTN to RB) and from the National Institute of Health (RO1 MH65151, RO1 AA017398 to YP).
Chapter 4

8 Role of anti-inflammatory compounds on HIV-1 gp120-mediated brain inflammation

This work is published and reproduced in this thesis with permission from Biomed Central: T Ashraf, W Jiang, T Hoque, J Henderson, C Wu and R Bendayan. Role of anti-inflammatory compounds in human immunodeficiency virus-1 glycoprotein120-mediated brain inflammation. 2014. Journal of Neuroinflammation. 11:91.

Author contributions:

Research design: T Ashraf (first author), W Jiang, T Hoque, J Henderson (collaborator), C Wu and R Bendayan (principle investigator).

Conducted experiments and data analysis: T Ashraf (figures 8-1, 8-2, 8-3, 8-4, 8-5, 8-6, 8-7 except animal surgery and qPCR); W Jiang (figures 8-1, 8-2, 8-5, 8-6); T Hoque (figures 8-3, 8-4); J Henderson (figure 8-3) and C Wu (figure 8-7, animal surgery).

Writing of the manuscript: T Ashraf (preparation of the manuscript drafts and responses to reviewer’s comments); T Hoque (editorial review, submission and image resolution improvement); J Henderson (editorial review) and R Bendayan (overall conceptual and editorial review of several manuscript drafts and responses to reviewer’s comments)
8.1 Abstract

Neuroinflammation is a common immune response associated with brain HIV-1 infection. Identifying therapeutic compounds that exhibit better brain permeability and can target signaling pathways involved in inflammation may benefit treatment of HIV-associated neurological complications. The objective of this study was to implement an *in vivo* model of brain inflammation by intracerebroventricular administration of the HIV-1 viral coat protein gp120 in rats and to examine anti-inflammatory properties of HIV adjuvant therapies such as minocycline, chloroquine and simvastatin.

Male Wistar rats were administered a single dose of gp120<sub>ADA</sub> (500 ng) daily for seven consecutive days, intracerebroventricularly, with or without prior intraperitoneal administration of minocycline, chloroquine or simvastatin. Maraviroc, a CCR5 antagonist, was administered intracerebroventricularly prior to gp120 administration for seven days as control. Real-time qPCR was used to assess gene expression of inflammatory markers in the frontal cortex, hippocampus and striatum. IL-1β and TNF-α secretion in CSF was measured applying ELISA. Protein expression of MAPKs (ERK1/2, JNKs and P38Ks) was detected using immunoblot analysis. Student’s *t*-test and ANOVA were applied to determine statistical significance.

In gp120<sub>ADA</sub>-injected rats, mRNA transcripts of IL-1β and iNOS were significantly elevated in the frontal cortex, striatum and hippocampus compared to saline or heat-inactivated gp120-injected controls. In CSF, a significant increase in TNF-α and IL-1β was detected. Maraviroc reduced upregulation of these markers suggesting that the interaction of R5-tropic gp120 to CCR5 chemokine receptor is critical for induction of an inflammatory response. Minocycline, chloroquine or simvastatin attenuated upregulation of IL-1β and iNOS transcripts in different brain regions. In CSF, minocycline suppressed TNF-α and IL-1β secretion, whereas chloroquine attenuated IL-1β secretion. In gp120-injected animals, activation of ERK1/2 and JNKs was observed in the hippocampus and ERK1/2 activation was significantly reduced by the anti-inflammatory agents.

Our data demonstrate that anti-inflammatory compounds can completely or partially reverse gp120-associated brain inflammation through an interaction with MAPK signaling pathways and
suggest their potential role in contributing towards the prevention and treatment of HIV-associated neurological complications.

8.2 Background

Neurocognitive impairment remains highly prevalent in HIV-1 infected individuals due to persistence of viral replication and associated inflammation in the brain (Heaton et al., 2011). Since the initiation of HAART, the development of HAD in HIV-1 infected patients has significantly decreased. However, milder forms of HAND are becoming more predominant in the post-HAART era, in part due to the longer life expectancy of infected individuals on treatment. Despite receiving treatment, up to 50% of HIV-1 infected patients continue to develop cognitive impairment, psychiatric illness and persistent fatigue (Heaton et al., 2011).

In response to HIV-1, resident microglia in the brain become activated and release inflammatory mediators that further trigger the activation of neighboring microglia and astrocytes and signal recruitment of peripheral monocytes from the systemic circulation (Ronaldson et al., 2008). Studies have demonstrated that virus-infected cells or glial cells can release various neurotoxic factors (AA, platelet activating factor, quinolinic acid, ROS, NO and glutamate) and a number of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-18, IFNα, IFNγ) (Perrella et al., 1992; Ronaldson and Bendayan, 2008; Ronaldson et al., 2008). Irreversible neuronal injury and loss is often caused by inflammation and oxidative stress associated with these mediators present in the systemic circulation that crosses the BBB and/or mediators secreted in the brain from glial cells. Detectable amounts of these cytokines in the CSF of patients with HAND have been reported (Perrella et al., 1992). In vitro studies in primary glial cell cultures also suggest that shed viral proteins (such as gp120, Tat and vpr) can induce secretion of pro-inflammatory cytokines. For example, previous work from our laboratory has demonstrated that R5-tropic gp120 can mediate secretion of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) by interacting with CCR5 chemokine receptor in primary cultures of human or rodent astrocytes (Ashraf et al., 2011; Ronaldson and Bendayan, 2006). HIV-1 proteins (such as gp120 and Tat) can also downregulate tight junction proteins expressed in brain microvessel endothelial cells which may facilitate the entry of the virus into the brain parenchyma (Kanmogne et al., 2005).
The treatment of brain HIV-1 infection remains challenging partly due to poor permeability of antiretroviral drugs across the BBB and into glial cells. One possible mechanism for the low brain permeability of these drugs is the functional expression of ATP-dependent, membrane-associated efflux transporters known as ABC transporters (P-gp, MRPs and Bcrp) at the BBB and in brain parenchyma (Bendayan et al., 2002). Several in vivo and in vitro studies have examined the role of these transporters in reducing the permeability of antiretroviral drugs into brain cellular compartments (Bendayan et al., 2002; Kaddoumi et al., 2007). For example, administration of P-gp specific inhibitor zosuquidar in macaques resulted in significant brain accumulation of nelfinavir (Kaddoumi et al., 2007). Evidence in the literature suggests that functional expression of these transporters in the brain is altered during HIV-1 infection. Langford et al. reported increased P-gp immunoreactivity in glial cells in brain autopsy tissues from patients with HIVE (Langford et al., 2004), whereas, Persidsky et al. reported a decreased P-gp expression in tissues obtained from HIVE patients and from the SCID mice model of HIVE (Persidsky et al., 2000). Furthermore, shed viral proteins (such as gp120 and Tat) and secreted pro-inflammatory cytokines during HIV-1 infection are also known to alter the expression of drug efflux transporters. Our previous work in rodent and human astrocytes suggests that gp120 can significantly downregulate P-gp functional expression (Ronaldson and Bendayan, 2006). We have also observed an increase in Mrp1 functional expression in response to gp120 in primary cultures of rat astrocytes (Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). However, whether gp120 can modulate the expression of these transporters in vivo in a similar manner is yet to be characterized.

Several classes of antiretrovirals have been reported to poorly permeate into the brain, whereas better permeable antiretroviral drugs can be associated with adverse side effects including neurotoxicity (Cavalcante et al., 2010). Due to the complexities associated with the treatment of brain HIV-1 infection and HIV-associated chronic secretion of inflammatory mediators, much interest has risen in identifying potential adjuvant therapies that can be used along with antiretroviral drugs. For example, minocycline, a second generation tetracycline derivative, has been considered as a potential candidate due to its versatile role in neuroprotection in different brain disease models (Meulendyke et al., 2012; Szeto et al., 2010). Chloroquine, an antimalarial drug and simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, have also
demonstrated an anti-inflammatory and anti-HIV effect at non-toxic concentrations in vitro or in vivo (Andras et al., 2008; Hagihara et al., 2000; Naarding et al., 2007; Nabatov et al., 2007). Furthermore, minocycline, chloroquine and simvastatin can inhibit MAPK signaling pathways (ERK1/2, JNKs and P38Ks) involved in generating inflammatory responses (Nikodemova et al., 2006). In a clinical trial minocycline treatment was found to be unsuccessful in improving the neurocognitive outcome in patients with cognitive impairment (Sacktor et al., 2011). On the contrary, in a SIV model early administration of minocycline was reported to be effective against striatal dopaminergic system dysfunction, suggesting that timely treatment initiation may have an effect on minocycline efficacy (Meulendyke et al., 2012). Based on these observations, we propose that early administration of these anti-inflammatory compounds may have the potential to reverse HIV-associated inflammatory response.

The objective of the present work was to implement an in vivo model of HIV-associated inflammation by ICV administration of viral coat protein gp120 and to investigate the regulation of drug transporters, tight junction proteins, signaling pathways and the potential anti-inflammatory effects of chloroquine, minocycline and simvastatin.

8.3 Materials and methods

8.3.1 Materials

HIV-1ADA gp120 full-length protein (subtype B; R5-tropic) and maraviroc were obtained from Immunodiagnostic Inc (Woburn, Massachusetts, United States) and the NIH AIDS Research and Reference Reagent program (Bethesda, Maryland, United States), respectively. ERK1/2 inhibitor U0126 and simvastatin were purchased from Cell Signaling (Whitby, Ontario, Canada) and Toronto Research Chemicals (Toronto, Ontario, Canada), respectively. Chloroquine, minocycline, JNK inhibitor SP600125, murine monoclonal AC-40 antibody against actin, rabbit polyclonal antibody against GFAP and HRP conjugated secondary antibodies (anti-mouse, anti-rabbit and anti-rat) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Alexa fluor 488 donkey anti-rabbit, anti-occludin, anti-zo1 and anti-claudin5 antibody, TRIzol™, DNAse I and Prolong gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, California, United States). Tissue-Tek cryo-optimum cutting temperature (OCT) formulation was purchased from Thermo Fisher Scientific (Waltham,
Massachusetts, United States). ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, P38K and phospho-P38K antibodies were obtained from Cell Signaling (Whitby, Ontario, Canada). Murine monoclonal C219 antibody against P-gp, rat monoclonal MRPI antibody and anti-BCRP (rat monoclonal) antibody were purchased from ID labs (London, Ontario, Canada), Kamiya Biomedical Company (Seattle, Washington, United States) and Abcam Inc (Boston, Massachusetts, United States), respectively. ELISA kits were obtained from R&D Systems (Minneapolis, Minnesota, United States). Guide cannula, dummy cannula, screws and dental cement for stereotaxic surgery were purchased from HRS Scientific (Montreal, Quebec, Canada). High capacity reverse transcriptase cDNA synthesis kit and SYBR Green Fastmix were obtained from Applied Biosystems (Foster City, California, United States) and Quanta Biosciences Inc (Gaithersburg, Maryland, United States), respectively.

8.3.2 Animals

This study was approved by the University of Toronto Animal Care Committee, Toronto, Ontario, Canada. Adult Wistar male rats (weighing approximately 250 grams; 12 weeks of age) were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Animals were housed with rodent chow and water ad libitum on a 12 hour light-dark cycle. All procedures were carried out in accordance with the guidelines of University of Toronto Animal Care Committee and the Canadian Council on Animal Care. The rats were randomly assigned to nine different groups: (1) wild-type (no surgery), (2) control (saline), (3) heat-inactivated gp120, (4) gp120, (5) maraviroc + gp120, (6) chloroquine + gp120, (7) minocycline + gp120, (8) simvastatin + gp120, (8) JNK inhibitor SP600125 + gp120, and (9) ERK1/2 inhibitor U0126 + gp120.

8.3.3 Animal surgery and ICV administration of gp120/maraviroc/signaling pathway inhibitors

Animals were anaesthetized with isoflurane (2 to 3%). The implantation of the guide cannula was performed according to previously published protocols using the following stereotaxic coordinates: 1 mm posterior to bregma, 1.5 mm lateral from midline and 3.5 mm ventral from the surface of the skull according to the Atlas of Paxinos and Watson (Bagetta et al., 1996; Paxinos G and Watson, 2006). Ketoprofen (5 mg/kg) was administered subcutaneously
during surgery for pain relief. Animals were allowed to recover for seven days after surgery. Following recovery, animals were administered a single dose of (100 ng or 300 ng or 500 ng) gp120\textsubscript{ADA} (R5-tropic; clade B) daily for seven consecutive days ICV using a 5 μl Hamilton syringe. gp120 was diluted into a sterile normal saline solution and administered in a volume of 2 μl (such as 250 ng/μl) over 2 minute period. Control animals received equal amount of saline. Heat-inactivated gp120 was also administered for seven days as an additional control group. Maraviroc, a selective CCR5 antagonist, was administered 30 minutes prior to gp120 administration for seven days [4 μl/4 minutes; 250 ng/μl in 1% dimethyl sulfoxide (DMSO)]. ERK1/2 inhibitor U0126 (7 μg) and JNK inhibitor SP600125 (10 μg) were administered ICV prior to gp120 administration at the same rate (4 μl/4 minutes; dissolved in 1.5% DMSO).

### 8.3.4 Intraperitoneal administration of minocycline, chloroquine and simvastatin

Minocycline (50 mg/kg loading dose followed by 25 mg/kg/day) or chloroquine (25 mg/kg/day) or simvastatin (1 mg/kg) was administered daily for seven consecutive days by a single intraperitoneal injection 30 minutes prior to ICV gp120 administration. Minocycline and chloroquine were dissolved in sterile water and simvastatin was dissolved in a 1% DMSO solution.

### 8.3.5 Brain tissue, brain capillary isolation and CSF collection

Twenty four hours after the last injection, animals were anaesthetized and CSF samples were collected by cisterna magna puncture according to previously published protocol (Jiang et al., 2009). Subsequently, anaesthetized animals were perfused through the left ventricle of the heart with 200 ml of saline solution and whole rat brains were harvested. For real-time qPCR and immunoblot analysis, brains were dissected on ice to isolate frontal cortex, hippocampus and striatum from both hemispheres. Samples were flash frozen in liquid nitrogen and kept at -80°C. Capillaries were also isolated from saline and gp120-injected rat brains following previously published protocol (Chan et al., 2013c). For immunohistochemical analysis, saline perfusion was followed by 180 ml of paraformaldehyde (4%) dissolved in PBS perfusion before collecting whole brains. These samples were stored in 50 ml paraformaldehyde (4%) at 4°C.
8.3.6 Quantitative real-time PCR

Real-time qPCR was applied to determine the gene expression of inflammatory markers according to previously described protocols followed by our laboratory (Hoque et al., 2012). Briefly, total RNA was extracted from brain regions using TRIzol™ (Invitrogen, Carlsbad, California, United States) reagent and the high capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, California, United States) was used to synthesize first-strand cDNA. Primer pairs for the rat IL-1β gene (5'-CTCAACTGTGAAATAGCAGCTTTC-3' and 5'-GGACAGCCCAAGTCAAGG-3'), rat TNF-α (5'-TCTTCTCATTGCTCGTG-3' and 5'-GATGAGAGGGAGCCCCATT-3'), rat iNOS (5'-CCAAGGTGACCTGAAAGAGG-3' and 5'-TTGATGCTTGTGACTCTTAGGG-3'), rat IL-6 (5'-CTTCACAAGTGGAGGCTTAAT-3' and 5'-ACAGTGCATCATCGCTGTTC-3') and the rat Cyclophilin B gene (housekeeping gene; 5'-GGAGATGGCACAGGAGGAA-3' and 5'-GCCCGTAGTGCTTCAGCTT-3') were designed using Primer Express 3 software (Applied Biosystems, Foster City, California, United States) and were validated for specificity and efficacy using LPS-treated rat astrocyte samples. Quantitative PCR was performed using SYBR Green Master Mix (Quanta Biosciences Inc, Gaithersburg, Maryland, United States) using an Eppendorf Real-time PCR System (Eppendorf Canada, Mississauga, Ontario, Canada). A relative quantification was performed by comparing the threshold cycle values of samples with serially diluted standards. Expression levels were normalized to housekeeping gene Cyclophilin B.

8.3.7 ELISA

Commercially available colorimetric sandwich ELISA kits for rat IL-1β and TNF-α were used to detect the secretion of these cytokines in CSF in the presence or absence of maraviroc, chloroquine, minocycline and simvastatin. CSF collected from control (no surgery) and ICV heat-inactivated gp120-administered animals was analyzed as an additional control group. The assays were performed according to the manufacturer’s instruction with slight modifications as previously described in our laboratory. Standard curves were generated using appropriate rat cytokines and absorbance read at 450 nm was converted to pg/ml. We confirmed the minimal detection level to be 2.5 pg/ml and 1.5 pg/ml for IL-1β and TNF-α, respectively.
8.3.8 Immunoblot Analysis

Immunoblotting was performed as described previously in our laboratory (Ronaldson and Bendayan, 2006). Tissue and capillary homogenates were prepared using a lysis buffer (1% (v/v) NP-40 in 20 mM tris, 150 mM NaCl, 5 mM EDTA at pH 7.5 containing 1 mM PMSF and 0.1% (v/v) PI cocktail). Tissues were sonicated for 10 seconds and centrifuged at 20,000 g for 10 minutes at 4°C to remove cellular debris. Protein concentrations of tissue or capillary homogenates were determined using Bradford’s protein assay (Bio-rad laboratories, California, United States). Total protein (1 μg, 5 μg to 50 μg) were separated on 7 to 12% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk for one hour, the membrane was probed for protein of interest with primary antibody [anti-P-gp (C219), 1:500; Mrp1, 1:500; Bcrp, 1:500; β-actin, 1:2000; ZO-1, 1:500; occludin, 1:500; claudin5, 1:250; GFAP, 1:1000; iNOS, 1:500; ERK1/2, 1:250; phospho-ERK1/2, 1:100; JNK, 1:250; phospho-JNK, 1:100; P38K, 1:250; phospho-P38K, 1:100]. β-Actin was used as loading control. HRP-conjugated secondary antibody was added after washes in tris-buffered saline with Tween. After further washing, bands were detected using enhanced chemiluminescent reagent (Thermo Fischer Scientific, Ontario, Canada). Densitometric analysis was performed in AlphaDigiDoc RT2 software (Alpha Innotech, San Leandro, California, United States) to quantify relative protein expression. The graphs represent relative density of the bands of interest normalized to corresponding β-actin.

8.3.9 Immunohistochemistry

Immunohistochemistry was performed according to previously published protocol (Hui et al., 2010). Briefly, paraformaldehyde-fixed samples were transferred to a 30% sucrose solution and kept overnight at 4°C before mounting with OCT compound. Coronal cryostat sections (25 μm) were prepared at Murine Imaging and Histology Facility (Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada). Sections were incubated in blocking buffer (5% goat serum and 0.2% Triton X-100 in 0.1 M PBS) for one hour followed by overnight incubation with primary antibody at 4°C (Anti-GFAP, 1:200). Following incubation, sections were washed thoroughly in PBS and incubated for two hours in Alexa-Fluor conjugated secondary antibody (1:200). After further washing, coverslips were mounted onto the specimen using a prolong gold anti-fade mounting medium with DAPI. Specimens were examined using a Nikon E1000
fluorescent microscope (Nikon Corp., Mississauga, Canada). Images were captured using the software SimplePCI (Compix, Inc., Imaging Systems, Sewickley, Pennsylvania, United States).

8.3.10 Data analysis

Student’s *t*-test was used to determine statistical significance between two groups. Multiple comparisons were performed using ANOVA and Bonferroni’s *post-hoc* analysis. A *P* value less than 0.05 was considered to be statistically significant. Data were analyzed using GraphPad Prism software (San Diego, California, United States). Samples collected from three to twelve animals per group were used.

8.4 Results

8.4.1 Gp120-mediated inflammatory response in the brain

Previous studies have demonstrated that ICV or region-specific administration of different strains of gp120 from 100 ng to 500 ng into rodents can induce an inflammatory response (Bagetta et al., 1999; Louboutin et al., 2010b). In our hands, administration of 100 ng to 300 ng HIV-1 gp120 ADA daily for seven days in adult male Wistar rats did not induce a significant inflammatory response in brain tissue (data not shown). Therefore, a dose of 500 ng was chosen. At this dose, we observed a significant upregulation of IL-1β, iNOS and TNF-α in different brain regions. Compared to saline-administered animals, in gp120 (500 ng)-injected rats, transcripts of IL-1β, iNOS and TNF-α were significantly elevated in the frontal cortex, whereas, elevated IL-1β and iNOS mRNA were observed in the hippocampus and striatum (Figure 8-1A-C). No significant change was observed in IL-6 transcript levels (data not shown). Heat-inactivated gp120 did not induce any changes in inflammatory markers and remained comparable to saline treatment (Figure 8-1A-C). ANOVA analysis performed for different regions in Figure 8-1 did not show a significant difference of IL-1β, iNOS and TNF-α transcripts between wild-type animals (no surgery) and saline-treated animals (Figure 8-1A-C).

Our previous work *in vitro* suggested that R5-tropic gp120 could interact with CCR5 chemokine receptors resulting in pro-inflammatory cytokine secretion (Ronaldson and Bendayan, 2006). In order to demonstrate that the inflammatory response observed in gp120-administered animals is mediated by the specific interaction of gp120 with CCR5 chemokine receptor *in vivo*, we
administered maraviroc, a CCR5 antagonist ICV prior to gp120 administration in rodents. Administration of maraviroc (1 μg) resulted in a downregulation of IL-1β, iNOS and TNF-α transcript levels in different brain regions when compared to only gp120-treated animals (Figure 8-1A-C).
**Figure 8-1** Effect of gp120 on the mRNA levels of (A) IL-1β, (B) iNOS and (C) TNF-α in different brain regions of ICV administered gp120 (500ng) rats along with a CCR5 antagonist, maraviroc (MVC). Wildtype (no surgery), control (saline) and heat inactivated (HI) gp120 injected animals were also analyzed. Results are expressed as mean±standard error of mean (SEM) (n=10 for saline and gp120 groups; n=3 for wildtype, HI gp120 and maraviroc treated groups). Asterisk represent data point significantly different from saline administered animals (** p<0.01; * p<0.05).
At the protein level, a significant increase in TNF-α and IL-1β was also detected in CSF samples collected from gp120-administered animals (Figure 8-2A and 8-2B). Consistent with the qPCR data, maraviroc treatment attenuated the gp120-mediated secretion of cytokines. Also, heat-inactivated gp120-mediated secretion of IL-1β and TNF-α was found to not be significantly different from saline-treated animals (Figure 8-2A and 8-2B).
**Figure 8-2** ELISA analysis of (A) IL-1β and (B) TNF-α secretion in the CSF of gp120 (500ng) administered rats in the presence of Maraviroc (MVC). CSF collected from saline and heat injected animals were used as control. Results are expressed as mean±SEM (n=10 for saline and gp120 groups; n≥3 for wildtype, HI gp120 and maraviroc treated groups). Asterisk represent data point significantly different from saline administered animals (**p<0.01; ***p<0.001).
8.4.2 Gp120-mediated activation of astrocytes

ICV administration of gp120 has previously been shown to result in astrocyte activation *in vivo* as demonstrated by an increase in GFAP expression, a cellular marker for astrocyte (Louboutin et al., 2010b). Therefore, in our model we investigated expression of GFAP using immunohistochemistry (Figure 8-3A). In gp120-administered animals, we observed an increase in GFAP staining in the hippocampus. This was further confirmed by immunoblotting analysis (Figure 8-3B).
Figure 8-3 (A) Immunohistochemical and (B) immunoblotting (upper panel) and densitometric analysis (lower panel) of GFAP in hippocampus of ICV administered gp120 rats compared to saline treated animals. A representative blot is shown. Results are expressed as mean±SEM. For immunoblotting, samples obtained from five different animals were used per group. Asterisk represent data point significantly different from saline administered animals (*p<0.05).
8.4.3  Effect of gp120 on tight junction proteins and drugs efflux transporters in brain capillaries

Previous studies have reported a breakdown of the BBB in models of brain HIV-1 infection in vitro and in vivo (Persidsky et al., 2000). In order to determine the integrity of the BBB in our in vivo model, we examined the expression of tight junction proteins (occludin, ZO-1, claudin5) as well as drug efflux transporters (P-gp, Mrp1 and Bcrp) in brain capillaries isolated from gp120-treated animals. We did not observe any significant changes in ZO-1, occludin, claudin5, P-gp, Mrp1 and Bcrp protein expression in brain capillaries isolated from saline or gp120-administered rats (data not shown).

8.4.4  Effect of gp120 on drug efflux transporter brain expression in brain regions

We have previously shown in vitro that gp120 exposure can significantly decrease P-gp protein expression and increase Mrp1 protein expression in primary cultures of rat astrocytes and/or HFAs. However, it was unknown if gp120 could alter the expression of these transporters in vivo. Applying immunoblot analysis, we detected significant changes in P-gp, Mrp1 and Bcrp protein expression in different brain regions of gp120-treated animals (Figure 8-4). Gp120 administration resulted in a significant decrease in P-gp protein expression in the frontal cortex and hippocampus (40 and 30%, respectively) (Figure 8-4A and 8-4B). In the striatum of gp120-treated animals, we did not detect any significant changes in P-gp protein expression compared to saline-administered animals (Figure 8-4C). A significant upregulation of Mrp1 was observed in hippocampus and striatum (67 and 68%, respectively) (Figure 8-4B and 8-4C), whereas, no significant change was detected in the frontal cortex (Figure 8-4A). Bcrp, another drug efflux transporter, was found to be upregulated in the frontal cortex (118% compared to saline) (Figure 8-4A) but not in the hippocampus and striatum (Figure 8-4B and 8-4C).
### B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Saline</th>
<th>gp120_500ng</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>170 kDa</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>42 kDa</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>190 kDa</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>42 kDa</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>72 kDa</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>42 kDa</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
</tbody>
</table>

**Hippocampus**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Saline</th>
<th>gp120_500ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
</tr>
<tr>
<td>Mrp1</td>
<td><img src="image21" alt="Image" /></td>
<td><img src="image22" alt="Image" /></td>
</tr>
<tr>
<td>Bcrp</td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 8-4 Immunoblot (upper panel) and densitometric analysis (lower panel) of drug efflux transporters (P-gp, Mrp1 and Bcrp) in (A) frontal cortex, (B) hippocampus and (C) striatum of ICV administered gp120 rats. Transporter over-expressing cell lines were used as positive controls. Representative blots are shown. Results are expressed as mean±SEM. Samples obtained from six different animals were used per group. Asterisk represent data point significantly different from saline administered animals (**p<0.01; * p<0.05).
8.4.5 Anti-inflammatory effects of chloroquine, minocycline and simvastatin

Animals were administered gp120 (500 ng) ICV with or without prior intraperitoneal administration of minocycline or chloroquine or simvastatin in order to assess their potential anti-inflammatory effect. Administration of minocycline or chloroquine partially or completely suppressed the gp120-induced upregulation of the inflammatory markers examined (Figure 8-5). In particular, administration of minocycline or chloroquine completely attenuated gp120-mediated upregulation of IL-1β and iNOS transcripts in all three brain regions (Figure 8-5A-C).
Figure 8-5 Effect of gp120 on the mRNA levels of (A) IL-1β, (B) iNOS and (C) TNF-α in different brain regions of ICV administered gp120 rats receiving simultaneous intraperitoneal injection of chloroquine or minocycline. Saline injected animals were used as control. Results are expressed as mean±SEM. Samples obtained from at least ten different animals were used per group. Asterisk represent data point significantly different from saline administered animals (*** p<0.001; **p<0.01; * p<0.05). Diamonds represent data point significantly different from gp120-treatment (♦♦♦ p<0.001; ♦♦p<0.01).
Minocycline was also effective in suppressing TNF-α transcripts in brain tissues and both TNF-α and IL-1β secretion in the CSF of gp120-administered animals, whereas chloroquine only attenuated IL-1β secretion (Figure 8-6A and 8-6B). Simvastatin attenuated both IL-1β and iNOS transcripts in the hippocampus and striatum (Figure 8-5A and 8-5B), but only suppressed iNOS in the frontal cortex (Figure 8-5B). At the protein level, however, simvastatin could not significantly suppress gp120-mediated IL-1β and TNF-α secretion in the CSF (Figure 8-6A and 8-6B).
**Figure 8-6** ELISA analysis of (A) IL-1β and (B) TNF-α secretion in the CSF of gp120 (500ng) administered rats receiving simultaneous intraperitoneal injection of chloroquine or minocycline or simvastatin. CSF collected from saline injected animals was used as control. Results are expressed as mean±SEM. Samples obtained from at least ten different animals were used per group. Asterisk represent data point significantly different from saline administered animals (*** p<0.001; ** p<0.01). Diamonds represent data point significantly different from gp120-treatment (♦♦♦ p<0.001).
8.4.6 Gp120-mediated activation of signaling pathways

The three main subfamilies of the MAPK pathway (ERK1/2, JNK and P38K) are known to be actively involved in regulating cytokine secretion (Kaminska, 2005). Therefore, we investigated the activation of ERK1/2, JNK and P38K in different brain regions of gp120-administered animals. In the hippocampus, we observed phosphorylation of ERK1/2 and JNKs (Figure 8-7A), however, we did not detect significant changes in ERK1/2, JNK or P38K phosphorylation in the frontal cortex and striatum (data not shown). Administration of minocycline and simvastatin significantly reduced ERK1/2 phosphorylation, whereas the effect of chloroquine was not found to be significant (Figure 8-7B). Gp120-mediated JNK phosphorylation was found to not be affected despite treatment with anti-inflammatory compounds (data not shown). However, in our in vitro model of primary cultures of rat astrocytes triggered with gp120, we detected attenuation of both ERK1/2 and JNKs in the presence of minocycline, chloroquine and simvastatin (data not shown). In order to further demonstrate that the gp120-mediated activation of these pathways can be attenuated in our model, inhibitors of ERK1/2 (U0126) and JNK (SP600125) pathways were administered ICV daily prior to gp120 administration and we confirmed significant inhibition of ERK1/2 and JNK phosphorylation, respectively (Figure 8-7C and 8-7D).
B

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>gp120_500ng</th>
<th>Chloroquine+ gp120_500ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-ERK1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simvastatin+ gp120_500ng</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-ERK1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minocycline+ gp120_500ng</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospho-ERK1/2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hippocampus

Expression of phosphorylated ERK1/2 (% Control)

Saline, gp120_500ng, Minocycline+gp120, CQ+gp120, Sim+gp120
Figure 8-7 Protein (upper panel) and densitometric analysis (lower panel) of (A) ERK1/2 and phospho-ERK1/2; JNK and phospho-JNK; P38K and phospho-P38K, (B) ERK1/2 and phospho-ERK1/2 in the presence of chloroquine, minocycline and simvastatin, (C) ERK1/2 and phospho-ERK1/2 in the presence of ERK1/2 inhibitor U0126; (D) JNK and phospho-JNK in the presence of SP600125 in the hippocampal tissue of gp120 administered rats. Representative blots are shown. Tissue collected from saline administered animals was used as control. Results are expressed as mean±SEM. Samples obtained from three to six different animals were used per group. Asterisk represent data point significantly different from saline administered animals (*** p<0.001; * p<0.05). Diamonds represent data point significantly different from gp120-treatment (♦ p<0.05).
8.5 Discussion

A number of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ) and oxidative stress markers (such as NO) are known to be elevated during brain HIV-1 infection. HIV-1 coat protein gp120 has previously been shown to generate an inflammatory response and oxidative stress both in vitro and in vivo (Bagetta et al., 1996; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). This viral protein is shed during HIV-1 replication and circulating levels of gp120 have been detected in plasma and in the CSF (Banks and Kastin, 1998; Banks et al., 1997; Cashion et al., 1999; Dohgu et al., 2012; Oh et al., 1992). Our laboratory has extensively investigated gp120-mediated release of pro-inflammatory cytokines and oxidative stress in primary cultures of rodent astrocytes as well as in HFAs exposed to viral isolates in vitro (Ashraf et al., 2011; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). In this study, we implemented an in vivo model of brain inflammation by ICV administration of gp120. In different models, doses of gp120 ranging from 100 to 500 ng (administered intracerebrally) have been shown to induce an inflammatory response (Louboutin et al., 2010b). In our model, ICV administration of 500 ng of HIV-1 gp120\textsubscript{ADA} resulted in a significant upregulation of different inflammatory markers in the frontal cortex, hippocampus and striatum. Heat-denatured gp120 failed to generate an inflammatory response, suggesting that a native gp120 structure is necessary to interact with chemokine receptors to induce an inflammatory response. We observed upregulation of IL-1β, iNOS and TNF-α in the frontal cortex of gp120-administered animals. In the hippocampus and striatum, IL-1β and iNOS transcripts were found to be elevated due to gp120 treatment. These markers are known to be upregulated during HIV-1 infection (Bagetta et al., 1999; Perrella et al., 1992). We also observed enhanced secretion of cytokines IL-1β and TNF-α in the CSF. Previously, increased GFAP expression has been observed in HIV-1 infected brain (Vanzani et al., 2006). In our model, we observed a significant increase in GFAP expression in the hippocampus, indicating activation of astrocytes in this region.

Interaction of gp120 and chemokine receptors has previously been reported in vitro (Ashraf et al., 2011; Ronaldson and Bendayan, 2006). Previous work from our laboratory has detected expression of CCR5 in both human and rat primary culture of astrocytes. Using neutralizing
antibodies against CCR5 and CXCR4 receptors, we have demonstrated that R5-tropic gp120 can interact with CCR5 chemokine receptors and result in pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) secretion in vitro (Ashraf et al., 2011; Ronaldson and Bendayan, 2006). Maraviroc is a potent and specific inhibitor of CCR5 that is clinically used to treat HIV-1 infection. Consistent with our in vitro findings, maraviroc attenuated the gp120-mediated increase in IL-1β, iNOS and TNF-α in our in vivo model suggesting that the mechanism of gp120-associated inflammation is CCR5-receptor dependent. These findings provide evidence of in vivo interaction of gp120 with CCR5 receptor and emphasize the critical role of this interaction in generating a brain inflammatory response.

Downregulation of tight junction proteins (such as occludin, ZO-1, claudin5) has been implicated in the pathogenesis of brain HIV-1 infection (Persidsky et al., 2000). A compromised BBB can facilitate entry of HIV-1 from the periphery, and can ultimately increase the spread of the virus in brain parenchymal cellular compartments. Although in vitro and in vivo studies have demonstrated that HIV-1 proteins (like gp120 and Tat) can downregulate tight junction proteins expressed in brain microvessel endothelial cells (Kanmogne et al., 2005; Louboutin et al., 2010a), we did not observe any significant change in tight junction proteins expression in our model. This lack of effect may be due to the dose, duration and/or strain of the gp120 treatment we used. Persidsky et al. reported that downregulation of tight junction proteins is associated with Rho activation in brain microvessel endothelial cells and this activation was mediated by monocyte migration across these cells (Persidsky et al., 2006a). Ricardo-Dukelow et al. demonstrated that HIV-1 infected macrophages upregulate a number of proteins in brain microvessel endothelial cells that may lead to BBB disruption (Ricardo-Dukelow et al., 2007). Therefore, lack of immune cell infiltration could be another potential reason for lack of change in tight junction protein expressions in our model. We also did not detect any significant changes in transporter protein expression in the capillaries.

Tight junction proteins form a physiological barrier at the brain capillary level, whereas a biochemical barrier also exists both at the BBB level and in brain parenchyma. This biochemical barrier primarily consists in the expression of drug efflux transporters and several metabolizing enzymes that restrict the bioavailability of a number of antiretroviral drugs into the brain. Modulation of these transporters by pathological stimuli has been previously reported (Hayashi
et al., 2006; Hayashi et al., 2005; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). Decreased P-gp expression at the gene and protein level has been reported in brain autopsy samples from patients with HIV-1 as well as in brain tissue collected from a SCID mice model of HIV-1 (Persidsky et al., 2000). In vitro, HIV-1 viral isolates or gp120-mediated downregulation of P-gp has also been reported by our laboratory in both rodent and human astrocytes (Ashraf et al., 2011; Ronaldson and Bendayan, 2006). Consistent with these data, in our in vivo model, we observed downregulation of this transport protein expression in the frontal cortex and hippocampus further confirming that gp120 has a down-regulatory effect on P-gp functional expression in brain parenchyma.

Altered functional expression of Mrp1 has also been reported in the context of HIV-1. Hayashi et al. reported Tat-induced upregulation of Mrp1, both at the gene and protein level in cultured astrocytes (Hayashi et al., 2006). In primary cultures of rat astrocytes triggered with gp120 or TNF-α, we also detected an increase in Mrp1 functional expression (Ronaldson et al., 2010; Ronaldson and Bendayan, 2008). In the present study, a significant upregulation of Mrp1 in the hippocampus and striatum was detected, further suggesting that gp120 exerts an up-regulatory effect on Mrp1 protein expression in vivo. In our hands, Bcrp was also found to be upregulated in the frontal cortex of gp120-treated animals. In contrast, IL-1β and TNF-α-mediated downregulation of Abcg2 has previously been reported in porcine brain microvessel endothelial cells (von Wedel-Parlow et al., 2009). These results indicate that, similar to other drug efflux transporters, Bcrp is susceptible to modulation by inflammatory cytokines. However, cytokine-mediated regulation of this transporter could be species and/or cell specific. Our in vivo data are consistent with previous in vitro findings in glial cells where exposure to gp120 resulted in a significant production of pro-inflammatory cytokines along with a downregulation of P-gp expression and an upregulation of Mrp1 expression (Ashraf et al., 2011; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). Together, these observations provide evidence that regulation of drug efflux transporters is highly complex during HIV-associated brain inflammation. Since a number of antiretroviral drugs are known substrates for these transporters, changes in the expression of these transporters in brain parenchyma may result in an altered distribution of antiretroviral drugs at cellular targets of HIV-1 infection.
To date, only a few studies have reported on the neuroprotective and anti-inflammatory potential of different compounds (such as minocycline, curcumin, neurosteroids) in attenuating a HIV-associated inflammatory response (Maingat et al., 2013; Meulendyke et al., 2012; Tang et al., 2009). The limitations of currently used antiretroviral drugs include lack of anti-inflammatory properties, poor brain permeability and neurotoxicity associated with better permeable drugs. Since neurological disorders are becoming more prevalent in HIV-1 infected patients and inflammation is a common immune response, identifying therapeutic compounds that can effectively permeate the BBB, exhibit anti-inflammatory properties, and are well tolerated may provide an additional option in preventing and/or treating HAND. For example, in a recent study, Maingat et al. reported efficacy of neurosteroid, dehydroepiandrosterone sulfate, in preventing inflammation and neurodegeneration as well as behavioral deficits in a FIV model (Maingat et al., 2013). Minocycline, a second generation tetracycline derivative, has been reported by many to have anti-inflammatory properties both in vitro and in vivo (Meulendyke et al., 2012; Ryu et al., 2004). In an Aβ injected rat model, minocycline has shown to successfully reverse the activation of glial cells (Ryu et al., 2004). In our study, minocycline also effectively inhibited gp120-mediated upregulation of different inflammatory markers in the frontal cortex, hippocampus and striatum and further prevented secretion of cytokines in the CSF. Recently, minocycline has been tested in a clinical trial in HIV-infected patients with cognitive impairment. Although this study reported that minocycline treatment was unsuccessful in improving neurocognitive function in patients with cognitive impairment (Sacktor et al., 2011), it is still inconclusive if administration of the drug at earlier stages of the infection could have a better clinical outcome. Since minocycline has been reported to have a safe profile when administered with antiretroviral drugs in HIV-infected individuals, it will be important to assess if early administration of antiretroviral drugs along with minocycline has any beneficial effects against HIV-associated neurocognitive disorders. Several clinical studies indicate earlier administration of antiretroviral drugs can decrease the incidence of neurological disorders (Ellis et al., 2011). In a SIV model, early administration of minocycline was reported to be effective against striatal dopaminergic system dysfunction, suggesting that timely treatment initiation may have an effect on minocycline efficacy (Meulendyke et al., 2012). Since antiretroviral drugs (with the exception of maraviroc) do not have anti-inflammatory properties, a combination
therapy including antiretroviral and anti-inflammatory compounds may prevent the development of HIV-associated cognitive deficits.

We also tested chloroquine, an antimalarial drug which exhibits anti-inflammatory and anti-HIV effects in vitro and in vivo. For example, chloroquine administration has been reported to reduce T-cell activation in chronic HIV-infected patients (Murray et al., 2010). In a rodent model, chloroquine prevented a bacterial toxin-induced intracerebral toxicity (Hagihara et al., 2000). In our gp120-administered model, chloroquine was able to suppress IL-1β and iNOS mRNA levels in the frontal cortex, hippocampus and striatum. Chloroquine was also effective in reversing gp120-mediated IL-1β release in the CSF. However, chloroquine was not able to inhibit gp120-mediated release of TNF-α. Clinical trials are necessary to determine whether chloroquine could be beneficial in reducing HIV-associated cognitive impairments, particularly in populations where malaria is also prevalent. Simvastatin, an HMG-CoA reductase inhibitor, is another compound that has demonstrated anti-inflammatory properties and neuroprotective effects (Andras et al., 2008; Tong et al., 2012). In a study by Andras et al., simvastatin prevented Aβ and HIV-1 Tat-induced upregulation of inflammatory genes in human brain microvessel endothelial cells (Andras et al., 2008). In another study, this drug attenuated oxidative stress and inflammation and also restored short-term and long-term memory in a transgenic mice model of Alzheimer’s disease (Tong et al., 2012). In our study, simvastatin was able to suppress IL-1β and iNOS in the hippocampus and striatum, but had minimal effect on IL-1β and TNF-α transcript levels in the frontal cortex. Also, the 1 mg/kg dose of simvastatin was not able to attenuate the secretion of cytokines in the CSF. In contrast to several other statins, simvastatin is known to have good permeability into the brain. However, in the periphery, simvastatin is highly metabolized by cytochrome P450 enzymes which can result in adverse drug-drug interaction with HIV-PIs making it a less ideal candidate for adjuvant therapy in HIV-infected individuals. Anti-inflammatory properties of statins that are not substrate of CYPs (rosuvastatin or pravastatin) or less potent inhibitors of CYPs (atorvastatin) should be considered in future studies.

Experimental data from various in vitro models suggest the involvement of different signal transduction pathways in the regulation of cytokine secretion and drug transporters during HIV-associated inflammation (Ashraf et al., 2011; Hayashi et al., 2006; Ronaldson et al., 2010).
Potential anti-inflammatory compounds are also known to target different signaling pathways to attenuate the inflammatory response. In particular, MAPKs are widely known to be involved in various pathological processes associated with HIV-1 infection (such as neurotoxicity, macrophage activation, viral replication) (Gong et al., 2011). Studies have further demonstrated that inhibition of these kinases can downregulate HIV-1 infection as well as decrease transcellular transport of HIV-1 across BBB in vitro (Dohgu and Banks, 2008; Dohgu et al., 2011; Gong et al., 2011). Therefore, we examined these pathways in our gp120-associated brain inflammation model and observed activation of ERK1/2 and JNKs in the hippocampus. The presence of minocycline or simvastatin decreased the gp120-mediated phosphorylation of ERK1/2 but not JNKs in the hippocampus. Since a number of studies suggest that chloroquine, simvastatin and minocycline can attenuate JNK activation in vitro or in vivo, we speculate that the lack of effect of these compounds on JNK phosphorylation could be time-dependent and could not be delineated in our in vivo model (Nikodemova et al., 2006). However, using specific inhibitors of ERK1/2 and JNK pathway, we were able to attenuate the gp120-mediated activation of these two pathways in vivo.

In summary, using a gp120-induced brain inflammation model in vivo, we have demonstrated that compounds with anti-inflammatory properties have the potential to prevent or reduce brain inflammation by interacting with signaling pathways. In our model, we have also observed altered expression of drug transporters due to gp120-mediated brain inflammation in vivo. The expression of ABC transporters expressed at the BBB, in part, regulate the permeability of antiretroviral drugs into the brain. In addition, drug transporters localized at brain parenchymal cellular compartments also contribute towards the drug disposition in CNS. Alteration of these transporters due to HIV-associated pathogenesis and/or antiretroviral therapy may change the therapeutic outcome. Many different factors determine the effectiveness of antiretroviral therapy in improving neurocognitive impairment. We propose that identification of compounds with anti-inflammatory properties will significantly benefit patients with neurocognitive impairments.

8.6 Conclusions

The major findings from our work revealed that agents such as minocycline, chloroquine and simvastatin which are known to display anti-inflammatory properties, are effective in reducing
brain inflammation triggered by the HIV-1 viral envelope protein, gp120 in an *in vivo* rodent model. Furthermore, we demonstrated that this effect is mediated, in part, through an interaction with the MAPK signaling pathway. Future studies are needed to determine the efficacy of the early administration of antiretroviral drugs along with adjuvant therapy against the progression of HIV-associated brain inflammation as well as cognitive deficits.

8.7 Acknowledgements

The authors thank Dr. Min Rui and Blake Ziegler for their assistance in animal work and brain capillary work, respectively. This work is supported by a grant from the Ontario HIV Treatment Network (OHTN). Dr. Bendayan is a Career Scientist of the OHTN, Ministry of Health of Ontario.
Chapter 5
Discussion, limitations, future directions and conclusion

9 Overall discussion

HIV-1 associated neurocognitive disorders are becoming prevalent in the post-cART era, whereas, the incidence of HAD has decreased (Heaton et al., 2011; Valcour, 2013; Vivithanaporn et al., 2010). About 50% of people living with HIV infection will develop HAND. Neurological symptoms commonly experienced by infected individuals are problems with learning efficiency, memory loss, poor concentration, chronic pain, depression, fatigue and motor abnormalities. These neurological deficits significantly impact the quality of life of infected individuals (Valcour, 2013; Vivithanaporn et al., 2010). The introduction of cART is allowing people with HIV-1 to live longer, but it is not sufficient to prevent development of neurological impairments. The sequestration of the virus in brain cellular reservoirs (i.e., astrocytes, microglia) allows the virus to evade antiretroviral therapy. Studies have demonstrated that virus-infected cells or neighboring astrocytes release a number of pro-inflammatory cytokines (i.e., TNF-α, IL-6, IL-1β), chemokines, ROS, NO and other neurotoxins (Alexaki et al., 2008; Persidsky and Gendelman, 2003). Elevated inflammatory mediators have been detected in brain autopsy samples from HIV-1 infected individuals and tissue samples from HIV-1 inoculated animal models (Persidsky et al., 1997; Persidsky et al., 1999). Shed viral proteins (such as gp120, Tat and vpr) can also generate inflammatory response and oxidative stress in brain macrophages, microglia and astrocytes (Guha et al., 2012; Mangino et al., 2012; Ronaldson and Bendayan, 2008).

Brain HIV-1 infection is difficult to treat since poor penetration of antiretroviral drugs has been reported across the BBB and BCSFB due to expression of drug efflux transporters. Expression of efflux transporters has also been detected in brain parenchymal cells that can act as a secondary barrier to drug permeability in HIV-1 cellular reservoirs (Bendayan et al., 2006; Dallas et al., 2004a; Dallas et al., 2004b; Ronaldson et al., 2004a). A number of antiretroviral drugs have been shown to be substrates of one or more ABC transporters. Our laboratory has previously demonstrated P-gp and other ABC transporter-mediated efflux of antiretroviral drugs in astrocyte and microglial cell systems (Dallas et al., 2004a; Dallas et al., 2004b; Ronaldson et al., 2004a).
Altered expression of ABC transporters has been previously reported in literature due to HIV-1 associated pathologies. In order to determine the effect of HIV-1 gp120 associated pathophysiological responses (i.e., inflammation, oxidative stress) in the regulation of drug transporters, our laboratory has examined primary cultures of rat astrocytes exposed to viral envelope protein gp120. During HIV-1 infection, gp120 is shed in the systemic circulation or the extracellular fluid from cells infected with HIV-1 (Oh et al., 1992). Our group has showed that R5 tropic HIV-1 gp120 can induce secretion of pro-inflammatory cytokines (IL-6, IL1-β and TNF-α) by interacting with CCR5 chemokine receptors in primary cultures of rat astrocytes and significantly decrease functional expression of P-gp (Ronaldson and Bendayan, 2006). The effect of different cytokines on P-gp expression was also examined and the results showed profound decrease in P-gp expression due to IL-6 exposure, whereas, TNF-α or IL-1β exposure resulted in a modest enhancement in this transporter expression (Ronaldson and Bendayan, 2006).

Another ABC transporter, Mrp1, was also found to be regulated by gp120. Previous work from our laboratory has reported an increase in functional expression of this transporter in response to gp120 treatment in primary cultures of rat astrocytes which correlated with an enhanced efflux of GSH and GSSG, implying a potential role of Mrp1 in regulating oxidative stress in astrocytes (Ronaldson and Bendayan, 2008). Whether Mrp1 is regulated by gp120-associated inflammation in astrocytes was not known. Therefore, we designed this part of our study to primarily investigate the regulation of Mrp1 during HIV-1 gp120-associated inflammation and identify which intracellular signaling pathways may be involved. TNF-α was the only cytokine identified to alter Mrp1 protein expression in our in vitro model, whereas, IL-1β and IL-6 did not have any significant effect. We observed that Mrp1 protein expression was increased in primary cultures of rat astrocytes treated with TNF-α. Increased transcript levels of Mrp1 were also detected after 6 hours of treatment with both gp120 and TNF-α (Ronaldson et al., 2010).

Previously, increased production of TNF-α and IL-6 as well as increased mRNA expression of MRP1 has been reported in human monocyte derived macrophages infected with HIV-1 BAL, a R5-tropic viral strain (Jorajuria et al., 2004). However, secretion of cytokines were found not to be correlated to altered expression of MRP1 in their system (Jorajuria et al., 2004). Inflammation-associated alteration of Mrp1 has been reported by other groups. Cherrington et al. has reported upregulation of hepatic Mrp1 in LPS administered Sprague-Dawley rats.
Lee and Piquette-Miller have reported cytokine-mediated upregulation of Mrp functional activity in hepatoma cell lines where exposure to IL-6 or IL-1β resulted in significant increase in Mrp1 transcript levels as well as functional activity (Lee and Piquette-Miller, 2001). However, IL-6 or IL-1β exposure did not alter Mrp1 expression in our system of cultured astrocytes. Since transporter expression, localization, activity, and regulation may vary between tissues, it is likely that the differences observed between our findings and the study of Lee and Piquette-Miller is due to tissue-specific regulation.

We have also confirmed the involvement of the JNK and NF-κB pathways in the regulation of Mrp1 in primary cultures of rat astrocytes. In order to evaluate the involvement of NF-κB signaling, we used two established inhibitors of this signaling pathway (i.e., SN50, BAY 11-7082) (Ronaldson et al., 2010). Similarly, we studied the role of JNK signaling using SP600125, an established and well-accepted inhibitor of this pathway (Ronaldson et al., 2010). We have demonstrated that gp120-mediated upregulation of Mrp1 was attenuated in the presence of SN50 or Bay-117082, two NF-κB inhibitors, suggesting the involvement of this transcription factor in Mrp1 regulation. However, the presence of SN50 did not block TNF-α-mediated Mrp1 upregulation indicating that this pathway does not directly regulate Mrp1 protein expression. In contrast, pretreatment with SP600125, an inhibitor of the JNK pathway, attenuated the observed TNF-α-mediated increase in Mrp1 protein expression. In addition, SP600125 treatment resulted in the attenuation of BCECF efflux from astrocytes suggesting that JNKs directly participate in the functional regulation of Mrp1 transporter during TNF-α exposure. Others have also investigated the involvement of signaling pathways in the regulation of Mrp1 in the context of HIV-1 infection. For example, Hayashi et al. demonstrated Tat induced upregulation of Mrp1 at the mRNA and protein level in cultured astrocytes with an involvement of the MAPK pathway (Hayashi et al., 2006). In summary, our results demonstrate the involvement of two intracellular signaling cascades in the regulation of Mrp1 functional expression in cultured rat astrocytes. In this study, we have demonstrated for the first time that NF-κB is not directly involved in the regulation of Mrp1, but mediates its effect by activating JNK pathway which in turn results in the release of TNF-α (Ronaldson et al., 2010).

We have also paralleled some of the key findings regarding cytokine secretion and functional expression of P-gp in HFAs (Ashraf et al., 2011). We have demonstrated that similar to rat...
astrocytes, R5-tropic gp120 induces secretion of IL-6, IL-1β and TNF-α in HFAs. Pretreatment with CCR5 neutralizing antibody attenuated cytokine secretion suggesting that cytokine production was mediated via gp120-CCR5 interactions. The regulation of P-gp expression by HIV-1 in human astrocytes was not known prior to the present study. Therefore, we investigated the effect of HIV-1 isolates on P-gp protein expression in our in vitro model. Exposure to macrophage (R5) or dual macrophage/T-lymphocyte (R5/X4)-tropic viral isolates decreased P-gp expression suggesting a downregulatory effect of HIV-1 on P-gp expression. A low number of astrocytes are known to be infected in vitro (3 to 19%). Therefore, the downregulation of P-gp expression could be due to inflammatory mediators released by astrocytes during HIV-1 exposure. Li et al. previously reported that HIV-1 can induce cytokine secretion in HFAs independent of infection. In their study, high levels of IL-6 were detected after astrocytes were treated with HIV-1. We speculate that profound downregulation of P-gp expression in our cultured HFAs could be due to HIV-associated cytokine release (Li et al., 2007). Previously, Gollapudi and Gupta reported upregulation of P-gp expression in HIV-1 infected T-cell line and in a monocytic cell line when subjected to HIV-1 infection (Gollapudi and Gupta, 1990). Since T-cells or monocytes harbour productive HIV-1 infection and astrocytes harbor latent infection, these discrepancies in the regulation of P-gp expression by HIV-1 could be due to different cell types. In HFAs, treatment with gp120 also reduced P-gp expression by 64% and increased cellular accumulation of digoxin, an established substrate of P-gp. Previously, decreased P-gp protein expression has been reported in brain autopsy samples from patients with HIVE and in brain tissue from SCID mice model of HIVE (Persidsky et al., 2000). Langford and colleagues used post-mortem tissues from patients who were on antiretroviral therapy and examined P-gp expression (Langford et al., 2004). An increased P-gp immunoreactivity in glial cells was detected in these brain autopsy tissues from patients with HIVE. However, of the 26 patients selected in this study, 16 were on antiretroviral therapy and for the remaining 10 patients, treatment information was not available. There was no comparison of P-gp expression between treatment-naive patients and patients on antiretroviral therapy (Langford et al., 2004). Data from a number of published studies in the literature including work from our own laboratory suggest that antiretroviral therapy can alter the expression of ABC transporters (Chandler et al., 2003; Zastre et al., 2009). Using HCMEC/D3 cell system, our group has demonstrated that in the presence of atazanavir or ritonavir, two HIV-PIs, a significant upregulation in P-gp functional
expression occurs (Zastre et al., 2009). Since antiretroviral drugs can modulate the expression of transporters in vitro and in vivo, it is still inconclusive if this increased P-gp immunoreactivity is due to therapy itself or due to HIV-associated pathogenesis. In both murine brain microvessel endothelial cells and astrocytes, HIV-1 Tat exposure resulted in an upregulation of P-gp expression (Hayashi et al., 2005). Since the role and mechanism of action of Tat and gp120 are different, we propose that different viral proteins may activate signaling pathways differently and in turn, have different effects on P-gp expression.

In HFAs, IL-6 treatment decreased P-gp expression by 50% after 12 hours of treatment. This decrease was not found to be as profound as previously observed in primary cultures of rat astrocytes (Ronaldson and Bendayan, 2006). However, endogenous levels of IL-6 differed between the culture supernatants of rat and human astrocytes. IL-6 was undetectable in primary cultures of rat astrocytes, whereas, approximately 182.36 pg/ml IL-6 was detected in the untreated cultured HFAs. Therefore, it is likely that these cells are continuously exposed to endogenous IL-6 levels and IL-6 mediated decrease in P-gp expression may differ between human and rat astrocytes due to elevated basal IL-6 levels. We have also conducted longer exposure and demonstrated that with prolonged exposure (36h and 48h) to IL-6, there was no change in P-gp expression. Previously, downregulation of P-gp expression due to IL-6 exposure have been reported in different cells or tissues suggesting that this cytokine may have a down-regulatory effect on P-gp expression. Lee and Piquette-Miller have shown that IL-6 exposure results in a reduced MDR1 gene expression and P-gp activity in human hepatocytes (Lee and Piquette-Miller, 2001). Increase in IL-6 serum levels was also found to be correlated with decrease P-gp protein expression in the intestine (Ogura et al., 2008). IL-6 induced downregulation of P-gp expression has also been demonstrated in the human brain microvascular endothelial cell line, HCMEC/D3 cells (Poller et al., 2010).

Results from our study in HFAs also demonstrated that gp120 or IL-6 mediated effect on P-gp was attenuated by SN50 suggesting involvement of NF-κB signaling in P-gp downregulation. Although, in primary cultures of HFAs, due to the limitation in obtaining human fetal brain tissue, we have not investigated the secretion of cytokines in the presence of an NF-κB inhibitor, we speculate that gp120 mediated secretion of IL-6 is regulated by NF-κB and therefore, by inhibiting NF-κB, we can attenuate P-gp downregulation.
Our results demonstrate that HIV-1 as well as the viral protein gp120 has a downregulatory effect on P-gp expression suggesting that astrocytes may be involved in antiretroviral drug sequestration within brain parenchyma during HIV-1 infection. Previous work done by our group in rodent astrocytes also support these findings where an increased accumulation of the HIV-PI, saquinavir, was observed in primary cultures of rat astrocytes treated with gp120 compared to untreated astrocytes (Ronaldson and Bendayan, 2006). The availability of antiretroviral drugs within the astrocytes may aid in limiting brain viral infection by preventing the latent virus from becoming infectious. However, increased sequestration of antiretroviral drugs into astrocytes may also lead to reduced concentrations at sites of cytopathic infection (i.e., microglia) and may contribute to the development of viral drug resistance. At present, it is not known whether these transporters are altered in microglia. Besides, other transporters of the ABC family are also known to be involved in antiretroviral drug transport. For example, MRP1 is also known to transport several PIs used in antiretroviral therapy (Dallas et al., 2004a; Janneh et al., 2005).

Using rodent astrocytes, we have shown that gp120 exposure results in an upregulation of Mrp1 expression in primary cultures of rat astrocytes (Ronaldson et al., 2010; Ronaldson and Bendayan, 2008). The regulation of these transporters during HIV-associated neuroinflammatory response will also contribute towards the overall antiretroviral drug permeability in astrocytes and microglia. Further in vivo studies are needed to clarify whether changes in transporter expression in astrocytes and microglia will affect antiretroviral drug distribution in the parenchyma in vivo. Overall, our data provide evidence that the regulation of ABC transporters is highly complex in HIV-associated brain inflammation and may result in altered brain permeability of antiretroviral drugs.

Our in vitro work suggest that gp120 can result in an inflammatory response in astrocytes and alters functional expression of drug efflux transporters. However, whether gp120 can interact with CCR5 receptor in a similar manner in vivo in order to generate an inflammatory response is not known. In addition, it has not been examined if gp120-associated inflammation can alter the expression of drug transporters in vivo. In order to address these questions, we implemented an in vivo rodent model of HIV-associated brain inflammation by administering HIV-1 gp120 into one of the lateral cerebral ventricles. In gp120-injected rats, transcripts of TNF-α, IL-1β and iNOS were found to be significantly elevated in different brain regions. A significant increase in
TNF-α and IL-1β secretion was also detected in the CSF (Ashraf et al., 2014). HIV-1 gp120 has previously been shown to generate similar inflammatory response in vivo (Bagetta et al., 1999; Corasaniti et al., 1998; Louboutin et al., 2010b). Previous studies have demonstrated gp120-mediated induction of IL-1β and TNF-α mRNA in the hypothalamus of gp120 administered rats (Ilyin and Plata-Salaman, 1997). Another study has reported IL-1β associated apoptotic cell death in the cortex of rodent brain after ICV administration of gp120 (Bagetta et al., 1999). Administration of maraviroc, a CCR5-specific antagonist, prior to gp120 treatment attenuated upregulation of these inflammatory markers in our model. Previously, we have confirmed that the interaction of R5-tropic gp120 to CCR5 receptor in primary cultures of rat and human astrocytes is critical for induction of an inflammatory response (Ashraf et al., 2011; Ronaldson and Bendayan, 2006). In our present study, for the first time, we have confirmed that similar interaction takes place in vivo and is necessary for generating gp120-mediated inflammatory response (Ashraf et al., 2014).

In our in vivo model, we have also observed an increase in GFAP expression in hippocampal lysates isolated from gp120-treated animals indicating activation of astrocytes. However, no significant change was observed in tight junction protein (ZO-1, occludin and claudin5) or drug transporter expressions in brain capillaries isolated from gp120 administered rats (Ashraf et al., 2014). A number of studies have previously shown downregulation of tight junction proteins in brain microvessel endothelial cells or in vivo models of HIV-1 or gp120-associated brain inflammation (Kanmogne et al., 2005). The lack of changes in tight junction proteins in our model is possibly due to the dose and duration of gp120 administration.

Following gp120 administration, changes in drug transporters were observed in different brain regions. Altered P-gp, Mrp1 and Bcrp were observed in different regions suggesting that gp120-associated inflammation can modulate the expression of antiretroviral drug transporters in vivo. In our previous work, we observed downregulation of P-gp and upregulation of Mrp1 in cultured astrocytes triggered with gp120 (Ronaldson et al., 2010). In our animal model, we observed downregulation of P-gp protein expression in frontal cortex and hippocampus confirming that gp120 has a down-regulatory effect on P-gp functional expression in brain parenchyma. Significant upregulation of Mrp1 in hippocampus and striatum was detected further suggesting that gp120 exerts an up-regulatory effect on Mrp1 protein expression in vivo. Bcrp expression
was also found to be upregulated in frontal cortex suggesting that similar to other drug efflux transporters, this transporter is also susceptible to gp120-associated brain inflammation.

Since a number of antiretroviral drugs penetrate poorly in the brain and lack anti-inflammatory properties, many different compounds are being investigated that can potentially be used as adjuvant therapy to prevent the development of HIV-1-associated neurocognitive deficits. In order to evaluate the potential efficacy of different anti-inflammatory compounds (minocycline, chloroquine and simvastatin) against inflammation in this in vivo rodent model, the animals were administered gp120 ICV with or without prior intraperitoneal administration of minocycline or chloroquine or simvastatin. Minocycline, a second generation tetracycline derivative, showed the most potency in reducing gp120-associated increase in inflammatory markers. Minocycline was successful in suppressing both TNF-α and IL-1β secretion in the CSF of gp120 administered animals. A number of other in vivo studies have shown protective effects of minocycline against brain HIV-1 infection. Minocycline significantly reduced encephalitis in SIV-infected pigtailed macaques (Zink et al., 2005). Suppressed CNS viral load and decreased CNS inflammatory markers (e.g. macrophage marker CD68, major histocompatibility complex class II, T-cell intracytoplasmic antigen 1, SIV glycoprotein 41, Aβ precursor protein, and activated p38) were observed after minocycline treatment (Zink et al., 2005). Striatal dopamine deficiency was also reversed by early administration of minocycline in SIV-infected macaques (Meulendyke et al., 2012). Using proton magnetic resonance spectroscopy in vivo and biomarkers in post-mortem tissues, it was reported that minocycline could attenuate a progressive decline in neuronal integrity, decrease glial activation and both CSF and plasma viral loads (Ratai et al., 2010). Decreased plasma virus and monocyte activation was also observed in SIV-infected macaques following minocycline administration (Campbell et al., 2011). However, in clinical trials, minocycline was not successful in improving neurocognitive outcome in patients with advanced cognitive impairments (Nakasujja et al., 2013; Sacktor et al., 2011). We speculate that administering minocycline in early stage of the infection may prevent development of HAND. In addition, it is still unclear if clinically prescribed doses of minocycline would achieve plasma and cellular levels that would be effective in suppressing HIV-1 replication and associated inflammation in the brain. Interaction with other antiretroviral drugs also has to be considered. One study reported that administration of minocycline altered the plasma concentration of
atazanavir, but did not have any significant effect on ritonavir plasma concentrations (DiCenzo et al., 2008). However, this study was limited due to the small sample size, short study duration and concomitant medications used by patients. It is yet to be examined whether long-term administration of minocycline with atazanavir and other PIs can influence treatment response. Other clinical trials with minocycline reported a safety profile for this drug when administered along with other antiretroviral drugs for 24 weeks (Sacktor et al., 2011). Further studies are needed to clarify the clinical role of early administration of minocycline in the treatment of HIV-associated neurological disorder.

In our animal model, chloroquine, an anti-malarial drug, was able to suppress IL-1β and iNOS mRNA levels in different brain regions and was also effective in reversing gp120-mediated IL-1β release in the CSF. Chloroquine has been previously reported to inhibit in vitro replication of HIV-1 by modulating glycosylation properties of gp120 (Savarino et al., 2004). In clinical trials, hydroxychloroquine has decreased plasma viral load. Hydroxychloroquine treatment has also been shown to decrease IL-1α and IL-6 secretion in human monocytes and T-cells (Sperber et al., 1993). In later stage of SIV-infected macaques, chloroquine treatment decreased activation of plasma dendritic cells and IFN-α secretion (Ma et al., 2012). In contrast, chloroquine transiently increased IFN-α secretion and did not provide any benefit against SIV infection in acute SIV infected macaques (Vaccari et al., 2014). In addition, in several clinical studies, chloroquine or hydroxychloroquine treatment failed to reduce viral load, CD4+ T-cell activation and plasma inflammatory markers (Engchanil et al., 2006; Paton et al., 2012; Routy et al., 2014). Therefore, it remains unclear whether chloroquine can confer anti-HIV effects and elicit a therapeutic response. Further studies are needed to investigate the effect of chloroquine used in conjunction with antiretroviral drugs against the development of HIV-1 associated neurocognitive disorder. If proven beneficial, the cost and availability of chloroquine will make it an ideal candidate for adjuvant therapy in resource-poor countries where both malaria and HIV-1 are prevalent.

Simvastatin, an HMG-CoA reductase inhibitor known to possess anti-inflammatory properties, administration did not demonstrate similar efficacy compared to minocycline or chloroquine. Although simvastatin attenuated transcripts of IL-1β and iNOS in the brain regions, it could not significantly suppress gp120-mediated IL-1β and TNF-α secretion in the CSF. This lack of effect
could be attributed to the dose of simvastatin used in our study, since higher doses of simvastatin have been reported to be successful in attenuating brain inflammation in other models of brain injury (Li et al., 2009). Many HIV-1 infected patients have dyslipidemia that may require statin treatment. However, a number of statins including simvastatin is metabolized by cytochrome P450 enzymes in the periphery and administering them with PIs can result in adverse drug-drug interactions. Statins that are not metabolized by cytochrome P450 enzymes (rosuvastatin or pravastatin) are known not to permeate well in the brain. Further studies are required to determine if statins can be beneficial in preventing HIV-1-associated cognitive deficits.

Our previous in vitro work demonstrated the involvement of MAPK pathway in the regulation of drug efflux transporters. We have reported involvement of JNK signaling pathway in the regulation of Mrp1 in primary cultures of rat astrocytes (Ronaldson et al., 2010). Previous work from our group also reported involvement of ERK1/2, JNKs and P38Ks in gp120-mediated downregulation of P-gp in cultured rodent astrocytes (unpublished work). Therefore, we investigated the activation of MAPK signaling pathways in different brain regions of gp120-administered animals. In our in vivo model, we observed activation of ERK1/2 and JNKs in the hippocampus (Ashraf et al., 2014). Interestingly, the presence of minocycline or simvastatin attenuated the gp120-mediated phosphorylation of ERK1/2 in the hippocampus. None of the compounds tested were able to attenuate JNK activation observed in the hippocampus in the gp120 treated animals. Although in primary cultures of rat astrocytes, we have shown attenuation of JNKs as well as ERK1/2 phosphorylation in the presence of all three compounds (Appendix C). Using specific inhibitors of ERK1/2 and JNK pathway, we were also able to attenuate the gp120-mediated activation of these two pathways in vivo (Ashraf et al., 2014). These findings further confirm that signaling pathways can be targeted by anti-inflammatory compounds or inhibitors in order to reverse inflammatory response observed during brain HIV-1 infection. Several studies have investigated the in vivo efficacy of MAPK pathway inhibitors in attenuating brain inflammation (Eggert et al., 2010; Munoz et al., 2007). In addition to MAPK pathways, other pathways have also been implicated. For example, treatment with statins has been reported to reduce mRNA expression of LPS-induced TNF-α and MCP1 in macrophage cell line via a PPARα and PPARγ dependent pathway (Yano et al., 2007). Therefore, the mechanisms of action
of these compounds need to be further assessed in cell culture models as well as in animal models that will provide future opportunities of testing them in clinical trials.

In summary, our results suggest that interaction of R5-tropic gp120 to CCR5 chemokine receptor *in vitro* or *in vivo* results in the activation of signaling pathways and secretion of cytokines. These cytokines can further activate other signaling pathways that can result in alteration of drug efflux transporters. Inhibitors of signaling pathways also prevented cytokine release and cytokine-mediated regulation of drug transporters. Both JNK and NF-κB pathways were found to be involved in gp120-mediated Mrp1 regulation *in vitro*. NF-κB pathway was involved in gp120-mediated downregulation of P-gp in human astrocytes. Administration of maraviroc or anti-inflammatory compounds prevented the release of these cytokines. *In vivo*, maraviroc, minocycline and chloroquine attenuated gp120-mediated release of IL-1β in the CSF. Maraviroc and minocycline also attenuated TNF-α release. The anti-inflammatory compounds also interacted with MAPK pathways *in vitro* or *in vivo*. Minocycline, chloroquine and simvastatin attenuated gp120-mediated activation of JNKs as well as ERK1/2 *in vitro*, whereas, minocycline and simvastatin decreased gp120-induced EKR1/2 phosphorylation *in vivo*. Signaling pathway inhibitors U0126 and SP600125 also prevented gp120-mediated activation of ERK1/2 and JNKs *in vivo*, respectively, indicating the potential of these inhibitors in reversing brain inflammation. Figure 9-1 summarizes our findings *in vitro* and *in vivo*. 
**Figure 9-1** A schematic summarizing gp120-mediated regulation of cytokine secretion and drug efflux transporters as well as effect of anti-inflammatory compounds *in vitro* or *in vivo*. 
10 Limitations

The use of rodent cell culture systems (i.e., primary cultures of rat astrocytes) is well-accepted as a useful model system for *in vitro* studies. A number of studies have examined the functional expression of drug efflux transporters in rodent cell culture systems. However, species specific differences may exist in the regulation of cytokine secretion or drug efflux transporters (Ashraf et al., 2011; Ronaldson and Bendayan, 2006). We have paralleled some key findings in HFA cultures to compare the regulation of P-gp by gp120-mediated cytokine secretion. However, due to the difficulty in obtaining human fetal brain tissue in a regular basis, it became difficult to perform a detailed investigation of the regulation of transporters and involvement of signaling pathways in the context of HIV-1 gp120-associated inflammation in human astrocytes. In addition, it is known that astrocytes in the adult brain are non-proliferative unless activated in response to infection or injury. Therefore, our astrocyte cultures obtained from neonatal rat brains or human fetal brain may not be reflective of adult astrocytes and *in vivo* studies may be undertaken to complement the data obtained in these cultured systems. Several studies have described techniques for isolation of primary cultures of human astrocytes from post-mortem or surgically resected adult brain tissue (De Groot et al., 1997; Verwer et al., 2007). The problem with the use of such protocols stems from the availability of brain tissue samples on a regular basis from which to prepare the cultures. Therefore, rodent neonatal brain and to a lesser extent, human fetal brain tissue still represent a widely used source for astrocytes due to their relative ease of collection and maintenance.

In our recent work, we have determined the effect of an individual HIV-1 protein i.e., gp120 on generating an inflammatory response in cultured astrocytes as well as in rodent brain. It is important to understand the role of individual viral proteins in the regulation of pro-inflammatory cytokines as well as drug efflux transporters. Our work with gp120 has allowed us to understand part of the pathology associated with HIV-1 in the brain. However, an HIV-1 infected model is necessary to understand the activation of signaling pathways, secretion of cytokines and regulation of transporters by the whole virus. Therefore, a better model to evaluate the mechanisms involved in inflammation and drug resistance in HIV-1 infection of the brain will be infectious animal models (i.e., SIV infected macaques).
Our in vitro model has allowed us to examine the secretion of cytokines and the regulation of these transporters after short-term exposures (i.e., 24 hours) only. Therefore, we have implemented a model to investigate these phenomena in vivo after 7-day exposure. We have observed a significant increase in gene and protein expression of inflammatory markers as well as alteration of transporter protein expression. However, we could not characterize the distribution of gp120 in our model after ICV administration. In order to determine the brain distribution of gp120, we have administered fluorescein isothiocyanate (FITC)-gp120 ICV in a volume of 2µl (250ng/µl) or 2.5 µl (1 µg/µl) or 5µl (1 µg/µl) in Wistar rats. After FITC-gp120 administration, animals were sacrificed after 13 minutes, 30 minutes and 2 hours. The collected brain tissue was processed for immunohistochemistry. We also collected different brain regions (frontal cortex, hippocampus and striatum) after FITC-gp120 ICV administration and used tissue homogenate to detect the presence of FITC using spectrophotometry compared to saline administered animals. We were not able to demonstrate the brain distribution of gp120 at these above mentioned doses. Previous studies with ICV administration of FITC conjugated proteins used significantly higher concentration of proteins (40 to 100 µg) in order to demonstrate their brain distribution (Frigerio et al., 2012). However, administration of gp120 in a higher concentration may lead to significant damage to the BBB and brain parenchyma rendering it difficult to determine the brain distribution of gp120.

11 Future directions

11.1 Regulation of ABC transporters at the BBB and in glial cells

Our work has demonstrated that P-gp is regulated by the NF-κB pathway in primary cultures of HFAs and Mrp1 is regulated by NF-κB and JNK pathways (Ronaldson and Bendayan, 2006). Our laboratory has also investigated the involvement of the MAPK pathway in the regulation of P-gp in cultured astrocytes triggered with gp120 (unpublished work). It is still not known whether gp120 can modulate the expression of these transporters in brain microvessel endothelial cells. In addition, regulation of these transporters in microglia, the primary site of HIV-1 in the brain, has not been examined. A number of other signaling pathways or transcription factors have been implicated in the regulation of transporters. Nrf2, a sensor of oxidative stress, has been
reported to be involved in regulating Mrp1 expression in mouse embryo fibroblasts (Hayashi et al., 2003). The Rho signaling has been reported to be involved in the regulation of P-gp (Zhong et al., 2010). In endothelial cells, intact lipid rafts were found to be involved in HIV-1 Tat-mediated activation of Rho signaling and upregulation of P-gp (Zhong et al., 2010). A number of nuclear receptors have been implicated in the regulation of ABC transporters. In particular, PXR and CAR are two well-known xenobiotic sensors that are capable of interacting with a wide spectrum of pharmaceutical agents including PIs (Urquhart et al., 2007). Work published by others and our laboratory show that PXR and CAR are actively involved in the regulation of drug efflux transporters in different tissues including intestine, liver and brain (Bauer et al., 2004; Bauer et al., 2008; Bauer et al., 2006; Chan et al., 2013a; Chan et al., 2011; Wang et al., 2010b). Our group has shown that in the presence of atazanavir or ritonavir, two HIV-Pis, a PXR dependent induction in P-gp functional expression occurs in HCMC/D3 cells (Chan et al., 2013b; Zastre et al., 2009). Several nuclear receptors (i.e., CAR, PPARγ, PPARα) have also been implicated in ABCG2 regulation (Hartz et al., 2010a; Hirai et al., 2007; Szatmari et al., 2006). Recent work from our laboratory also demonstrated that PPARα-selective agonists (i.e., clofibrate, GW7647) can significantly induce ABCG2 mRNA and protein expression in HCMC/D3 cells, whereas pharmacological inhibitors (i.e., MK886, GW6471) prevent this induction (Hoque et al., 2012). The involvement of these nuclear receptors in the regulation of ABC transporters in astrocytes and microglia are not understood and further studies are needed to elucidate their role.

11.2 Effect of anti-inflammatory compounds on HIV-1 gp120 associated cognitive deficits

In order to demonstrate that early administration of anti-inflammatory compounds can prevent the development of HIV-1 gp120 associated cognitive deficits, our laboratory has recently implemented an in vivo model of HIV-1 gp120 associated cognitive deficits by ICV administration of gp120 in Wistar male rats. The learning and memory components are currently being characterized in this model using Morris water maze. Once characterized, the efficacy of different anti-inflammatory compounds (i.e., minocycline, chloroquine) in the prevention of cognitive impairments will be assessed in this model. Several other compounds have been reported to have anti-inflammatory properties. For example neurosteroid,
dehydroepiandrosterone sulfate has been reported to successfully prevent inflammation and behavioral deficits in a FIV model (Maingat et al., 2009). Another study reported that administration of anthraquinone derivative from rhubarb, emodin, showed anti-inflammatory properties by inhibiting NO and PGE2 production and downregulating COX-2 and iNOS expression in caco-2 cells (Choi et al., 2013). Several signaling pathway inhibitors have also been demonstrated to have neuroprotective properties. For example, CEP-1347, an inhibitor of multilineage kinase, has shown to have reduced microgliosis and neuronal loss in a SCID mice model of HIVE (Eggert et al., 2010). A brain penetrable inhibitor of P38α, MW01-2-069A-SRM, has been demonstrated to suppress pro-inflammatory cytokine upregulation as well as behavioral deficits in an Alzheimer’s disease mouse model (Munoz et al., 2007). In order to prevent or delay the progression of HIV-1-associated neurological disorder, it is critical to identify compounds that have anti-inflammatory properties and penetrate well into the brain.

11.3 Permeability of antiretroviral drugs during HIV-1 gp120-associated brain inflammation in vivo

Our laboratory has previously demonstrated that gp120-mediated inflammation can alter the functional expression of P-gp and Mrp1 in vitro (Ashraf et al., 2011; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008). Recently, we have also demonstrated that administration of gp120 in vivo resulted in altered P-gp, Mrp1 and Bcrp in different brain regions (Ashraf et al., 2014). However, it is yet to be demonstrated whether the altered expression of these transporters affect antiretroviral drug distribution in vivo. In our implemented gp120-associated brain inflammation model, we did not detect changes in transporter expression at the level of brain capillaries and anticipate that drug permeability will not be altered. Lack of changes at the brain capillaries could be due to the moderate inflammatory response observed in our model. We speculate that in the gp120-associated cognitive deficit model there will be significant damages to the BBB which will allow investigating the permeability of antiretroviral drugs in vivo during HIV-associated neuropathological condition.
12 Conclusion

Limited drug penetration in the brain remains a major obstacle to pharmacotherapy for brain HIV-1 infection. The expression of ABC transporters at the BBB as well as in brain HIV-1 cellular targets (i.e., microglia, astrocytes) determines the permeability and disposition of antiretroviral drugs in the brain. Our work has demonstrated that HIV-1 gp120-associated inflammation can change the expression of P-gp and Mrp1 in astrocytes suggesting that antiretroviral drug permeability may be significantly altered at brain parenchymal cellular compartments during brain HIV-1 infection. Our current findings also suggest a role of different signaling pathways in the regulation of cytokines and drug efflux transporters. We have shown that agents such as minocycline, chloroquine and simvastatin are effective in reducing brain inflammation triggered by HIV-1 gp120 in vivo and this effect is mediated, in part, through an interaction with the MAPK signaling pathway. Future studies are needed to determine the efficacy of the early administration of antiretroviral drugs along with adjuvant therapy against the progression of HIV-associated brain inflammation as well as cognitive deficits. In order to successfully treat brain HIV-1 infection, a more comprehensive understanding of antiretroviral drug transporters and anti-inflammatory compounds as well as their regulatory mechanisms in the context of HIV-1 infection is required both in vitro and in vivo.


Cherrington NJ, Hartley DP, Li N, Johnson DR and Klaassen CD (2002) Organ Distribution of Multidrug Resistance Proteins 1, 2, and 3 (Mrp1, 2, and 3) mRNA and Hepatic Induction of Mrp3 by Constitutive Androstane Receptor Activators in Rats. *J Pharmacol Exp Ther* 300:97-104.


Leslie EM, Deeley RG and Cole SP (2001) Toxicological Relevance of the Multidrug Resistance Protein 1, MRP1 (ABCC1) and Related Transporters. *Toxicology* 167:3-23.


Appendices

Appendix A: Data not shown in chapter 2

Figure A 1: Immunoblot analysis of P-gp in primary cultures of HFAs treated with TNF-α (0.5 and 10ng/ml). MDR1 overexpressing MDCK-MDR1 cell lysate was used as positive control. Whole cell lysates (50 µg) from primary cultures of HFAs were resolved on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Results are expressed as mean ± SD of three separate experiments. Asterisks represent data points that are significantly different from control (*p<0.05).
Appendix B: Data not shown in chapter 5

A. IL1\( \beta \) relative mRNA expression (% Control) (Normalized to cyclophilin)

B. IL6 relative mRNA expression (% control) (Normalized to cyclophilin)
Figure B 1: Effect of two different doses of gp120 (300ng or 500 ng) on the mRNA levels of A) IL-1β, B) IL-6, C) iNOS and D) TNF-α in different brain regions of Wistar rats after ICV administration of gp120. Saline injected animals were used as control. Results are expressed as mean±SEM. (*) represent data point significantly different from control (p<0.05). Samples obtained from at least six different animals were analyzed per group.
A. Serum

![Graph showing IL-1β levels in Saline and gp120_600ng]

B. Serum

![Graph showing IL-6 levels in Saline and gp120 (500ng)]
Figure B 2: ELISA analysis of A) IL-1β, B) IL-6 and C) TNF-α in the serum of gp120 (500ng) administered rats. Serum collected from saline injected animals were used as control. Results are expressed as mean±SEM. Samples obtained from at least eight different animals were used per group.
Figure B 3: ELISA analysis of IL-6 secretion in the CSF of gp120 (500ng) administered rats. CSF collected from saline injected animals were used as control. Results are expressed as mean±SEM. Samples obtained from at least eight different animals were used per group.
Brain capillaries

**Figure B 4:** Immunoblot (upper panel) and densitometric analysis (lower panel) of ZO-1, occludin and claudin 5 in rat brain capillaries isolated from HIV-1 gp120 or saline administered rats. Caco-2 cell lysate was used as control. Results are expressed as mean±SEM. Samples obtained from eight different animals were used per group.
Figure B 5: Immunoblot (upper panel) and densitometric analysis (lower panel) of P-gp, Mrp1 and Bcrp in rat brain capillaries isolated from HIV-1 gp120 or saline administered rats. Transporter overexpressing cell lines were used as positive controls. Results are expressed as mean±SEM. Samples obtained from eight different animals were used per group.
Figure B 6: Immunoblot (upper panel) and densitometric analysis (lower panel) of Mrp1 in hippocampus of ICV administered gp120 rats in the presence or absence of JNK inhibitor, SP600125. Hela-MRP1 cell lysate was used as positive control. Representative blots are shown. Results are expressed as mean±SEM. Samples obtained from six different animals were used per group. Asterisk represent data point significantly different from saline administered animals (*p<0.05). Diamonds represent data point significantly different from gp120-treatment (♦♦ ♦♦ p<0.01).
Figure B 7: Immunoblot (upper panel) and densitometric analysis (lower panel) of Mrp1 in hippocampus of ICV administered gp120 rats with or without minocycline treatment. Hela-MRP1 cell lysate was used as positive control. Representative blots are shown. Results are expressed as mean±SEM. Samples obtained from six different animals were used per group. Asterisk represent data point significantly different from saline administered animals (*p<0.05). Diamonds represent data point significantly different from gp120-treatment (♦ p<0.05).
**Figure B 8:** Immunoblot (upper panel) and densitometric analysis (lower panel) of ERK1/2 and phospho-ERK1/2 in primary cultures of rat astrocytes treated with gp120 (1nM) in the presence or absence of 1 µM chloroquine, 100 nM simvastatin and 60 µM minocycline. Results are expressed as mean±SEM. Samples obtained from three different experiments were used per group. Asterisk represent data point significantly different from control (*p<0.05) (CQ= chloroquine, mino= minocycline, sim=simvastatin).
Figure B 9: Immunoblot (upper panel) and densitometric analysis (lower panel) of JNK and phospho-JNK in primary cultures of rat astrocytes treated with gp120 (1nM) in the presence or absence of 1 µM chloroquine, 100 nM simvastatin and 60 µM minocycline. Results are expressed as mean±SEM. Samples obtained from three different experiments were used per group. Asterisk represent data point significantly different from control (*p<0.05) (CQ= chloroquine, mino= minocycline, sim=simvastatin).
List of publications


Copyright Acknowledgements

Copyright clearance for sections 1.1, 1.2, 1.3, 1.5, 1.6, 1.7, 1.9.3; figures 1-1, 1-2 and tables 1-1, 1-2 and 1-3:

BENTHAM SCIENCE PUBLISHERS LICENSE
TERMS AND CONDITIONS
Sep 08, 2014

This is a License Agreement between Tamima Ashraf ("You") and Bentham Science Publishers ("Bentham Science Publishers") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Bentham Science Publishers, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number: 3464290277235
License date: Sep 08, 2014
Licensed content publisher: Bentham Science Publishers
Licensed content publication: Current Pharmaceutical Design
Licensed content title: Role of CNS Transporters in the Pharmacotherapy of HIV-1 Associated Neurological Disorders
Licensed copyright line: Copyright © 2014, Eureka Science Ltd.
Licensed content author: Tamima Ashraf, Kevin Robillard, Gary N.Y. Chan and Reina Bendayan
Licensed content date: 28 February, 2014
Type of Use: Thesis/Dissertation
Requestor type: Author of requested content
Format: Print, Electronic
Portion: chapter/article
Rights for: Main product
Duration of use: Life of current/future editions
Creation of copies for the disabled: no
With minor editing privileges: no
In the following language(s) with incidental promotional use The lifetime unit quantity of new product The requesting person/organization is: Title of your thesis / dissertation Expected completion date Expected size (number of pages) Billing Type Billing address

Original language of publication no 0 to 499 Tamima Ashraf, university of Toronto Regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: Implications in HIV-1 neuropathogenesis and its treatment Nov 2014 220 Invoice 1055 Southdown road, unit 403 Mississauga, ON L5J0A3 Canada

Total

0.00 USD

Terms and Conditions

STANDARD TERMS AND CONDITIONS FOR REPRODUCTION OF MATERIAL

Introduction

The publisher for this copyrighted material is Bentham Science Publishers Ltd. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your CCC account and that are available at any time at http://myaccount.copyright.com.

Limited License

Publisher hereby grants to you a non-exclusive license to use this material. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process; any form of republication must be completed within 180 days from the date hereof (although copies prepared before then may be distributed thereafter); and any electronic posting is limited to a period of 180 days.
Geographic Rights: Scope
Licenses may be exercised anywhere in the world.

Altering/Modifying Material: Not Permitted
You may not alter or modify the material in any manner, nor may you translate the material into another language without permission from the Publisher.

Reservation of Rights
Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

License Contingent on Payment
While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

Copyright Notice: Disclaimer
It is a condition of this license that on all copies you republish, display or distribute you (i) provide full bibliographical information for the licensed material, including but not limited to author name, title and publication date of the work, volume and number if applicable, publisher name (Eureka Science Ltd.), page reference, (ii) include the statement, "Reprinted by permission of Eureka Science Ltd."); and (iii) include the copyright notice that appears in
the work as published by Eureka Science Ltd..

**Warranties: None**
Publisher makes no representations or warranties with respect to the licensed material.

**Indemnity**
You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

**No Transfer of License**
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

**No Amendment Except in Writing**
This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

**Objection to Contrary Terms**
Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

**Jurisdiction: Not Required**
This license transaction shall be governed by and construed in accordance with the laws of the United Arab Emirates. You hereby agree to submit to the jurisdiction of the federal and state courts located in Dubai for purposes of resolving any disputes that may arise in
connection with this licensing transaction.

Other Terms and Conditions: None

v1.0

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
This is a License Agreement between Tamima Ashraf ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

- **License Number**: 3471391187232
- **License date**: Sep 17, 2014
- **Licensed content publisher**: John Wiley and Sons
- **Licensed content publication**: Wiley Books
- **Licensed content title**: Drug Transporters: Molecular Characterization and Role in Drug Disposition, 2nd Edition
- **Book title**: None
- **Licensed copyright line**: Copyright © 2014, John Wiley and Sons
- **Licensed content author**: Guofeng You (Editor), Marilyn E. Morris (Editor), Binghe Wang (Series Editor)
- **Licensed content date**: Aug 1, 2014
- **Type of use**: Dissertation/Thesis
- **Requestor type**: University/Academic
- **Format**: Print and electronic
- **Portion**: Text extract
- **Number of Pages**: 3
- **Will you be translating?**: No
- **Title of your thesis / dissertation**: Regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: Implications in HIV-1 neuropathogenesis and its treatment
- **Expected completion date**: Nov 2014
TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking accept in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication),
translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA,
FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.

- These terms and conditions together with CCC’s Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC’s Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC’s Billing and Payment terms and conditions.

- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

- This Agreement shall be governed by and construed in accordance with the laws of
the State of New York, USA, without regards to such states conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses:: Creative Commons Attribution (CC-BY) license Creative Commons Attribution Non-Commercial (CC-BY-NC) license and Creative Commons Attribution Non-Commercial-NoDerivs (CC-BY-NC-ND) License. The license type is clearly identified on the article.

Copyright in any research article in a journal published as Open Access under a Creative Commons License is retained by the author(s). Authors grant Wiley a license to publish the article and identify itself as the original publisher. Authors also grant any third party the right to use the article freely as long as its integrity is maintained and its original authors, citation details and publisher are identified as follows: [Title of Article/Author/Journal Title and Volume/Issue. Copyright (c) [year] [copyright owner as specified in the Journal]. Links to the final article on Wiley’s website are encouraged where applicable.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-commercial re-use of an open access article, as long as the author is properly attributed.

The Creative Commons Attribution License does not affect the moral rights of authors,
including without limitation the right not to have their work subjected to derogatory treatment. It also does not affect any other rights held by authors or third parties in the article, including without limitation the rights of privacy and publicity. Use of the article must not assert or imply, whether implicitly or explicitly, any connection with, endorsement or sponsorship of such use by the author, publisher or any other party associated with the article.

For any reuse or distribution, users must include the copyright notice and make clear to others that the article is made available under a Creative Commons Attribution license, linking to the relevant Creative Commons web page.

To the fullest extent permitted by applicable law, the article is made available as is and without representation or warranties of any kind whether express, implied, statutory or otherwise and including, without limitation, warranties of title, merchantability, fitness for a particular purpose, non-infringement, absence of defects, accuracy, or the presence or absence of errors.

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC) License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes. (see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by non-commercial users
For non-commercial and non-promotional purposes, individual users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles, as well as adapt, translate, text- and data-mine the content subject to the following conditions:

- The authors' moral rights are not compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be impugned).

- Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.

- If article content is copied, downloaded or otherwise reused for non-commercial research and education purposes, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on Wiley Online Library) should be maintained. Copyright notices and disclaimers must not be deleted.

- Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

Use by commercial "for-profit" organisations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:

Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing:

- Copying, downloading or posting by a site or service that incorporates advertising with such content;

- The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)

- Use of article content (other than normal quotations with appropriate citation) by
for-profit organisations for promotional purposes

- Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;

- Use for the purposes of monetary reward by means of sale, resale, licence, loan, transfer or other form of commercial exploitation such as marketing products

- Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com

Further details can be found on Wiley Online Library
http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
Copyright clearance for figure 1-3 and 1-4:

Your permission request falls within the STM signatory guidelines, found here: http://www.stm-assoc.org/permissions-guidelines/. Permission is automatically granted. Please be sure to acknowledge the original source of the Elsevier material.

If you have any additional questions, feel free to contact us.

Thank you,
Laura

Laura Stingelin
Permissions Helpdesk Associate
Elsevier
1600 John F. Kennedy Boulevard
Suite 1800
Philadelphia, PA 19103-2899
T: (215) 239-3867
F: (215) 239-3805
Copyright clearance for figure 1-6:

September 8, 2014
Tanima Ashraf
Pharmaceutical Sciences Department
University of Toronto
1055 Southdown Road, Unit 403
Mississauga, ON L5J 0A3
Canada

Email: tanima.ashraf@utoronto.ca

Dear Tanima Ashraf:

This is to grant you permission to include the following figure in your thesis entitled “Regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: Implications in HIV-1 neuropathogenesis and its treatment” for the University of Toronto:

Figure 1 from Shannon Dallas, David S. Miller, and Reina Bendayan, Multidrug Resistance-Associated Proteins: Expression and Function in the Central Nervous System, *Pharmacol Rev* June 2006 58:140-161

Permission to reproduce the figure is granted for worldwide use in all languages, translations, and editions, and in any format or medium including print and electronic. The authors and the source of the materials must be cited in full, including the article title, journal title, volume, year, and page numbers.

Sincerely yours,

Richard Dodenhoff
Journals Director
Copyright clearance for figure 1-8:

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS
Oct 28, 2014

This is a License Agreement between Tamima Ashraf ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

<table>
<thead>
<tr>
<th>License Number</th>
<th>3464281374110</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Sep 08, 2014</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>John Wiley and Sons</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>GLIA</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Regulation of ABC membrane transporters in glial cells: Relevance to the pharmacotherapy of brain HIV-1 infection</td>
</tr>
<tr>
<td>Licensed copyright line</td>
<td>Copyright © 2008 Wiley-Liss, Inc.</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Patrick T. Ronaldson, Yuri Persidsky, Reina Bendayan</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>Jul 22, 2008</td>
</tr>
<tr>
<td>Start page</td>
<td>1711</td>
</tr>
<tr>
<td>End page</td>
<td>1735</td>
</tr>
<tr>
<td>Type of use</td>
<td>Dissertation/Thesis</td>
</tr>
<tr>
<td>Requestor type</td>
<td>University/Academic</td>
</tr>
<tr>
<td>Format</td>
<td>Print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>Figure/table</td>
</tr>
<tr>
<td>Number of figures/tables</td>
<td>1</td>
</tr>
<tr>
<td>Original Wiley figure/table number(s)</td>
<td>Figure 6</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Title of your thesis / dissertation</td>
<td>Regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: Implications in HIV-1 neuropathogenesis and its treatment</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Nov 2014</td>
</tr>
</tbody>
</table>

248
TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking accept in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the
Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY
NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY’s prior written consent.

- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.

- These terms and conditions together with CCC’s Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties’ successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC’s Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC’s Billing and Payment terms and conditions.

- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state’s conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent
jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

**WILEY OPEN ACCESS TERMS AND CONDITIONS**

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses: Creative Commons Attribution (CC-BY) license Creative Commons Attribution Non-Commercial (CC-BY-NC) license and Creative Commons Attribution Non-Commercial-NoDerivs (CC-BY-NC-ND) License. The license type is clearly identified on the article.

Copyright in any research article in a journal published as Open Access under a Creative Commons License is retained by the author(s). Authors grant Wiley a license to publish the article and identify itself as the original publisher. Authors also grant any third party the right to use the article freely as long as its integrity is maintained and its original authors, citation details and publisher are identified as follows: [Title of Article/Author/Journal Title and Volume/Issue. Copyright (c) [year] [copyright owner as specified in the Journal]. Links to the final article on Wiley’s website are encouraged where applicable.

**The Creative Commons Attribution License**

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-commercial re-use of an open access article, as long as the author is properly attributed.

The Creative Commons Attribution License does not affect the moral rights of authors, including without limitation the right not to have their work subjected to derogatory treatment. It also does not affect any other rights held by authors or third parties in the article, including without limitation the rights of privacy and publicity. Use of the article must not assert or imply, whether implicitly or explicitly, any connection with, endorsement or sponsorship of such use by the author, publisher or any other party associated with the article.

For any reuse or distribution, users must include the copyright notice and make clear to others that the article is made available under a Creative Commons Attribution license, linking to the relevant Creative Commons web page.

To the fullest extent permitted by applicable law, the article is made available as is and without representation or warranties of any kind whether express, implied, statutory or otherwise and including, without limitation, warranties of title, merchantability, fitness for a
particular purpose, non-infringement, absence of defects, accuracy, or the presence or absence of errors.

**Creative Commons Attribution Non-Commercial License**

The Creative Commons Attribution Non-Commercial (CC-BY-NC) License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes. (see below)

**Creative Commons Attribution-Non-Commercial-NoDerivs License**

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

**Use by non-commercial users**

For non-commercial and non-promotional purposes, individual users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles, as well as adapt, translate, text- and data-mine the content subject to the following conditions:

- The authors' moral rights are not compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be impugned).

- Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.

- If article content is copied, downloaded or otherwise reused for non-commercial research and education purposes, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on Wiley Online Library) should be maintained. Copyright notices and disclaimers must not be deleted.

- Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

**Use by commercial "for-profit" organisations**

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes
requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:

- Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;

- Copying, downloading or posting by a site or service that incorporates advertising with such content;

- The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)

- Use of article content (other than normal quotations with appropriate citation) by for-profit organisations for promotional purposes

- Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;

- Use for the purposes of monetary reward by means of sale, resale, licence, loan, transfer or other form of commercial exploitation such as marketing products

- Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com

Further details can be found on Wiley Online Library
http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
Copyright clearance for chapter 2:

September 8, 2014

Tamina Ashraf
Pharmaceutical Sciences Department
University of Toronto
1055 Southdown Road, Unit 403
Mississauga, ON L5J 0A3
Canada

Email: tamima.ashraf@utoronto.ca

Dear Tamima Ashraf:

This is to grant you permission to include the following article in your thesis entitled “Regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: Implications in HIV-1 neuropathogenesis and its treatment” for the University of Toronto:

Patrick T. Ronaldson, Tamima Ashraf, and Reina Bendayan, Regulation of Multidrug Resistance Protein 1 by Tumor Necrosis Factor α in Cultured Glial Cells: Involvement of Nuclear Factor-κB and c-Jun N-Terminal Kinase Signaling Pathways, Mol Pharmacol. April 2010 77:644-659

On the first page of each copy of this article, please add the following:

Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved.

In addition, the original copyright line published with the paper must be shown on the copies included with your thesis.

Sincerely yours,

Richard Dodekoff
Journals Director

Copyright clearance for chapter 3:

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS
Oct 28, 2014
This is a License Agreement between Tamima Ashraf ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

<table>
<thead>
<tr>
<th>License Number</th>
<th>3464271107922</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Sep 08, 2014</td>
</tr>
<tr>
<td>Licensed content</td>
<td>John Wiley and Sons</td>
</tr>
<tr>
<td>publisher</td>
<td></td>
</tr>
<tr>
<td>Licensed content</td>
<td>Journal of Neuroscience Research</td>
</tr>
<tr>
<td>publication</td>
<td></td>
</tr>
<tr>
<td>Licensed content</td>
<td>Regulation of P-glycoprotein by human immunodeficiency virus-1</td>
</tr>
<tr>
<td>title</td>
<td>in primary cultures of human fetal astrocytes</td>
</tr>
<tr>
<td>Licensed copyright</td>
<td>Copyright © 2011 Wiley-Liss, Inc.</td>
</tr>
<tr>
<td>line</td>
<td></td>
</tr>
<tr>
<td>Licensed content</td>
<td>Tamima Ashraf, Patrick T. Ronaldson, Yuri Persidsky, Reina Bendayan</td>
</tr>
<tr>
<td>author</td>
<td></td>
</tr>
<tr>
<td>Licensed content</td>
<td>Aug 8, 2011</td>
</tr>
<tr>
<td>date</td>
<td>1773</td>
</tr>
<tr>
<td>Start page</td>
<td>1782</td>
</tr>
<tr>
<td>End page</td>
<td></td>
</tr>
<tr>
<td>Type of use</td>
<td>Dissertation/Thesis</td>
</tr>
<tr>
<td>Requestor type</td>
<td>Author of this Wiley article</td>
</tr>
<tr>
<td>Format</td>
<td>Print</td>
</tr>
<tr>
<td>Portion</td>
<td>Full article</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Title of your thesis / dissertation</td>
<td>Regulation of pro-inflammatory cytokines and drug efflux transporters by signal transduction pathways in glial cells: Implications in HIV-1 neuropathogenesis and its treatment</td>
</tr>
<tr>
<td>Expected completion date</td>
<td>Nov 2014</td>
</tr>
<tr>
<td>Expected size (number of pages)</td>
<td>220</td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>

Terms and Conditions
TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking accept in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley
Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

- **NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.**

- **WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.**

- **You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.**

- **IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.**

- **Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and**
the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY’s prior written consent.

- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.

- These terms and conditions together with CCC’s Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties’ successors, legal representatives, and authorized assigns.

- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC’s Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC’s Billing and Payment terms and conditions.

- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state’s conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.
WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses:: Creative Commons Attribution (CC-BY) license Creative Commons Attribution Non-Commercial (CC-BY-NC) license and Creative Commons Attribution Non-Commercial-NoDerivs (CC-BY-NC-ND) License. The license type is clearly identified on the article.

Copyright in any research article in a journal published as Open Access under a Creative Commons License is retained by the author(s). Authors grant Wiley a license to publish the article and identify itself as the original publisher. Authors also grant any third party the right to use the article freely as long as its integrity is maintained and its original authors, citation details and publisher are identified as follows: [Title of Article/Author/Journal Title and Volume/Issue. Copyright (c) [year] [copyright owner as specified in the Journal]. Links to the final article on Wiley’s website are encouraged where applicable.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-commercial re-use of an open access article, as long as the author is properly attributed.

The Creative Commons Attribution License does not affect the moral rights of authors, including without limitation the right not to have their work subjected to derogatory treatment. It also does not affect any other rights held by authors or third parties in the article, including without limitation the rights of privacy and publicity. Use of the article must not assert or imply, whether implicitly or explicitly, any connection with, endorsement or sponsorship of such use by the author, publisher or any other party associated with the article.

For any reuse or distribution, users must include the copyright notice and make clear to others that the article is made available under a Creative Commons Attribution license, linking to the relevant Creative Commons web page.

To the fullest extent permitted by applicable law, the article is made available as is and without representation or warranties of any kind whether express, implied, statutory or otherwise and including, without limitation, warranties of title, merchantability, fitness for a particular purpose, non-infringement, absence of defects, accuracy, or the presence or absence of errors.

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC) License permits use,
distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes. (see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by non-commercial users

For non-commercial and non-promotional purposes, individual users may access, download, copy, display and redistribute to colleagues Wiley Open Access articles, as well as adapt, translate, text- and data-mine the content subject to the following conditions:

- The authors' moral rights are not compromised. These rights include the right of "paternity" (also known as "attribution" - the right for the author to be identified as such) and "integrity" (the right for the author not to have the work altered in such a way that the author's reputation or integrity may be impugned).

- Where content in the article is identified as belonging to a third party, it is the obligation of the user to ensure that any reuse complies with the copyright policies of the owner of that content.

- If article content is copied, downloaded or otherwise reused for non-commercial research and education purposes, a link to the appropriate bibliographic citation (authors, journal, article title, volume, issue, page numbers, DOI and the link to the definitive published version on Wiley Online Library) should be maintained. Copyright notices and disclaimers must not be deleted.

- Any translations, for which a prior translation agreement with Wiley has not been agreed, must prominently display the statement: "This is an unofficial translation of an article that appeared in a Wiley publication. The publisher has not endorsed this translation."

Use by commercial "for-profit" organisations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee. Commercial purposes include:

- Copying or downloading of articles, or linking to such articles for further redistribution, sale or licensing;
• Copying, downloading or posting by a site or service that incorporates advertising with such content;

• The inclusion or incorporation of article content in other works or services (other than normal quotations with an appropriate citation) that is then available for sale or licensing, for a fee (for example, a compilation produced for marketing purposes, inclusion in a sales pack)

• Use of article content (other than normal quotations with appropriate citation) by for-profit organisations for promotional purposes

• Linking to article content in e-mails redistributed for promotional, marketing or educational purposes;

• Use for the purposes of monetary reward by means of sale, resale, licence, loan, transfer or other form of commercial exploitation such as marketing products

• Print reprints of Wiley Open Access articles can be purchased from: corporatesales@wiley.com

Further details can be found on Wiley Online Library http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

Copyright clearance for chapter 4:

Manuscript: Role of anti-inflammatory compounds in human immunodeficiency virus-1 glycoprotein120-mediated brain inflammation

Authors: Tamima Ashraf, Wenlei Jiang, Md Tozammel Hoque, Jeffrey Henderson, Chiping Wu and Reina Bendayan

Received: 7 March 2014

Accepted: 15 April 2014