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Tat protein induced neurocognitive dysfunction

by

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Declaration

I, Khayelihle Brian Makhathini, student number: 210553995 hereby declare that the dissertation/thesis entitled:

Tat protein induced neurocognitive dysfunction

Is the result of my own investigation and research and that it has not been submitted in part or full for any other degree or to any other University or Tertiary Institution. Where use was made of the work of others, it is duly acknowledged. The research done in this study was carried out under the supervision of Prof W.M.U. Daniels and Dr M. Mabandla

WMU Daniels | MV Mabandla | KB Makhathini

Date

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"People of integrity are genuine and consistently living an authentic life"

Dr Cindy Trimm

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Abbreviations

ANI: asymptomatic neurocognitive impairment

AP-1: activator protein-1

Apaf-1: Apoptotic protease activating factor-1

ART: antiretroviral treatment

BBB: blood brain barrier

CAD: caspase-activated DNase

cAMP: Cyclic adenosine monophosphate

CCR5: C-C chemokine receptor type-5

CD4: cluster of differentiation-4

CNS: central nervous system

CXCR4: Chemokine receptor type-4

DNA: Deoxyribonucleic acid

EAAT-1: extracellular excitatory amino acid transporters-1

ENase: endonucleases

Env: envelope

ERK: Extracellular signal-regulated kinases

Fas/CD95: Fas ligand

Gag: Group-specific antigen

Gp: glycoprotein

HAART: highly active anti-retroviral therapy

HAD: HIV associated dementia

HAND: HIV associated neurocognitive disorder

HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome

i.p.: intraperitoneally

IDU: injection drug users

IFN-ɔ: interferon gamma

IL-1ɓ: Interleukin-1 beta

MAPK: Mitogen-activated protein kinases

MND: mild neurocognitive disorder

MSM: men who have sex with men

Nef: Negative regulator factor

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NMDA: N-Methyl-D-aspartic acid

PA: Picolinic acid

PCR: polymerase chain reaction

Pol: polymerase

Rev: Regulator of viral expression

RNA: Ribonucleic acid

SACEMA: South African Centre for Epidemiological Modeling and Analysis

SW: sex workers

TAR: Tat associated region

Tat: Trans-Activator of Transcription

TBF: ϐ-Transforming growth factor-beta

TNF-Ŭ: Tumor necrosis factor-alpha

TRAIL: tumour necrosis factor-related apoptosis-induced ligand

UNAIDS: United Nations Programmed acquired immunodeficiency syndrome

Vif: Viral infectivity

Vpr: Viral protein R

Vpu: Viral protein U

Abstract

Human immunodeficiency virus type 1 (HIV-1) is a major health threat in South Africa. Studies have shown that HIV affects the central nervous system (CNS), and can initiate a progressive neurodegenerative process culminating in HIV associated dementia. Tat protein and gp120 are two viral proteins that have been linked to the neurotoxic effects of HIV. The present study used an animal model to study the effects of tat protein on the brain. The study also investigated the ability of picolinic acid to block the damaging effects of Tat protein.

Sprague-Dawley rats were divided into four groups. The control group received a bilateral injection of saline (100µl), and the experimental group received a bilateral injection of tat protein (5µg/100µl) into the hippocampus. The other two groups of rats were treated similarly, but also received picolinic acid at a concentration of 3.36mg/3ml, injected intraperitoneally (i.p) before the intrahippocampal injections. Two tests were performed to assess the cognitive behaviour of all the animals, namely the light/dark box and Morris water maze. After the behavioural tests, the brains of animals were collected for evaluating the activity of the enzyme caspase 3 by various means. Some brains were used to determine caspase 3 mRNA transcriptions by polymerase chain reaction; others were used to measure the expression of caspase 3 protein by Western blotting techniques, while the rest of the brains were used for flow cytometry analysis where a caspase 3 specific staining kit was used.

Our behavioural results indicated that tat protein caused impairment in learning and memory in the experimental group, when compared to controls. The group that received both tat protein and picolinic acid showed a significant improvement in learning and memory in Morris water maze test, in comparison to the tat protein treated group.

Our gene expression data showed a significant up regulation of caspase 3 gene in the tat protein treated group, compared to controls. This result was supported by the Western blot data that showed significantly increased caspase 3 protein expression in the tat protein treated group. While these increases in caspase 3 expression strongly point to an apoptotic mode of cell death in the hippocampus of animals that were treated with tat protein, our flow cytometry results were less convincing with marginal levels of caspase 3 staining being observed. Both increases in gene and protein expression were inhibited in animals that were pretreated with picolinic acid.

Our data led us to conclude that tat protein can cause cognitive abnormalities through toxic sequelae that may include apoptosis. It is therefore likely that this viral protein may be one of the etiological factors of HIV associated neurocognitive impairment. Our results further suggest that picolinic acid may be considered as an adjunct therapy for HAND.

Chapter 1

Literature review

1. Scope of the HIV problem

HIV and AIDS (acquired immunodeficiency syndrome) has become a serious problem in South Africa and worldwide (UNAIDS, 2011). Recently the UNAIDS reported that approximately 34 million people are living with HIV, and about 30 million people have died of AIDS-related causes since the beginning of the epidemic (Table 1, UNAIDS, 2011). Global statistics suggest that 3.4 million children have been diagnosed with HIV in 2010, of which 390 000 were new infections.

Table 1: HIV Prevalence and Incidence by Region

Region	**Total no. (%) living with HIV at the end of 2010**	**Newly infected in 2010**	**Adult prevalence % in 2010**
Global total	**34 million (100)**	**2.7 million**	**0.8**
Sub-Saharan Africa	22.9 million (67)	1.9 million	5.0
South/South-East Asia	4.0 million (12)	270 000	0.3
Eastern Europe/ Central Asia	1.5 million (4)	160 000	0.9
Latin America	1.5 million (4)	100 000	0.4
North America	1.3 million (4)	58 000	0.6
Western/Central Europe	840 000 (2)	30000	0.2
East Asia	790 000 (2)	88 000	0.1
Middle East/ North Africa	470 000 (1)	59 000	0.2
Caribbean	200 000 (0.6)	12 000	0.9
Oceania	54 000 (0.2)	3 300	0.3

UNAIDS Report on the Global HIV/AIDS Epidemic 2011, Kaiser Family Foundation.

The number of AIDS orphans were estimated to be approximately 16.6 million i.e. children who have lost one or both parents due to HIV, with most of them living in sub-Saharan Africa (89%) (UNAIDS, 2011; UNAIDS Kaiser Family Foundation, 2011). While 1.8 million AIDS-related deaths have been documented for 2010, the overall number is on a declining trajectory, with a decreased of 21% reported since 2005. This reduction is mainly attributed to the roll-out of antiretroviral treatment (ART).

Sub-Saharan Africa is the region that has the highest prevalence rate of people living with HIV. In this region there are 9 countries where more than 10% of adults are estimated to be HIV-positive; with South Africa having the highest score (UNAIDS Kaiser Family Foundation Report, 2011).

Table 2: HIV prevalence estimates and the number of people living with HIV, 2002–2011

Year	**Population 15-49 years infected with HIV**			**Incident Adult 15-49 years**	**Estimate of HIV infected population (millions)**
	% of woman	**% of the population**	**% of the Total population**		
2002	17.7	16.2	9.6	1.59	4.37
2003	18.0	16.2	9.7	1.58	4.49
2004	18.1	16.2	9.8	1.63	4.59
2005	18.3	16.2	9.9	1.73	4.69
2006	18.9	16.6	10.2	2.11	4.87
2007	18.9	16.5	10.2	1.54	4.95
2008	18.9	16.4	10.3	1.43	5.02
2009	19.1	16.4	10.4	1.45	5.13
2010	19.3	16.5	10.5	1.43	5.26
2011	19.4	16.6	10.6	1.38	5.38

(Statistics South Africa, 2011)

According to Statistics South Africa (2011) there are more than 50 million people in the country. Of this 5.38 million people are HIV positive, the majority being between the ages of 15 and 49 (Table 2). The total prevalence of HIV infection is approximated to be 10.6% (Table 2, Mid-year population estimates, 2011).

The South African National AIDS Council (2011) investigated the characteristics of the new cases that present with HIV infection and found those men who have sex with men (MSM), sex workers (SW) and their clients, and injection drug users (IDU) to be the most prominent (Table 3). Worryingly, it is estimated that half of homosexual men living in Johannesburg, are HIV positive. These findings are supported by studies of the South African Centre for Epidemiological Modeling and Analysis (SACEMA) that indicated 9.2% of new HIV infections to be associated with MSM. SACEMA also estimated that 19.8% of all new HIV infections are interrelated with sex work. Other reports from the South African National AIDS Council (2011) and the Medical Research Council of South Africa (Parry, 2008) showed that 86% of South Africans who inject illicit drugs, share injection equipment. In addition, these persons also practice unsafe sex. The scope and enormity of the HIV/AIDS problem is therefore obvious and this justifies its present top position on the South African health and research agenda.

Table 3. Percent of new infections attributed to sex workers (SW), injection drug users (IDU) and homosexual men (MSM)

	Percent of new HIV infections, group only	**Percent of new infections, group and their partners/clients**
SW	5.5%	19.8%
IDU	1.1%	1.3%
MSM	7.9%	9.2%
Total	**14.5%**	**30.3**

(South African National AIDS Council, 2011)

2. Human immunodeficiency virus (HIV)

2.1. The virus structure

Human immunodeficiency virus (HIV) belongs to the retrovirus family. The virus has an outer envelope (outer membrane), embedding an RNA genome. This RNA genome is surrounded by a capsid, forming a nucleus that also accommodates viral proteins vital for its replication. Between the envelope and capsid (cytosol) core and matrix proteins are found (Figure 1; Mattson *et al*., 2005; Kaul *et al*., 2001). The HIV genome has three main regions namely env, pol and gag (Figure 1). They code for a range of viral proteins. For example, *env* gene encodes for the glycoprotein gp160 which is cleaved to form two important envelope proteins, gp120 and gp41 (Figure 1; Nath and Geiger, 1998; van de Bovenkamp *et al*., 2002; Mattson *et al*., 2005). Gp120 is responsible for the spikes that can be seen on the surface of the virus, and is the protein that interacts with CD4 and CCR5 binding proteins on T-helper cells (Kaul and Lipton, 1999; Kashou and Agarwal, 2011). Gp41 is a trans-membrane glycoprotein that anchors these spikes in the envelope (Figure 1), and facilitates fusion and internalisation of the virus into the host cell (El-Kharroubi *et al*., 1998). *Pol* gene codes for the enzyme reverse transcriptase that converts viral RNA to DNA (Mattson *et al*., 2005), as well as the protease and intergrase enzymes that are important in viral replication. Group specific antigen (Gag) proteins are integral to the capsid, matrix and nucleocapsid. There are also at least six regulatory proteins namely tat, rev, nef, vif, vpu and vpr (Mattson *et al*., 2005). Tat and rev are nuclear proteins, while nef may be found in both the nucleus and the cytoplasm of the host cell. Usually tat is actively released in the extracellular environment, where it may affect adjacent cells (van de Bovenkamp *et al*., 2002; Mattson *et al*., 2005).

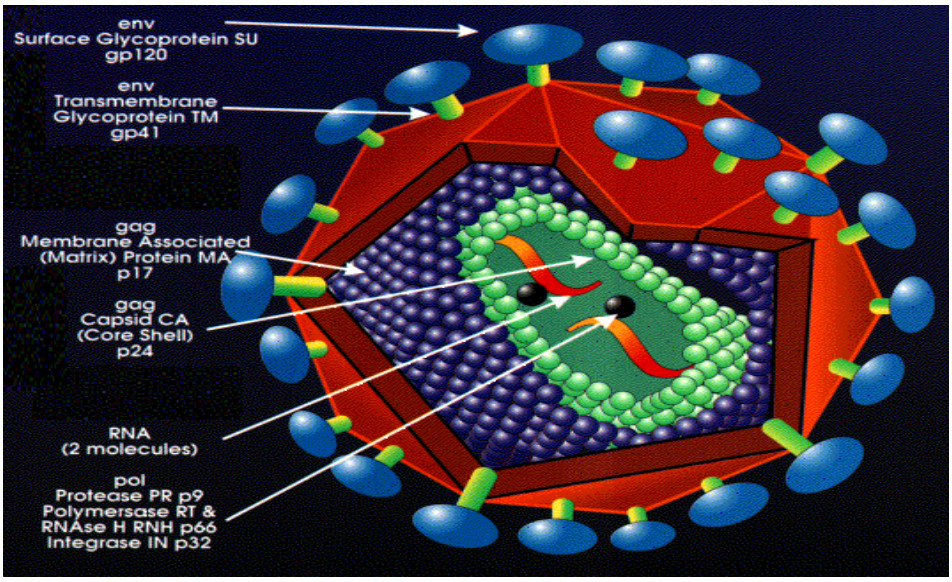


Figure 1: The structure of HIV and proteins that are involved in replication (El-Kharroubi *et al*., 1998)

2.2. HIV replication

HIV-1 mainly targets cells of the immune system. HIV-1-infected T-lymphocytes have been detected within 4-6 weeks after HIV-1 inoculation (Ghafouri *et al*., 2006). However, post-mortem studies have indicated the presence of HIV in tissue of the central nervous system (CNS) as well (Kaul *et al*., 2005).

Cells that become infected with HIV possess receptors that facilitate binding of HIV-1 to the cell membrane (Ghafouri *et al*., 2006; Wayengera, 2010). Chemokine receptors, such as CXCR4, are important for HIV-1 entry into the lymphocytes, while CCR5 performs a similar function for monocytes, macrophages and microglia. Entry of the virus into the cell is followed by a series of steps that include reverse transcription of viral RNA, nuclear entry of double-stranded viral DNA, viral integration into host genome, viral genomic replication, viral packaging and finally budding and exit (Figure 2 and Figure 3; Wayengera, 2010).

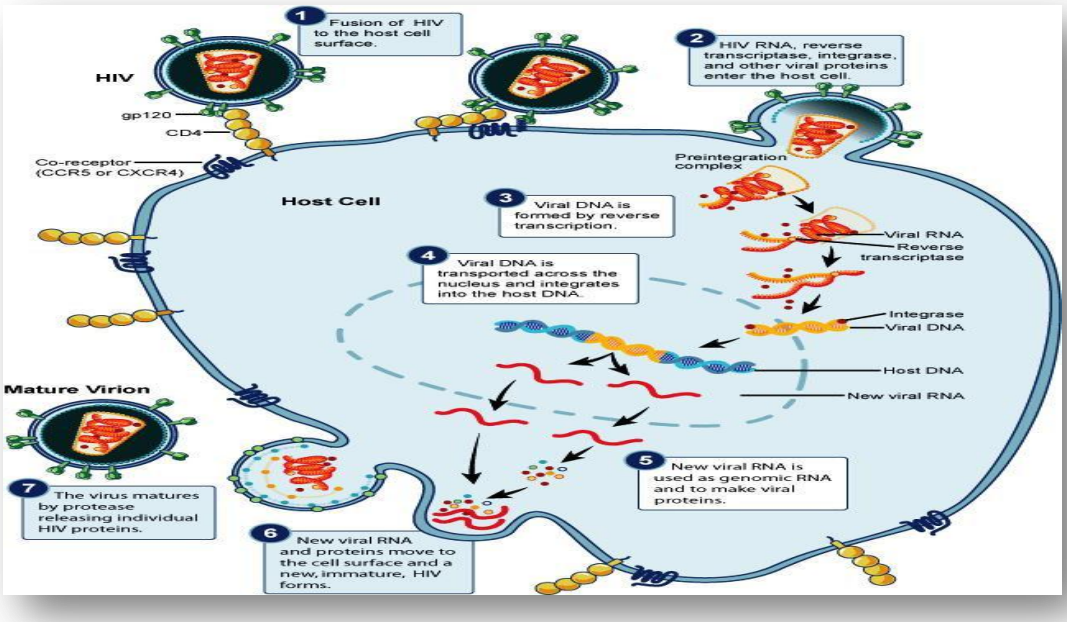


Figure 2: An illustration of the HIV replication process (Wayengera, 2010)

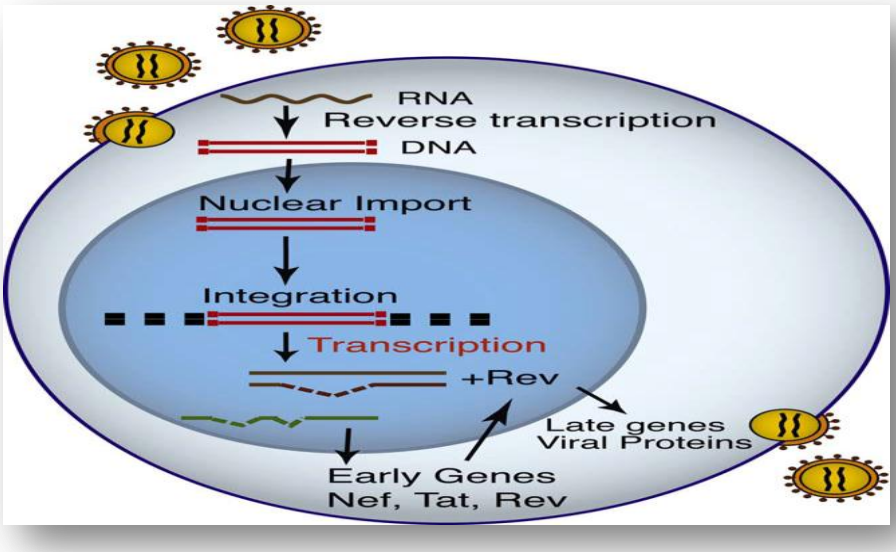


Figure 3: This figure shows the replication of the HIV DNA material in the host cell (Wu and Marsh, 2003)

2.3. HIV and hippocampus dysfunction

HIV crosses the blood brain barrier (BBB) soon after the person is infected. Previous studies revealed that brain structures such as the basal ganglia, deep white matter, subcortical and fronto-striatal areas of the brain are affected by HIV (Castelo *et al*., 2007). Neuroimaging data has shown a decrease or volume loss in the cortical or subcortical region, and hypertrophy is apparent in the basal ganglia, especially the putamen (Castelo *et al.,* 2007; Cohen *et al.,* 2010).

The hippocampus is known for its important role in learning and memory, and also spatial navigation (Castelo *et al.,* 2007; Venkatesan *et al*., 2007). Loss of memory, poor attention and visuospatial functioning, have been observed in patients infected with HIV (Cohen *et al.*, 2010). These observations strongly implicated the hippocampus in HIV associated neurocognitive impairment (Antinori *et al*., 2007).

2.4. HIV – associated neurocognitive impairments

The term HIV dementia was introduced by Navia and Price (1987) to describe the mild memory loss, motor slowing and incapacitating cognitive collapse that they observed in HIV patients. More recently, the classification of these symptoms was revised and the disorder is now referred to as HIV associated neurocognitive disturbance (HAND), consisting of three syndromes: asymptomatic neurocognitive impairment (ANI); mild neurocognitive disorder (MND); and HIV associated dementia (HAD) (Antinori *et al*., 2007). ANI doesn't interfere with daily work and doesn't meet any requirement for dementia. MND produces at least mild interference which may result in unwanted disruptions in normal working, homemaking, or social functioning (Antinori *et al*., 2007). HAD on the other hand, is characterised by marked interference in day-to-day functioning. The extent of this disorder includes the loss of learning and memory, recalling and motor deficits.

3. Treatment of HIV associated dementia (HAD)

The treatment of HAD is complicated by the presence of the blood brain barrier, that limits the penetration of drugs into the CNS (Saksena and Smith, 2005; Li *et al*., 2009; Joska *et al*., 2011). Although the administration of highly active anti-retroviral therapy (HAART) has improved the quality of life of many HIV patients, its overall efficacy for neuroAIDS remains unsatisfactory (Joska *et al*., 2011). Studies have shown that in the pre-HAART era dramatic volumetric brain

abnormalities were seen in people infected with HIV (Cohen *et al.*, 2007; Castole *et al*., 2010). There is some indication that reduced subcortical volumes and metabolite abnormalities can be found in HIV-infected patients on HAART, and patient on HAART are still experiencing cortical atrophy problem (Cohen *et al.*, 2007). Questions as to why HAD continues to prevail in the era of HAART, despite considerable success of HAART regimens and protease inhibitors in particular, are continuously being asked (Saksena and Smith, 2005; Joska *et al*., 2011).

In general, current treatment strategies are based on combination therapy that include nucleotide/nucleoside reverse transcriptase inhibitors (eg. lamivudine and stavudine), and non-nucleotide/nucleoside reverse transcriptase inhibitors (eg. nevirapine and efavirenz) (Saksena and Smith, 2005; Joska *et al*., 2011). These drugs usually act by binding directly to the active site of the reverse transcriptase enzyme, and so prevent the replication of HIV (Saksena and Smith, 2005).

HIV proteases are the enzymes that cleave protein molecules into smaller fragments, and at the same time they are responsible for both viral replication within the cell, and release of mature viral particles from an infected cell (De Clercq, 2004; Gills *et al*., 2007). There are now protease inhibitors for HIV (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir), and these inhibitors are more effective if they are given with food, and also in combination with other drugs (De Clercq, 2004; Gills *et al.*, 2007).

Other pharmacological approaches have also been investigated in the search for better management of HAD. For instance, NMDA receptor blockers, chemokines, chemokine-receptor and cytokine-receptor antagonists, p38 MAPK inhibitors, caspase inhibitors and antioxidants (free-radical scavengers or other inhibitors of excessive nitric oxide or reactive oxygen species) have been examined as possible adjunct treatments for the HIV syndrome (Kaul *et al*., 2001). Similarly, β-chemokines that act as antagonists of CXCR4 and CCR5 have been proposed to be useful to treat HIV, since it has been shown to prevent gp120-induced neuronal apoptosis in *in vitro* studies (Kaul *et al*., 1999; Kaul *et al*., 2001).

There are 3 other drugs that are currently under study for use in patients that live with AIDS dementia. They are nimodipine (an L-type calcium-channel antagonist), and memantine and nitroglycerin (both NMDA-receptor antagonists) (Lipton and Gendelman, 1995; Parsons *et al*., 2007). These novel approaches are mostly in the experimental stage and their true benefit for HAD remains to be established. Furthermore, the blood brain barrier impedes entry of the drugs

into the brain, thereby hindering the obtaining of effective doses to combat HIV. It is therefore imperative that the search for better therapeutic interventions continues.

Activation of the kynurenine pathway in the brain has been associated with inflammatory neurological diseases (Heyes *et al*., 1992). Basically this biochemical pathway entails the breakdown of L-tryptophan to quinolinic acid, and high levels of quinolinic acid have been found in the cerebrospinal fluid or post-mortem brain tissue of HIV-infected individuals (Heyes *et al*., 1992, Sanni *et al*., 1998; Guillemin *et al.*, 2005). Picolinic acid is another pyridine compound that is produced along with quinolinic acid during L-tryptophan catabolism (Grant *et al.*, 2009). While quinolinic acid is noted for its harmful effects to neurons, picolinic acid on the other hand has been reported to have more of a neuroprotective role (Grant *et al.*, 2009).

4. HIV-1 neurophathogenesis in the CNS

The blood–brain barrier (BBB) (Figure 4) is composed of a tightly bound layer of capillary endothelial cells that separates the CNS from the periphery (Gloor *et al.*, 2001; Ghafouri *et al*., 2006). The BBB maintains homeostasis within the CNS by regulating the selective influx of substances and cells into the brain (Gloor *et al*., 2001; Gendelman *et al*., 1994; Ghafouri *et al*., 2006).

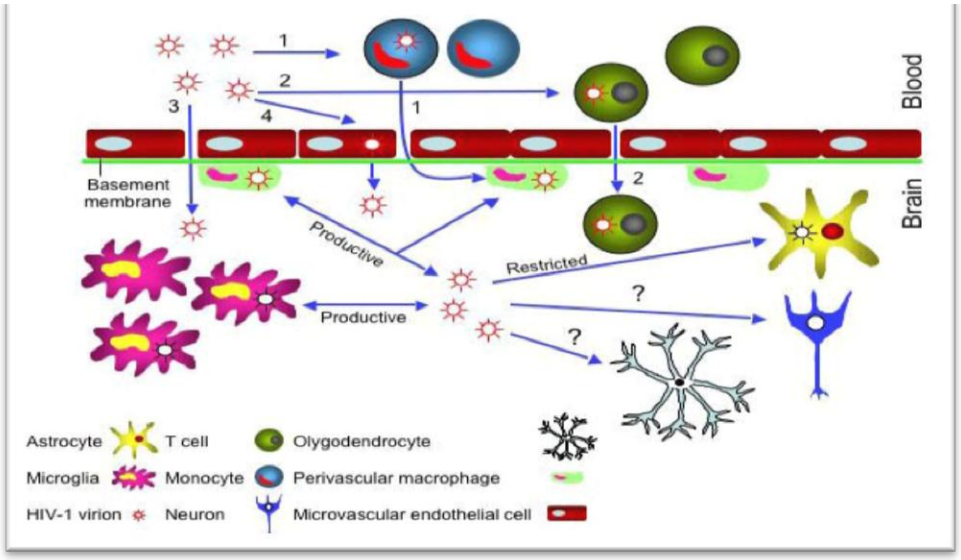


Figure 4: The corrosion of the BBB by HIV-1 in the brain (Ghafouri *et al*., 2006)

How HIV-1 affects oligodendrocytes and neurons is still unclear. It has been suggested that HIV-1 can influence these cells in two ways, a direct or an indirect mechanism. The direct pathway refers to the release of HIV proteins directly into neuronal cells, while the indirect pathway involves the secretion of cytokines and neurotoxins by HIV- infected astrocytes or microglial cells into the surrounding intercellular space (Rappaport *et al*., 1999; Ghafouri *et al*., 2006).

Despite the barrier property of the BBB, HIV-1 manages to enter into the brain. This occurs in one of four ways:

1) the virus can infect monocytes that can migrate into the brain and subsequently differentiate into perivascular macrophages,

2) the virus can infect CD4+ T cells in the periphery and the infected T cells can cross the BBB,

3) the virus can degrade the BBB and directly enter the brain where it infects astrocytes and microglia, or

4) the virus can enter by trans-cytosis of brain microvascular endothelial cells (Figure 4).

In the brain macrophages and microglia serve as scavengers and sentinel cells, non-specifically eliminating foreign material and secreting trophic factors that contribute to the maintenance of homeostasis in the CNS (Kaul *et al*., 2005). However, persistent release of neurotrophins may in themselves lead to deleterious consequences in the brain (Rappaport *et al*., 1999; Kaul *et al*., 2005). In general, the damaging effects of these trophic factors are mediated through the activation of cytokines such as TNF-β and TNF-α (Rappaport *et al*., 1999). In addition to these mechanisms, infected microglial cells may also release HIV-1 related proteins such as tat protein, which in turn will have toxic effects in the brain. It has been proposed that this mechanism may in particular have relevance to the development of HAD (Figure 5; Kaul *et al*., 2001).

Astrocytes do not have CD4 receptors and therefore it is unlikely that HIV-1 infects these cells directly. However, astrocytes do express other HIV-1 co-receptors such as CXCR4 and CCR5 that may facilitate entry of the virus (Kaul *et al*., 2001; Ghafouri *et al*., 2006). Astrocytes may also be activated by HIV proteins (e.g. tat protein and gp120), as well as inflammatory cytokines such as TNF-α, IL-1β and interferon-γ (IFN-γ) (Kaul *et al*., 2001; Ghafouri *et al*., 2006).

Malfunction of astrocytes has been associated with significant increases in the intracellular calcium concentration of neurons, with concomitant dysregulation in glutamate handling. These neurochemical changes can result in glutamate toxicity, and the eventual death of neurons (Figure 5; Kaul *et al.*, 2001).

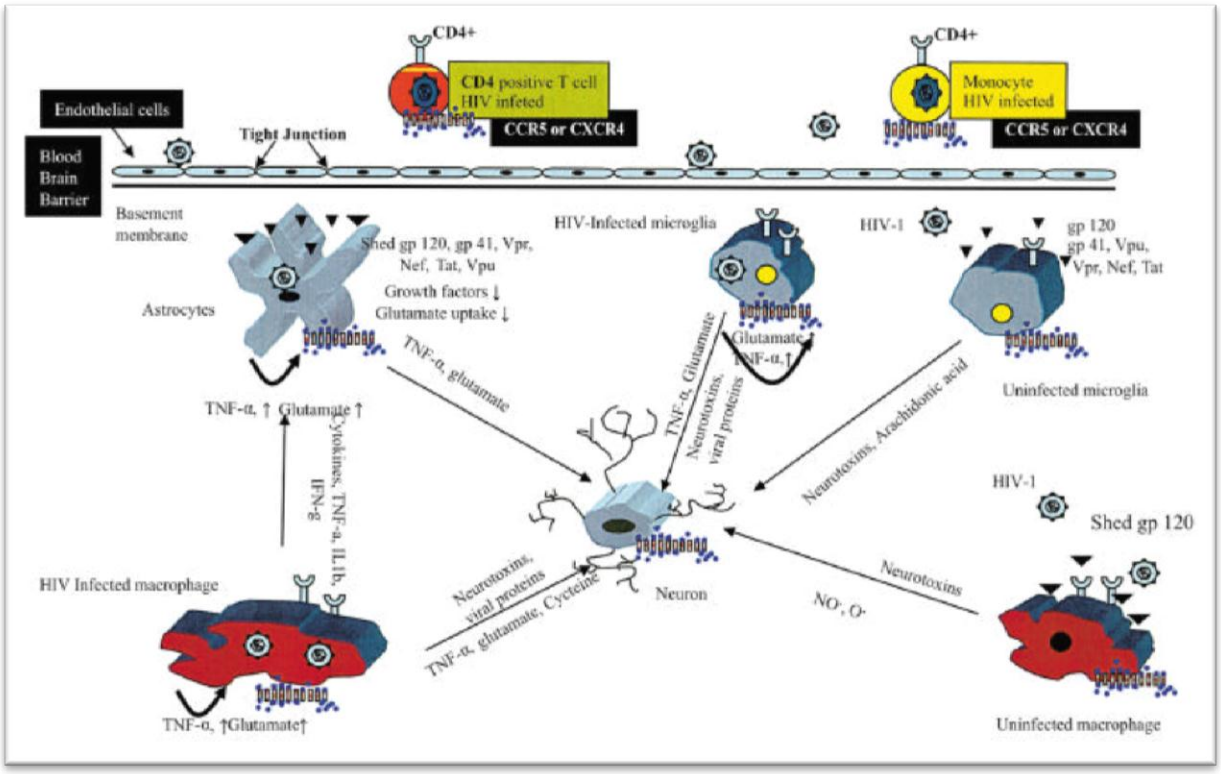
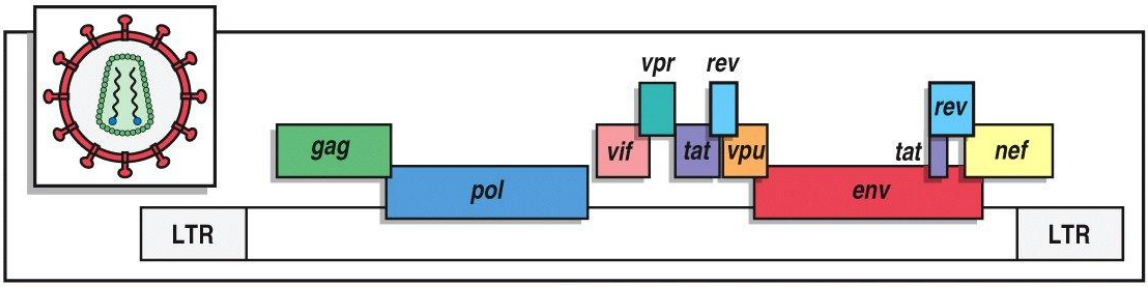


Figure 5: Diagram showing the release of HIV proteins and cytokines by activated microglia and macrophages during HIV-induced neurotoxicity (Pocernicha *et al*., 2005).

5. The effects of HIV-1 proteins in the brain

Studies have suggested that HAD can result from the damaging effects of viral proteins on brain cells. Proteins such as gp120 and tat protein have been shown to have toxic properties, while others (vif, vpr, vpu and nef) have been suggested to be important in establishing interactions between the virus and human host cells (Figure 6; Ghafouri *et al*., 2006; Li *et al*., 2009; Romani *et al*., 2010). The present study chose to focus on the effects of tat protein and therefore subsequent discussions will be limited to the effects of this viral protein only.



Gene		Gene product/function
gag	Group-specific antigen	Core proteins and matrix proteins
pol	Polymerase	Reverse transcriptase, protease, and integrase enzymes
env	Envelope	Transmembrane glycoproteins. gp120 binds CD4 and CCR5; gp41 is required for virus fusion and internalization
tat	Transactivator	Positive regulator of transcription
rev	Regulator of viral expression	Allows export of unspliced and partly spliced transcripts from nucleus
vif	Viral infectivity	Affects particle infectivity
vpr	Viral protein R	Transport of DNA to nucleus. Augments virion production. Cell cycle arrest
vpu	Viral protein U	Promotes intracellular degradation of CD4 and enhances release of virus from cell membrane
nef	Negative-regulation factor	Augments viral replication *in vivo* and *in vitro*. Decreases CD4, MHC class I and II expression

Figure 11-24 Immunobiology, 6/e. (© Garland Science 2005)

Figure 6: This figure depicts the overall genomestructure of HIV-1. The products of the various genes and their functions are also shown.

Tat is a highly basic protein, non-specific and consequently has the ability to bind to many different RNAs (Ruben *et al*., 1989; Li *et al*., 2009). Tat protein is produced early in HIV-1 infection, signifying its important role in regulating transcription of the viral genome especially via the transactivation of TAR (Tat associated region) (Campbell *et al*., 2005; Li *et al*., 2009). Tat protein exists either in an 86 or 101/ 104 amino acid length form (Figure 7). The tat gene consists of 2 exons, the first coding amino acids 1-72 and the second exon coding for amino acids 73-101/104 (Figure 7; Jeang *et al*., 1999; Li *et al*., 2009). The first exon of the tat gene codes for 5 functional regions; the first region has 1–21 amino acids and is responsible for the biological activity of tat protein.

The second region is a cysteine-rich region (amino acids 22–37). Residues 14–31 play an important role in viral infectiveness? (Jeang *et al*., 1999; Campbell *et al*., 2005), while the cysteine residue at position 31 is responsible for binding to the N-methyl-D-aspartic acid (NMDA) receptor on neurons. This cysteine is therefore instrumental in its neurotoxic effects (Li *et al*. 2008; 2009). Thirdly is the basic region (amino acids 49–57), and fourthly a glutamine-rich region (amino acids 58–72). The third and fourth regions together are responsible for binding and nuclear localization, and uptake of tat protein by cells (Campbell *et al*., 2005; Li *et al*., 2009).

The second exon of the tat gene codes for amino acids 73-101/104. This part of tat protein is important for transactivation, trans repression of transcription factors such as AP-1 and NF-kB, and virus replication (Li *et al*. 2008; 2009).

1	2	3	4	5	6
1-21 Pro rich	22-37 Cys rich	38-48	49-59 Basic	60-72 Glu rich	73-101/104

First Exon	2nd Exon

Figure 7: This figure depicts the division of tat protein into various regions. The figure also shows which of the two exons of the tat gene codes for the respective regions of tat protein (Li *et al*., 2009).

6. Apoptosis

One of the mechanisms by which HIV-1 infection may cause neurocognitive impairment is proposed to be via the induction of apoptosis in the brain. There are two pathways that may initiate apoptosis, namely an extrinsic and an intrinsic pathway.

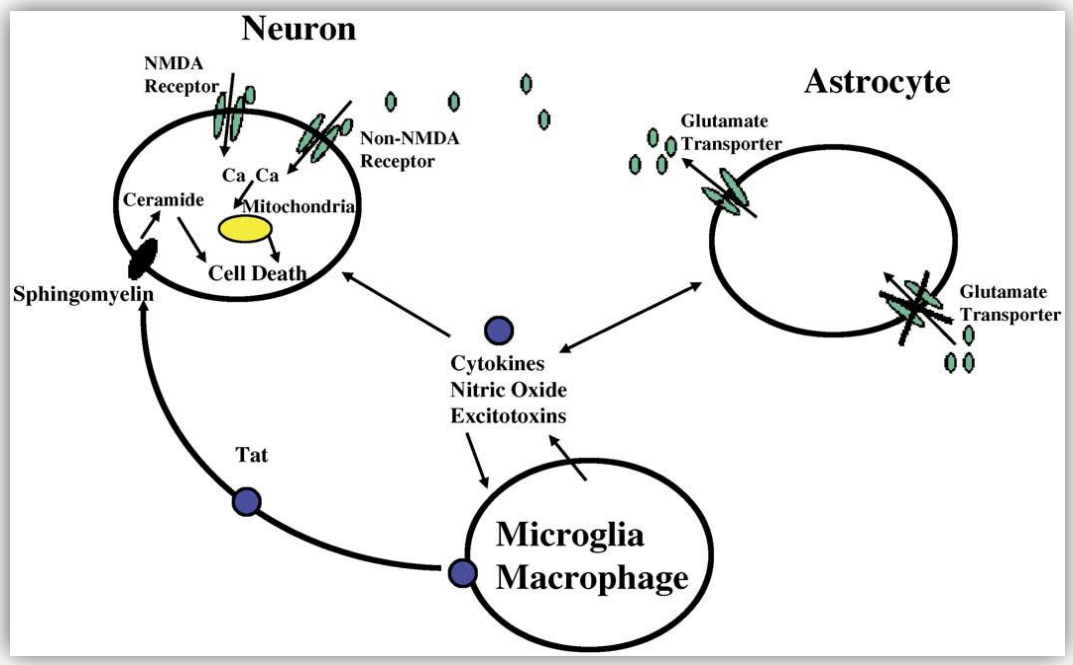


Figure 8: This illustration shows the relationship between tat protein function, the extrinsic pathway that mediates cell death through activation of cytokines, and the intrinsic pathway which involves malfunction of the mitochondrion (Pocernicha *et al*., 2005).

Figure 9 shows how the extrinsic pathway may be initiated through activation of cell-death receptors such as Fas/CD95. The activation of these receptors leads to the subsequent activation of a number of signalling proteins that converge on the enzyme caspase-8. The function of caspase-8 is to cleave procaspase-3, resulting in the production of caspase-3, one of the major enzymes responsible for the induction of apoptosis (Li *et al*., 2005; Pocernicha *et al*., 2005; Romani *et al*., 2010).

The intrinsic pathway starts at the level of the mitochondrion, where apoptosis-inducing compounds such as cytochrome c, are released resulting in the activation of caspase-9. In turn caspase-9 activates caspase-3 (Figure 9; Li *et al*., 2005; Paquette *et al*., 2005; Ruwanpura *et al*., 2008; Romani *et al*., 2010).

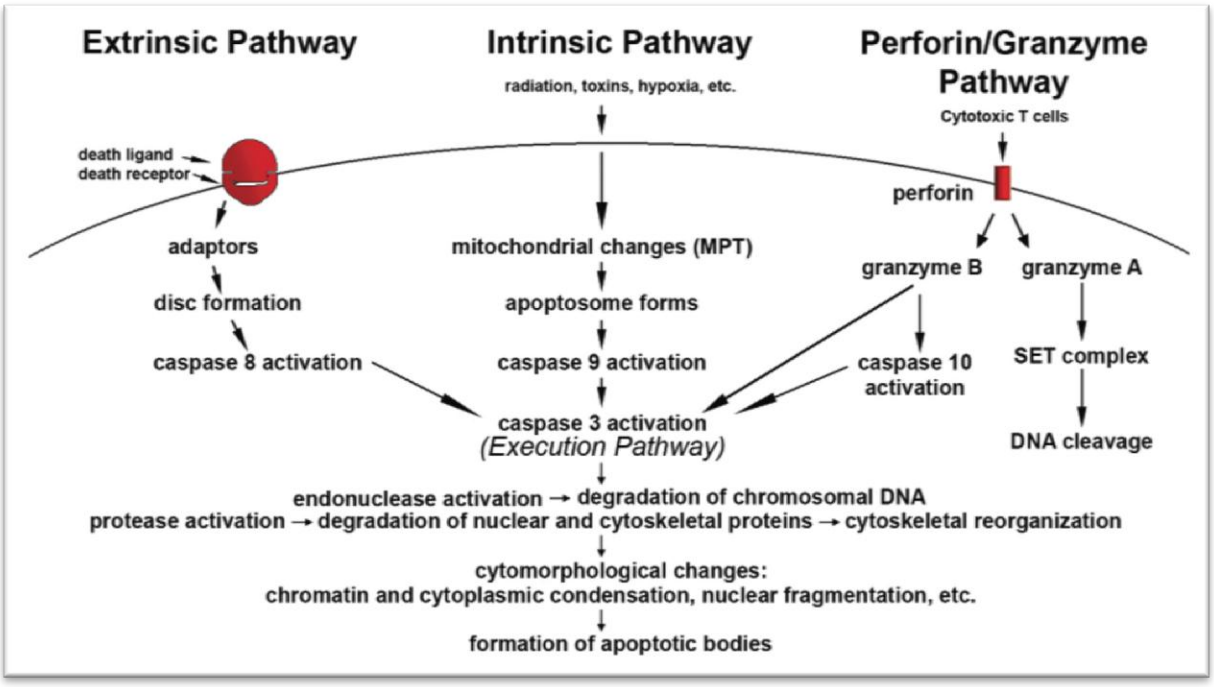


Figure 9: This structure show the complex activation of neurochemical that caused the cell death in the brain that leads to apoptosis (Elmore, 2007)

Besides the extrinsic and intrinsic pathways, stimulation of other pathways may also lead to apoptosis, for example those involving the activity of caspase 6. Together these pathways reflect the complexity of apoptosis, and the myriad of signalling pathways that participate in this mode of cell death (Elmore, 2007; Kantar *et al*., 2011). Nevertheless, it seems as if caspase-3 is fundamental to many pathways, and its activation is central to the final execution of the apoptotic process. Caspase-3 is responsible for the activation of the nuclease known as caspase-activated DNase (CAD) or endonuclease (ENase), whose function is to cleave DNA into 180-200 base pair fragments, one of the hall marks of apoptosis (Li *et al*., 2005; Elmore, 2007; Kanwar *et al*., 2011).

It has been proposed that tat protein may induce apoptosis by upregulating tumour necrosis factor-related apoptosis-induced ligand (TRAIL), and eventually activate caspase-3 (Huang *et al*., 2005). Studies showing tat protein to upregulate Fas, Fas ligand (FasL/CD95L) and TNF-α (Song *et al*., 2006) supported this proposal. Tat protein not only initiated the extrinsic pathway, but reports indicating its activation of the intrinsic pathway have also been documented. For instance, it has been suggested that tat protein interacts with the two non-polymerized subunits of

microtubules (α and β-tubulin dimers), as well as polymerized microtubules, and this interaction may lead to mitochondrial induction of apoptosis (Figure 10; Romani *et al*., 2010). Apparently it is the change in polymerization and depolymerisation microtubule stabilization that facilitates the release of pro-apoptotic proteins e.g. cytochrome c, to induce the intrinsic pathway (Campbell *et al*., 2004; Romani *et al*., 2010).

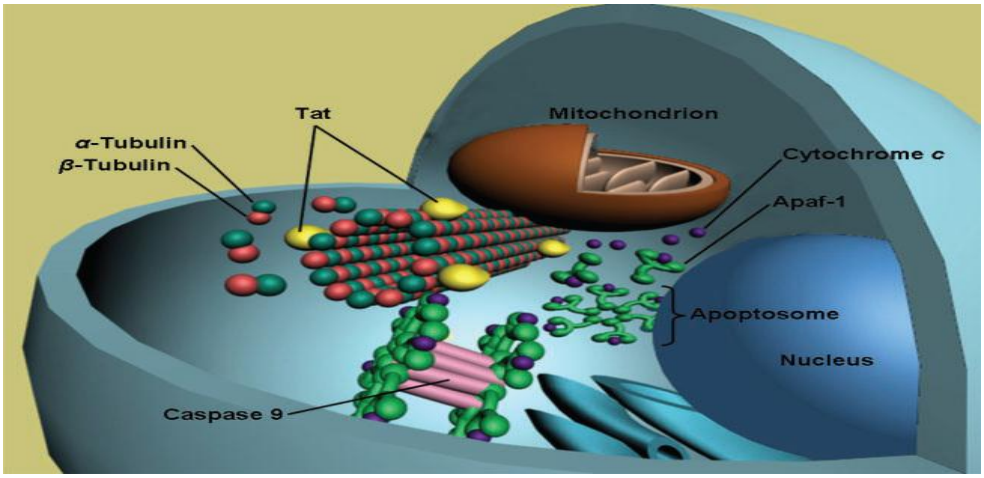


Figure 10: A diagram showing the induction of apoptosis by tat protein altering the microtubule structure within a cell. This disturbance in cytoskeletal stability leads to the activation of the intrinsic pathway of apoptosis (Romani *et al*., 2010).

7. Other mechanisms of tat-mediated cell death

It has been shown that tat protein alters calcium homeostasis within neurons by interfering with calcium regulating systems in the plasma membrane and endoplasmic reticulum (Haughey and Mattson, 2002; Sharma and Bhattacharya, 2009). Abnormal increases in intracellular concentrations of calcium allow this ion to bind to calmodulin, leading to the activation of a number of signalling cascades, resulting in the inappropriate production of cAMP, or activation of nitric oxide synthase and protein kinase C. Previous studies have linked these compounds to cell death (Lipton, 2004; Li *et al*., 2005; Cross *et al*., 2011).

In addition a recent study by Gupta *et al*. (2010), has demonstrated that tat protein may also cause neuronal damage by disrupting glutamate homeostasis in the brain. Glutamate is the major excitatory neurotransmitter in the central nervous system (Lipton, 2004), and has been associated with neuronal cell death primarily due to its excessive activation of N-methyl-D-aspartate

(NMDA)-type glutamate receptors. Overstimulation of these receptors results in extreme Ca^{2+} influx through this ion channel (Lipton, 2004; Cross *et al*., 2011).

Alternatively, tat-mediated glutamate excitoxicity may stem from the interaction of tat protein with glutamate transporters located on glial cells. To prevent excitotoxicity, the proper functioning of Na^{+}-dependent glutamate transporters, or extracellular excitatory amino acid transporters (EAAT-1 and EAAT-2) on astrocytes, and EAAT-3 on neurones is important for maintaining a low concentration of glutamate in the brain (Figure 11; Chrétien *et al*., 2002; Cross *et al*., 2011). Tat protein has been shown to specifically derail the normal functioning of the cystine/glutamate antiporter through the activation of stress-related signalling pathways such as p38 and p42/44 (ERK) (Gupta *et al*., 2010).

Also, during HIV replication, the virus consumes substantial amounts of cysteine, thereby increasing the activity of the antiporter and subsequently increasing glutamate release into the extracellular space (Cross *et al*., 2011). Simultaneously, the shortage of available cysteine disturbs glutathione synthesis leading to glutathione depletion and the eventual accumulation of pro-oxidative substances. These oxidants in turn impair the function of other EAATs, blocking extracellular glutamate uptake and consequently promote glutamate-mediated excitotoxicity (Figure 11). It is interesting to note that the presence of oxidative stress has been observed in the brains and cerebrospinal fluid of HAD patients (Bottiggi *et al*., 2007; Romani *et al*., 2010).

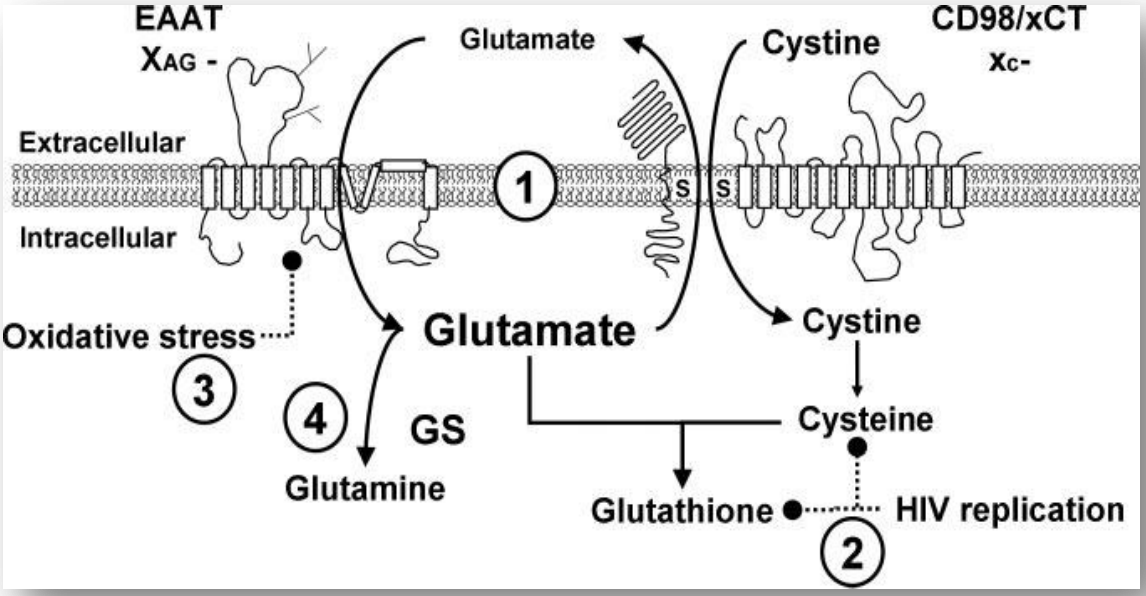


Figure 11: A diagram illustrating the involvement of glutamate transporters in the regulation of the extracellular concentration of glutamate in the brain. The diagram also indicates possible areas of interference in glutamate homeostasis during HIV replication (Cross *et al*., 2011).

8. Models to study cognitive function in rodents

The hippocampus is one of the limbic structures of the brain that plays a prominent role in cognitive processes such as learning, memory storage and spatial navigation. Not surprisingly it is also the brain area that is first to show damage in people suffering from cognitive impairments (Colombo and Broadbent, 2000). Because of its devastating effects on the quality of life, rodent models have been developed to study the pathophysiology of disorders associated with abnormalities in learning and memory. Rodent models can reflect HIV-1 pathogenesis and are also helpful in testing the therapeutic measures or vaccination of HIV (Gorantla *et Al.*, 2012). As much as HIV-1 does not infect rodent cells, mice and rat models can be used to mimic the viral infection by using transgenic rodents to express viral genes, or by transplanting HIV-1 infected cells into immunodeficient rodent tissues (Gorantla *et Al.*, 2012).

In experiments focusing on HIV-associated cognitive impairments, animal models have been created by injecting HIV-infected monocytes into the basal ganglia or hippocampus of the

animal (Wang *et al*., 1999; Zink *et al*., 1999; Li *et al*., 2009). After the intracranial injections the changes in cognitive behavior of the animal was assessed with the use of 2 apparatuses that have been designed to measure learning and memory in rodents. These are the Morris water maze test and the light/dark box test.

8.1. The Morris water maze test

This test assesses the exploratory and spatial memory of rodents (Morris, 1981). The maze consists of a circular pool, 1m in diameter, in which a hidden platform is located in one of the imaginary quadrants. The water is usually opaque so that the platform is not easily visible in the water. Also, the temperature of the water is kept between 22-25°C so that the animal is not exposed to hypothermic stress, once it's placed in the water.

The test entails 3 training sessions, usually on 3 consecutive days, with a test trial on the 4^{th} day following the last training session. During the training sessions the animal is allowed to explore the water maze in order to find the hidden platform. The animal is given a maximum of 120s for this challenge. Failing to find the platform, the animal is guided to it, permitted to stay at least 10s on the platform to familiarise it with the position of the platform within the maze. For each of the subsequent training sessions, the animal is place in a different starting quadrant. To do the test the rodent is placed in any quadrant other than where the hidden platform is located, with the animal facing the wall of the water maze.

The parameter recorded is the time taken for the rat to reach the hidden platform. Control animals usually learn the position of the hidden platform quickly, and hence the time period required to locate the hidden platform decreases progressively with each training session. On the other hand, cognitively impaired animals struggle to learn the position of the hidden platform, and consequently these animals require a significant longer time to locate it.

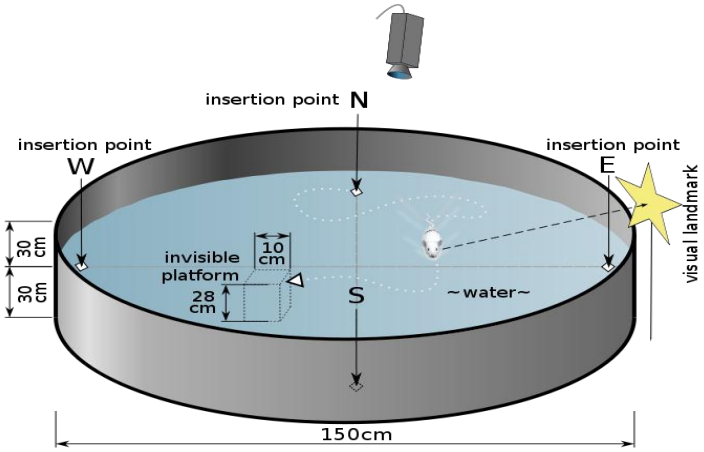


Figure 12: This diagram shows the dimensions of the Morris water maze (Morris, 1981)

8.2. The Light/Dark Box

This test was designed by Boizot in 1985 to primarily assess the spatial memory and navigation skills of rodents. During this test the animals learn a path that leads them from the fearful light compartment of the light/dark box, to the more preferred dark compartment. During the testing phase, the learned path is made more complex by the insertion of obstacles. The obstacles consisted of adding compartment dividers with different positions of holes at the bottom. While the initial divider has a hole in the center, the position of the holes in these extra dividers is towards the left and right corners respectively. This arrangement increased the difficulty of the new pathway for the animal to find its way to the dark compartment. The ability of the animal to navigate its way i.e. time taken to reach the dark compartment, is then documented. Control rats have no difficulty in achieving this goal, while impaired animals either do not find their way or use a much longer time to reach the dark compartment.

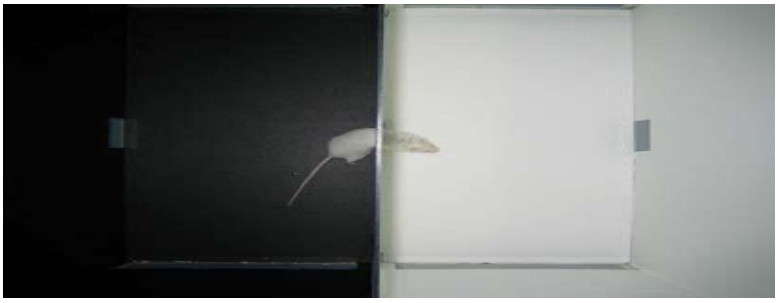


Figure 13: A picture of the light/box test with its two different compartments (Boizot, 1985)

9. Aim of the present study

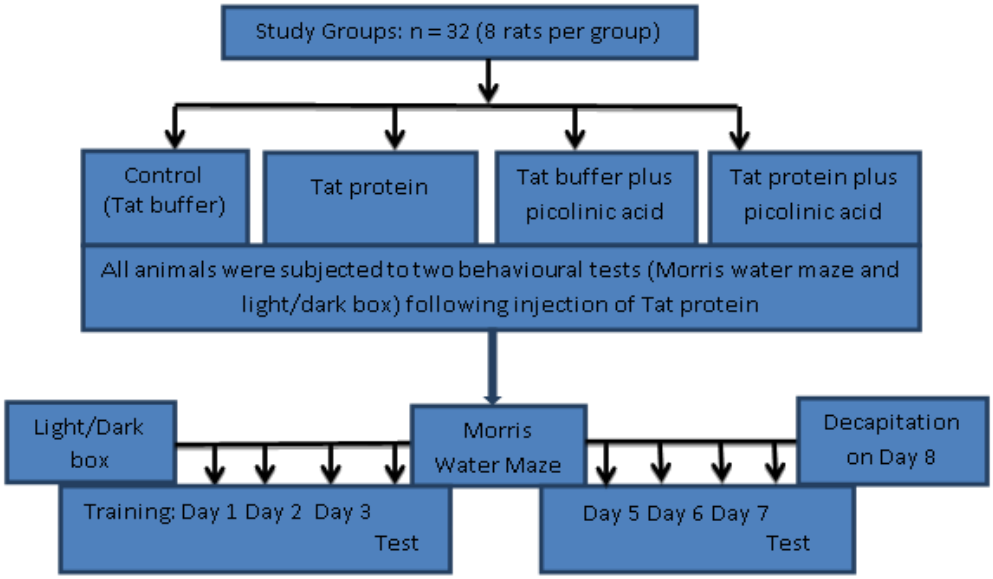
The overall aim of the present study was to develop a rodent model that displayed aspects of HIV-associated cognitive impairment. We then wanted to use this model to investigate whether picolinic acid could be considered as an adjunct treatment to assist in reversing the neuronal damage accompanying HIV infection. Briefly our approach was to

- inject tat protein directly into the hippocampus of rats using stereotaxic techniques;
- identify any cognitive behavioral change that may occur following the tat protein injection, using the Morris water maze and the light/dark box tests;
- assess the extent of apoptotic cell death in the hippocampus induced by the injection of tat protein; and
- evaluate the potential of picolinic acid to counter the damaging effects of tat protein.

Chapter 2

Methodology

1. Experimental design



The overall objective of the present study was to inject tat protein into the hippocampus of rats to induce cognitive impairment comparable to HIV-associated dementia, and to investigate the ability of picolinic acid to normalize tat protein-induced changes in brain function.

In order to achieve this objective a total of 32 rats were randomly divided into four groups namely,

(1) a control group that was treated with 3ml of saline intraperitoneally prior to receiving a bilateral injection of 100µl of tat buffer solution into the dorsal hippocampus (n=8);

(2) a group that was treated with saline but received a bilateral injection of 5µg of tat protein dissolved in a volume of 100µl tat buffer solution, intrahippocampally (n=8);

(3) a group that received an intraperitoneal injection of picolinic acid 10mg/kg before being treated with tat protein (n=8), and

(4) a group that were treated with picolinic acid before receiving bilateral injections of tat buffer solution in their dorsal hippocampi (n=8).

The following day (24hours later), all animals were subjected to behavioral tests (the light/dark box and Morris water maze tests) to assess their learning and memory abilities. The animals were decapitated 8 days after the intrahippocampal injections, their hippocampi dissected and snap frozen in liquid nitrogen and stored at -80ºC. These tissues were later subjected to further neurochemical analysis that included flow cytometry, real time PCR and Western blotting.
Ethical approval for all experimental procedures was obtained from the Animal Ethics Sub-committee of University of KwaZulu-Natal (Ethical clearance number 067/11).

2. Materials

2.1. Animals

Male Sprague-Dawley rats (250-300g) were used in this study. Animals were provided by and housed in the Biomedical Research Center of the university, and were kept under laboratory conditions that included a room temperature of 25ºC and humidity of 70%. The day/night cycle was changed to lights on at 11am/lights off at 11pm in order to perform the behavioral test during the dark period of the cycle when the animals are naturally more active. The animals had free access to food and water.

2.2.Materials

Tat protein was produced by one of our colleagues as previously described (Ramautar *et al*., 2012). The tat protein was expressed in BL21 *E. coli* B cells, and subsequently isolated and purified by ion exchange and affinity chromatographic techniques. Tat protein was injected bilaterally into the hippocampus of animals at a concentration of 1µg/20µl. A volume of 100µl was injected into each hemisphere of the rat. Picolinic acid was obtained from (Sigma-Aldrich, St Louis, USA) and administered at a concentration of 3.36mg/3ml i.e. a dose of 10mg/kg intraperitoneally to the animals. All other general drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, USA). The source of specialised chemicals for the various neurochemical assays is indicated in the relevant sections below.

3. Methods

3.1. Surgical technique

A stereotaxic procedure was adopted to inject the tat protein into the dorsal hippocampus. Briefly, animals were anaesthetized with a combination treatment of pentobarbital (60mg/kg, i.p), followed by atropine sulfate (0.3mg/kg, i.p.). After full anaesthesia has been reached, the skin above the head was shaved and disinfected. The head of the rat was positioned in a David Kopf® stereotaxic apparatus. This was followed by an incision in skin covering the skull. Subcutaneous tissue was removed and the position for the sites of injection clearly marked on the skull. These were at the following coordinates: Anterior-Posterior (AP) = −3.7mm posterior to bregma, Medio-Lateral (ML) = ±2.6mm left or right to the midline, and Dorsal-Ventral (DV) = -3.2mm from the surface of the skull. After the piercing of the dura mater, a Hamilton Syringe was used to infuse the tat protein into the CA1-2 region of the dorsal hippocampus. A total volume of tat (100µl) was carefully dispensed into the brain over a period of 15 minutes.

After the infusion period a further 3 minutes elapsed before the needle was slowly retracted from the brain. This additional time period was allowed to facilitate maximum diffusion of the Tat protein into the hippocampal tissue. Once the needle was completely withdrawn, the skin was sutured. Animals were then returned to their home cages for recovery. Control animals were subjected to the same procedure, but received 100µl of Tat buffer solution.

Tat protein sticks to the wall of glass. This necessitated the Hamilton syringe first to be pre-treated according to methods described previously (Wang *et al*., 1999; Maragos *et al*., 2003). Briefly the Hamilton syringe was pre-treated with a solution of dimethyldichlorosilane and then sequentially rinsed with deionized water, absolute alcohol and again with deionized water. It was then dried in a 60 °C oven for at least 1hour and then cooled to room temperature. The syringe was then ready for experimental use.

3.2. Behavioral Tests

The animals were subjected to two behavioral tests to assess the deficits in cognition namely the light/dark box test and the Morris water maze. These tests determine the learning and recall ability of rats, and cover exploratory, navigation, spatial and contextual memory. Abnormalities in these aspects of memory in the rat have been suggested to reflect some of the neurocognitive symptoms of the HIV patient such as poor concentration, slowness in thinking, difficult in

remembering or ability to learn (impaired registering, recall and recognition of previous information). A more detailed description and explanation of these tests have been provided in Section 8 of Chapter 1 - the Literature Review.

3.*3. Gene expression (Real-Time PCR)*

Total ribonucleic acid (RNA) was isolated from previously stored snap frozen hippocampal tissue. The hippocampal tissue weighing between 20-50mg was homogenized in 175µl of QIAzollysis buffer (Qiagen, Bio-Rad, SA) using a sonicater (12000 Hz for 10 seconds). The RNA was extracted as per manufacturer's instructions. In-depth details of the procedure areattached as Addendum A. The purity of the RNA was determined using a Nano-drop® ND-100 Spectrophotometer (Thermo Scientific, SA) with the purity of RNA taken at the absorbance ratio of A260/A280. RNA was considered as pure if the absorbance ratio ranged between 1.7 and 2.5.

The isolated RNA was used to synthesize cDNA using the iScrip™ cDNA Synthesis Kit (Bio-RAD, SA), as per manufacturer's instruction. The RNA was converted to cDNA using the Gene-Amp 9700 Thermocycler (Applied Biosystems, California, USA), with the following parameters: melting point 25°C for 5 minutes, 40°C for 30 minutes, 85°C for 5 minutes and hold at 4°C. The cDNA was then stored at –20 °C until further analysis (Addendum B)

The primers used were designed and produced commercially (Inqaba, SA) and their sequence is shown in Table 4. The expression of caspase 3 gene was subsequently assessed using a light cycler (Roche Diagnostics). The Light Cycler-master mix consisted of the following: 5.4µl water, 1.6µl $MgCl_2$ (3 mM), 1.0µl forward primer (1.0 µM), 1.0µl reverse primer (1.0 µM) and 1.0µl Fast Start SYBR Green I; (Roche Diagnostics, SA) and 1.0µl cDNA. RNA was quantified using the Light Cycler under the following PCR conditions: one cycle consisting of 95 °C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, 60°C for 1 minute and 72°C for 10 seconds with a single fluorescence measurement (Mackraj *et al*., 2008; Zhen-Hong *et al*., 2011). This was followed by a final cooling step to 40°C. A dilution series of CASP-3 gene and GAPDH amplicons were used to construct a standard curve. Quantitative analysis of the data was analyzed using Light Cycler software 4.1 (Roche Diagnostics). Melting curve analysis was done to detect the presence of a single specific product.

Table 4. Sequences of primers for real-time qPCR (Inqaba, SA)

Target gene Primers (5′-3′)	**(nt)**	**PP**	**bp**
Caspase-3 F5′-GGTGCCACTATGAATTTGAAATTAC-3′	25	1216-1240	25
R5′-CCAACTCTTCATTTCCACAG-3′	20	1401-1382	20

3.4. Western Blot

The hippocampal tissue of 4 animals per group was homogenized in 600µl of RIPA buffer (8.76g NaCl, 10ml Tris pH 7.2 (1M Tris), 10ml 10% SDS, 1ml Triton X100, 10g Na-deoxycholate and 10ml of 0.5M EDTA). The protein lysate was stored at -80°C. The protein concentration was quantified using the Bradford method reagent (50 mg Coomassie Brilliant Blue G250, 50 ml methanol, 100 ml 85% Phosphoric acid and 850 ml distilled water). Samples were diluted in a ratio of 1:2 with a sample buffer (3.55ml distilled water, 1.25ml 0.5 Tris-HCl pH 6.8, 2.5ml glycerol, 2ml 10% SDS and 0.2ml 0.5% Bromophenol blue), 50µl of β-mercaptoethanol was added and heated to 95°C for 4minutes.

The electrophoresis apparatus was set up and filled with 1x running buffer (30.3 g Tris base, 144g glycine and 10g SDS). The protein samples were loaded up to 25µl (20 µg) protein in each well. (Note: The volume of protein samples varied to adjust to similar protein concentrations being loaded). The samples were run on the SDS-PAGE gel at 200V (400 mA) for about 45 to 50 minutes or until the bottom most marker band reached the bottom of the gel. Proteins separated on the gels were then transferred to nitrocellulose membranes using the Turbo blot apparatus (Bio-Rad, SA). Transfer required 30 minutes with this method. The membranes were subsequently blocked in non-fat milk (5g fat free milk powder in 100 ml TBS-T), for 2 hours. Membranes were then incubated overnight with the caspase 3 primary antibody in TBS-T (1:1000, Cell Signaling Technology, USA)after which they were washed three times with TBS-Tween. The membranes were then incubated with anti-rabbit IgG HRP-linked antibody that served as the secondary antibody (1:1000, Cell Signaling Technology, USA) for 2 hours and wash two times with TBS-Tween and again with plain TBS. The chemiluminescence peroxidase substrate-3 reagents were added to the membranes and these were finally viewed on a Chemi-doc system (Bio-Rad, SA).

3.5. Flow Cytometry

3.5.1. Tissue preparation protocol for flow cytometry:

For flow cytometric analyses, hippocampal tissue had to be converted into a cell suspension. The frozen hippocampal tissue was cut into 2mm pieces, placed on the grid part of a Medicon tube and 1ml of PBS was added to the tissue. The Medicon tube was then placed into the Medi-machine (Becton Dickinson, SA) and the tissue homogenized for 30 seconds. 1ml of homogenate was drawn from the Medicon tube using a clean syringe.

The homogenate was filtered through Falcon filters into Falcon tubes and centrifuged for 10 minutes at 4500 r.p.m. The supernatant was discarded and the palette suspended in 1ml of PBS. A cell count was done using a standard Nieubauer haemocytometer. This was necessary to ensure that at least $1x10^6$ cells were present in each sample tube for analysis.

3.5.2. Preparation of sample for flow cytometric analysis

The $1x10^6$ cell suspension was again centrifuged for 10 minutes at 4500r.p.m and the resulting supernatant discarded. The pellet was now resuspended in 0.5ml of cytofix/cytoperm solution (Becton Dickinson, SA) and incubatedon ice for 20minutes. After this the resuspended cells were washed with 0.5ml of Perm/wash (Becton Dickinson, SA). This step was repeated. The cells were then incubated with polyclonal rabbit anti-active caspase-3 antibody (Caspase 3 kit, The Scientific Group, SA) in the dark for 30-50 minute at room temperature. The cell suspension was then centrifuged (10 minutes at 4500 r.p.m.), washed with 1ml of BD Perm /wash, and finally resuspended in 0.5ml of BD Perm/wash for analysis on a FACS Calibur flow cytometric machine (Becton Dickinson, SA). Details of the PE Active Caspase-3 Apoptosis Kit can be found in Addendum C.

4. Statistics

All results are presented as mean ±SEM. The data was analyzed using the software program Graph Pad Prism (version 5, San Diego, California, USA). Data was subjected to either ANOVA with repeated measures or ANOVA where appropriate. This was followed by Tukey's multiple comparison test. Differences were considered significant when the p value was less than 0.05.

Chapter 3

3. Results

3.1. Behavioral test (Light/dark box)

Animals were subjected to three days of learning trials and a test trial was performed on the 4th day. The group that was injected with tat buffer showed good learning and memory ability when these animals were tested in the light/dark box (Figure 14). Already on the second trial day the time to move into the dark compartment was significantly reduced when compared to trial day 1 (56.8 ± 2.5 vs 17.5 ± 5.9; $p < 0.05$). This significant decrease in time to find the dark compartment prevailed on trial day 3, as well on the test day (Figure 14; trail 3 = 7.1 ± 1.4; Test = 12.9 ± 2.0; $p < 0.05$).

Unlike the tat buffer-treated animals, the group that received tat protein showed poor learning and memory ability (Figure 14). While there was a decrease in time taken to enter the dark compartment between trial 1 and 2 (56.25 ±2.498vs43.38 ± 5.982, $p > 0.05$), this change was not significant. Similarly, a further reduction in time on trial day 3 (37.75 ± 8.845; $p > 0.05$) still did not reach significance when compared to either trial day 1 or 2. When the animals were tested on day 4, they required a comparable amount of time to enter the dark compartment as trial day 2 and 3. There were therefore no significant differences in the time taken for the animals that were treated with tat protein to enter the dark compartment when assessed on the respective days (Figure 14).

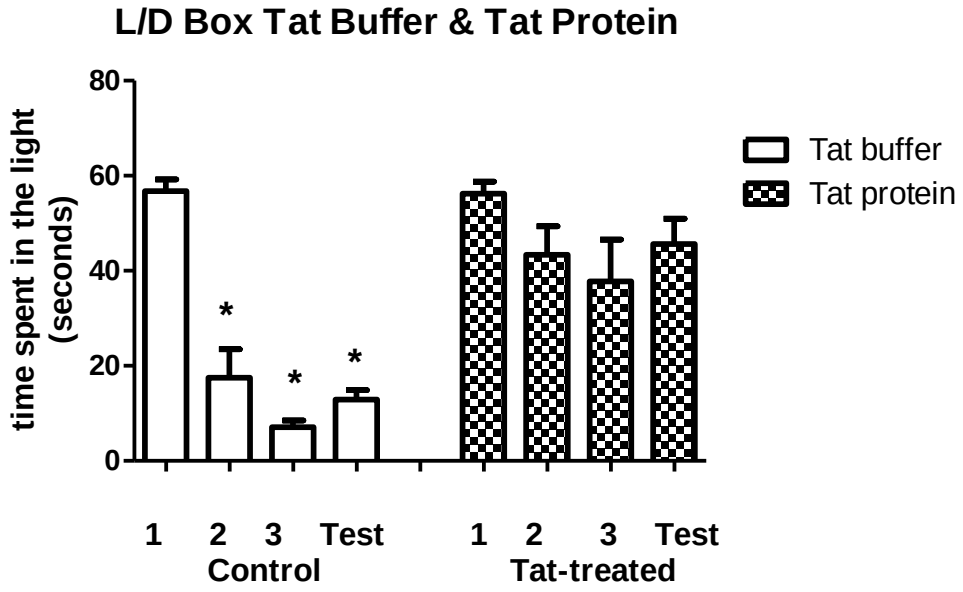


Figure 14: This graph represents the time spent by tat buffer (control) and tat protein treated animals to move from the light compartment into the dark compartment of a light/dark box. Values are the mean ± SEM of 8 animals.

*p<0.05, significantly different from trial 1 (ANOVA with repeated measures followed by Tukey's multiple comparison test).

3.2. Morris water maze test

This test assesses explorative and spatial learning and memory. The group that was injected with tat buffer showed good learning and memory capability when tested in the Morris water maze. The time spent by these animals to find the hidden platform during trial 2 was significantly reduced when compared to trail 1 (Figure 15; trial 2 = 94.63 ± 12.59vs trial 1 30.88 ± 7.222; $p < 0.05$). This significant decrease in time spent to find the hidden platform was again observed during trail 3, as well as during the test trial on day 4 (Figure 15; trial 319.13 ± 7.376; test = 23.88 ± 4.223; $p < 0.05$).

In contrast, rats that were treated with tat protein demonstrated poor learning and memory ability. The differences in time spent to find the hidden platform between the various trails as

well as the test trial were not significant (Figure 15; trial 1 = 87.63 ± 13.91; trial 2 = 100.3 ± 13.90; trial 3 = 60.00 ± 15.63; test = 76.50 ± 14.05; $p > 0.05$).

Tat protein treated rats that also received picolinic acid showed a partial recovery in their learning and memory ability when tested on the Morris water maze (Figure 16). While there were no significant differences between the times spent to find the hidden platform during the 3 learning trials, a significant change was observed between the times spent to find the hidden platform during trial 2 versus the test trial. (Figure 16; trial 2 = 87.80 ± 11.99vs test =32.00 ± 13.11; $p < 0.05$).

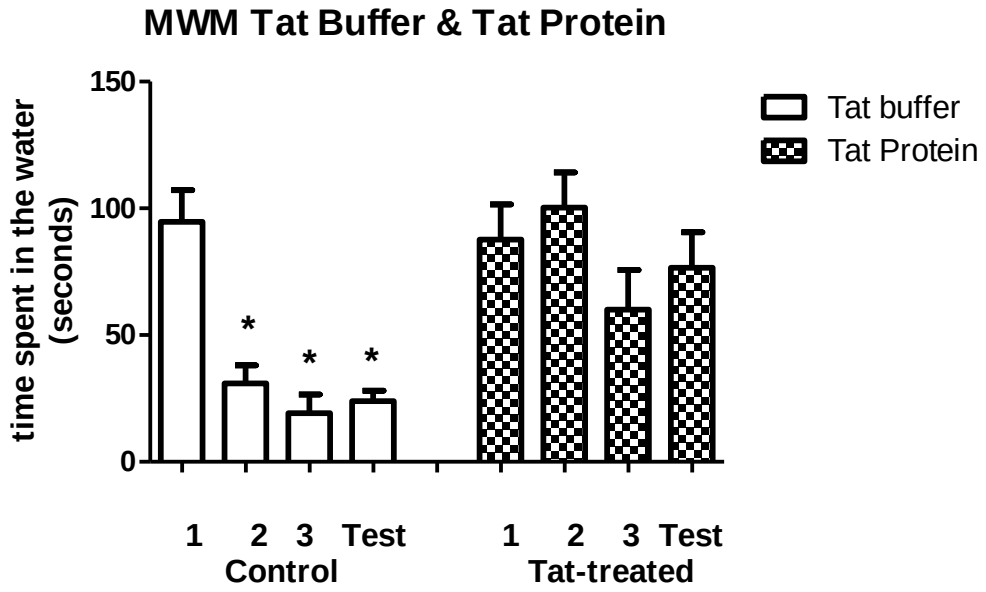


Figure 15: This graph represents the time spent by tat buffer (control) and tat protein treated animals to find the hidden platform during a 3-day trial / next-day test session in a Morris water maze. The values are the mean ± SEM of 8 animals.

*$p<0.05$, significantly different from trial 1 (ANOVA with repeated measures followed by Tukey's multiple comparison test).

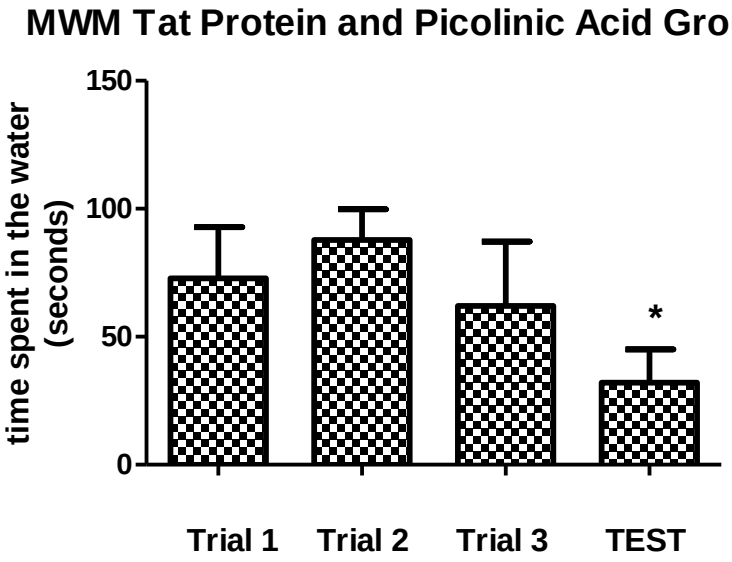


Figure 16: This graph represents the time spent by animals that were treated with tat protein plus picolinic acid during a 3-day trial / next-day test session in a Morris water maze. The values are the mean ± SEM of 5 animals.

*$p<0.05$, significantly different from trial 2 (ANOVA with repeated measures followed by Tukey's multiple comparison test).

Previous studies from our own laboratory (Ramautar *et al*., 2012) and others (Li *et al*., 2005; Paquette *et al*., 2005; Ruwanpura *et al*., 2008; Romani *et al*., 2010) have shown that tat protein may result in apoptotic cell death. In our study we subsequently focused on caspase 3 activity and expression levels, since it is one of the executioner enzymes central to apoptosis.

3.3. Real-time PCR

Real-time PCR was used to determine the effects of tat protein on the transcription levels of the caspase 3 gene in the hippocampus. Our data showed that injection of tat protein led to a significant increase in caspase 3 mRNA levels when compared to the tat buffer treated control group (Figure 17). This increase was not seen in the tat protein group treated with picolinic acid that showed caspase 3 mRNA levels similar to control animals (Figure 17).

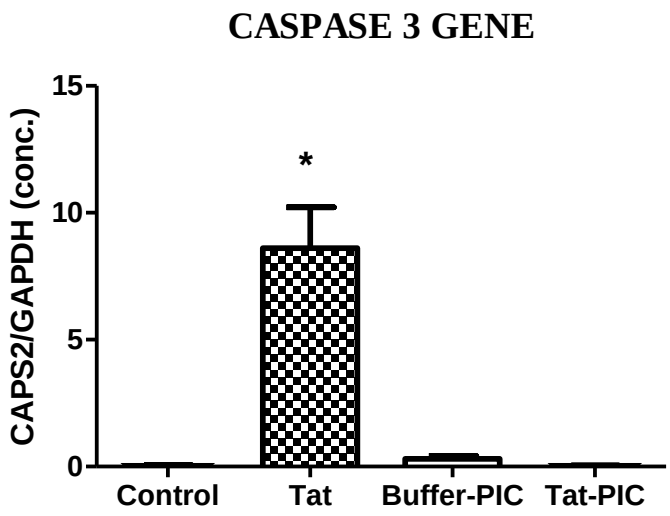


Figure 17: This graph represents the expression of mRNA of caspase 3 gene in the hippocampus of rats treated with tat buffer, tat protein, picolinic acid alone or tat protein and picolinic acid. Values are the mean ± SEM of 3 animals. The experiment was done twice.

*$p<0.05$, significantly different from control, picolinic acid and tat+picolinic acid groups (ANOVA followed by Tukey's multiple comparison test).

3.4. Western blot

Western blot analysis was used to correlate our real time PCR results, i.e. mRNA transcription levels, with protein expression levels. Here visual inspection of the Western blot showed that tat protein stimulated both pro-active and active caspase 3 expression levels (Figure 18). Treatment with picolinic acid prevented this increase, especially in the case of active caspase 3, where its expression was significantly reduced (Figure 18). Statistical analysis of the intensity bands showed that the increase in active caspase 3 expressions was significantly reduced by picolinic acid treatment (Figure 19).

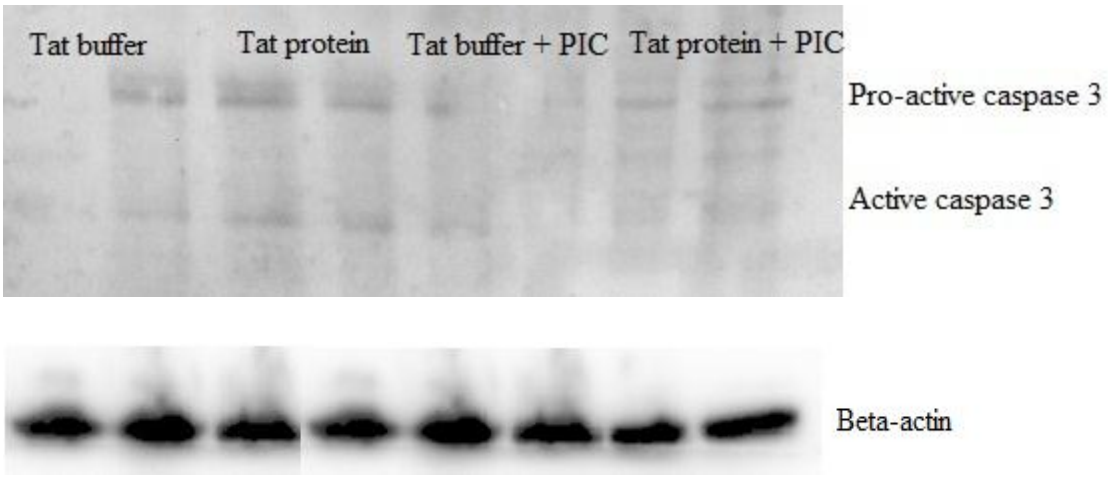


Figure 18: A representative Western blot of 3 experiments showing pro-caspase 3 and active caspase 3 staining in tat buffer treated, tat protein treated, tat buffer plus picolinic acid treated and tat protein plus picolinic acid treated animals.

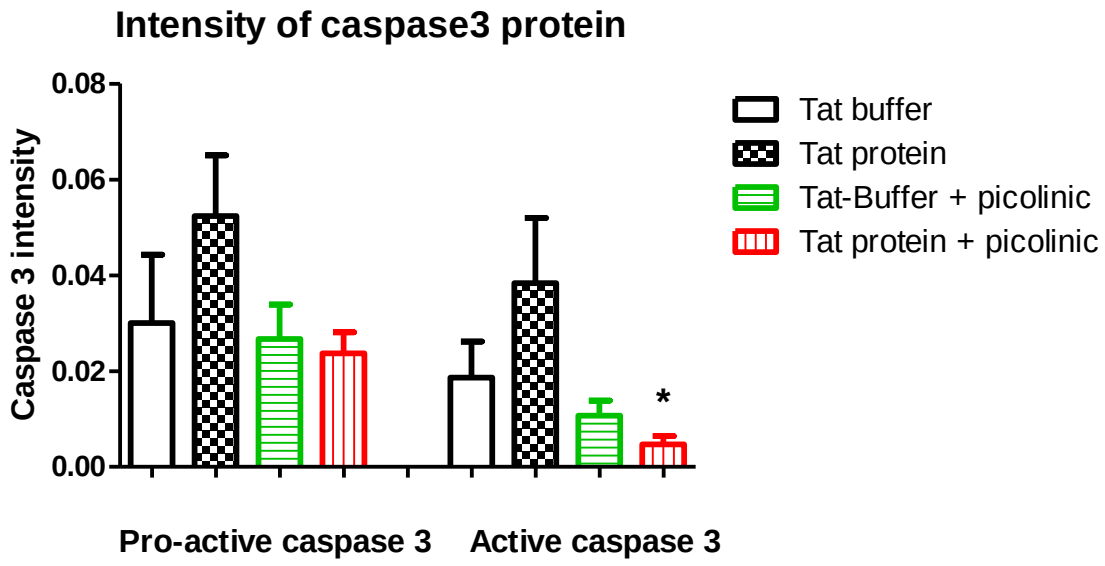


Figure 19: A graph showing the mean intensities of 3 Western blots of the pro-active caspase 3 and active caspase 3 bands of the various treated groups.

*$p<0.05$, significantly different from tat protein treated group (ANOVA followed by Tukey's multiple comparison test).

3.5. Flow cytometry

Flow cytometric techniques were used to assess intracellular expression of caspase 3 in the cells of the hippocampus. Figure 20 suggested the tat protein treated group to show higher caspase 3 staining than the group that was treated with tat buffer, while the group of rats treated with tat buffer plus picolinic acid showed an even greater intensity of staining. However, statistical analysis of the means of the results of 3 animals showed that these increases were not significantly different (Figure 21).

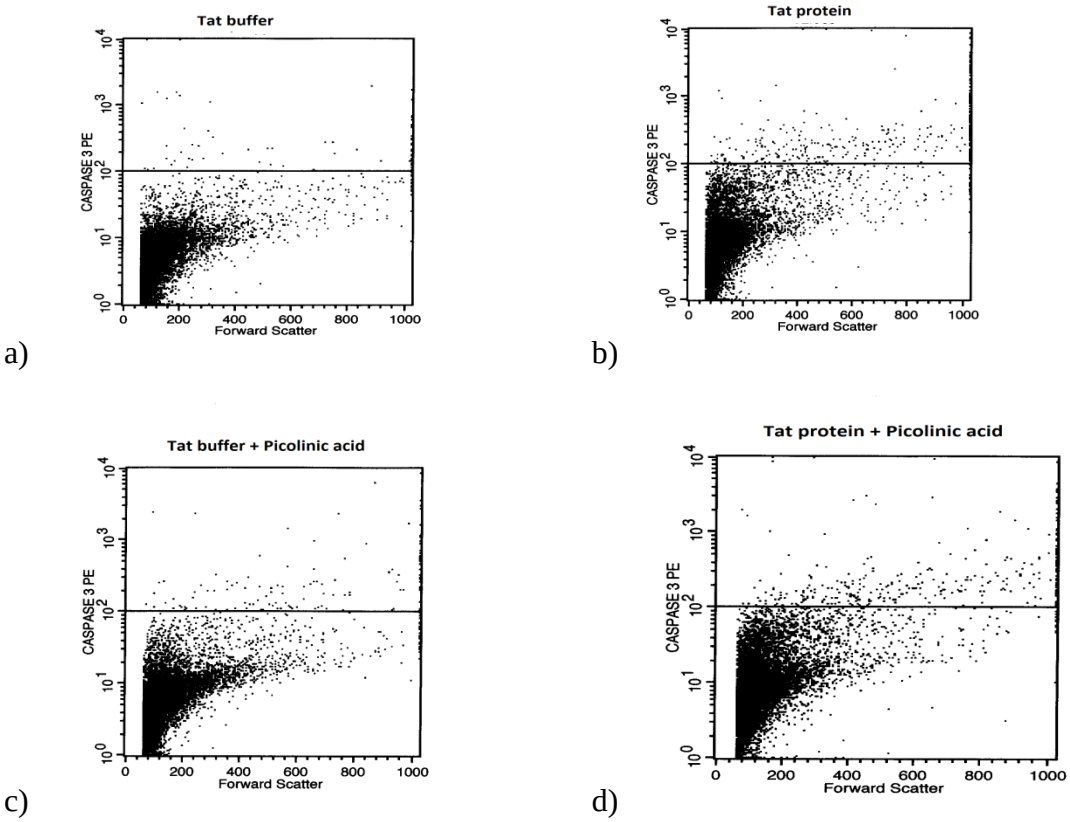


Figure 20: Representative flow cytometry scatter graphs of one animal showing the level of caspase 3 active cells, a) Tat buffer group; b) Tat protein treated group, c) Tat buffer plus picolinic acid, and d) Tat protein plus picolinic acid.

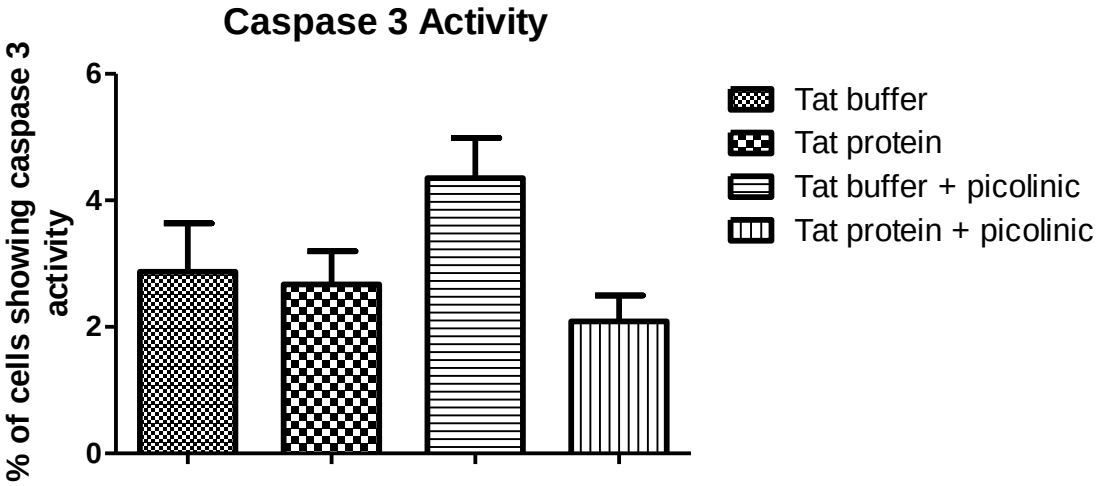


Figure 21: This graph represents the caspase 3 staining in hippocampal cells obtained from tat buffer treated, tat protein treated, tat buffer plus picolinic acid treated and tat protein plus picolinic acid treated animals. Values are the mean ± SEM of 3 animals.

Chapter 4

Discussion

Recent studies showed that more than 60% of HIV-infected individuals go on to develop HIV associated neurocognitive disorder (HAND) (Bansal *et al*., 2000; Grant, 2008; Singh *et al*., 2010). This alarming statistic has been a primary driver for current studies to continue their search for elucidating the mechanism by which the virus causes this disorder. In the present study we tested the hypothesis that the viral protein, tat, could lead to neuronal cell death, thereby leading to the development of cognitive disabilities. In doing so, we injected tat protein into the hippocampus of rats and assessed the effects thereof on the learning and memory ability of the animal. Simultaneously, we examined whether picolinic acid could block the deleterious effects of tat protein. Our study specifically investigated apoptotic cell death and particularly focused on the expression of the executioner enzyme caspase 3.

In brief our findings were:

1. Injection of tat buffer into the hippocampus has no effect on learning and memory,
2. Injection of tat protein into the hippocampus impaired learning and memory,
3. Tat protein-treated animals showed upregulation of the caspase 3 gene as exhibited by PCR,
4. Western blot analysis showed increased expression of caspase 3 enzyme in the tat protein-treated group,
5. Assessment of the level of apoptotic death by flow cytometry provided differences between the various groups of animals, but these were not significant, and
6. Pre-treatment with picolinic acid significantly prevented the deleterious effects of tat protein.

HIV-tat protein is a trans-activator protein that has previously been implicated in the development of neurocognitive impairment (Li *et al*., 2009; Carey *et al.,* 2012). In our study, injection of tat protein into the hippocampus also led to difficulties in learning and memory as assessed by two paradigms, namely the Morris water maze and light/dark box. The Morris water maze is the most popular test that is used to determine hippocampal-dependent spatial-based learning and memory function in rodents (Self *et al*., 2009; Fitting *et al*., 2013). For example,

Self *et al*. (2009) employed the Morris water maze to assess the behavioral changes in animals that were exposed to binge-like alcohol consumption prior to tat protein treatment. In these experiments the authors showed that rats that received alcohol and tat protein performed significantly poorer in the Morris water maze compared to all other experimental groups. Likewise, Fitting *et al.* (2013) demonstrated how increased expression of tat protein in transgenic mice caused a suppression of long term potentiation in Schaffer collateral - CA1 pyramidal cell synapses, and this suppression was associated with deficits in learning and memory. Similar to these previous studies, our results also indicated a decline in learning and memory in the Morris water maze in those rats that were treated with tat protein.

The finding of tat-mediated cognitive impairment as observed in the Morris water maze, was confirmed by data obtained from the light/dark box experiments. The light/dark box is used to test anxiety-like behavior and the explorative or navigation skill movement from the light compartment to the dark compartment (Boutin and Hoscoët, 2003). The fact that tat protein-treat animals required a significantly longer time to reach the dark compartment than the tat buffer controls supported the notion of tat protein disrupting normal learning and memory processes in these animals.

It is well known that the hippocampus is one of the important parts of the brain that is responsible for learning and memory. Thus, the negative effects of tat protein on cognitive function can be attributed to the protein having direct toxic effects on hippocampal neurons. In the present study the injection of tat protein directly into the hippocampus was toxic to the cells as suggested by increased caspase 3 transcription and expression, an enzyme closely associated with apoptotic cell death (Elmore, 2007). Our results are in agreement with those of others also reporting neurotoxic effects of tat protein on hippocampal cells followed by learning and memory impairment (Li *et al*., 2009; Carey *et al*., 2012). These authors used genetically engineered mice to achieve an increase in the expression of tat protein in the hippocampus, and demonstrated that this elevation in tat levels could cause abnormalities in cognitive behavior. Additional evidence for tat protein-induced toxicity stems from both in vitro and in vivo experiments. Exposing hippocampal slices to tat protein resulted in pyramidal cell loss in the CA3 region that was accompanied by a concomitant decrease in microtubule associated protein-2 staining (Magaros *et al*., 2003). This result correlated well with the reduction in microtubule

associated protein-2 staining that these authors observed in the hippocampal tissue from patients suffering from HIV encephalitis (Magaros *et al*., 2003).

Since caspase 3 expression was raised in the hippocampus of tat protein-treated rats, it is likely that damage to hippocampal cells in our experiments was mostly due to apoptosis. This result is plausible as in vitro studies have shown tat protein to induce caspase 3 driven apoptosis in CEM-GFP cells (Ramautar *et al.*, 2012), as well as human enterocytes (Buccigrossi *et al*., 2011). Furthermore, a recent study by Gibellini *et al.* (2012) has shown that tat protein may induce the expression of different cytokines such asIL-1, IL-6, M-CSF, IL-10, TNF-α, and RANKL that affect neuronal cells negatively. It is well known that some of these cytokines are initiators of apoptotic pathways (Sei *et al.*, 1995; Nagata, 1996).

Apoptosis can occur at least via two pathways, namely the extrinsic or the intrinsic pathway. The extrinsic pathway causes cell death through the upregulation of cytokines, chemokines and death-inducing receptors (e.g. FAS/CD95 receptor). Cell death via the intrinsic pathway involves the release of apoptosis-inducing molecules such as cytochrome c from the mitochondrion, followed by the activation of APAF-1 and the executioner enzymes caspases 9 and caspase 3 (Li *et al*., 2005; Paquette *et al*., 2005; Ruwanpura *et al*., 2008; Romani *et al*., 2010). These enzymes are responsible for the cleavage of DNA, the formation of apoptotic bodies and eventually cell death.

Apoptotic cell death is not the only strategy that tat protein could have used to induce cell death. For instance, tat protein may cause abnormal increases in intracellular concentrations of calcium, facilitating this ion to bind to calmodulin with subsequent activation of a number of signalling cascades. As such, inappropriate levels of cAMP are produced or there may be undesired activation of nitric oxide synthase and protein kinase C enzymes, leading to cell death (Lipton, 2004; Li *et al*., 2005; Cross *et al*., 2011). In addition, tat protein has been shown to increase oxidative stress (Pocernich *et al*., 2005). Reactive oxygen species are toxic oxygen moieties, such as hydroxyl radical, peroxyl radical, superoxide anion, hydrogen peroxide, and peroxynitrite, and all of them have been associated with the oxidation of proteins and DNA, and the peroxidation of lipids. Oxidative stress can also induce the production of ceramide, sphingomyelin and4-hydroxynonenal. High levels of these compounds have been found in the CSF and brain tissue of HIV patients (Pocernich *et al*., 2005).

Pre-treating animals with picolinic acid prior to tat protein administration blocked the detrimental effects of tat protein with respect to learning and memory behavior, and the transcription and expression of caspase 3. This result was in line with an earlier in vitro study where PA was shown to inhibit transactivation and apoptosis in CEM-GFP cells (Ramautar *et al*., 2012).

Picolinic acid occurs naturally in the body albeit in small concentrations. It is a by-product of the kynurenine pathway where tryptophan is converted to quinolinic acid (Grant *et al*., 2009). Picolinic acid can be found in primary neurons and astrocytes. It is also present in CSF at low concentrations. Picolinic acid is a metal chelator and can bind cations such as zinc, manganese, copper and iron (Grant *et al.*, 2009).

One of the main functional components of tat protein is its zinc-finger moiety that is responsible for transactivation of virus and cellular genes (Davies *et al*., 2010; Ramautar *et al*., 2012). Amino acids 1-72 of the tat protein form five regions that are responsible for the biological activity of tat protein. These amino acids are coded by the first exon of the tat protein gene. The two regions that are rich in cysteine residues form the binding sites for zinc (Campbell and Loret, 2009).

Since picolinic acid is a potent metal chelator, one of the mechanisms by which it can inhibit the action of tat protein, is to sequester its zinc ions. In vitro studies have attributed the prevention of tat protein-induced apoptosis in CEM-GFP cells to the ability of picolinic acid to competitively remove zinc from the zinc-moieties in tat protein (Ramautar *et al*., 2012). This explanation is plausible as other specific metal chelators have also been used to inhibit HIV transcription (Debebe *et al.,* 2007). These authors demonstrated how iron, a clinically approved oral iron chelator blocked HIV-1 transcription by decreasing the expression of cyclin-dependent kinase 2, a regulator of the cell cycle that is essential for tat-dependent transcription (Debebe *et al*., 2007).

Alternatively picolinic acid could also have interfered with the neurotoxic effects of tat protein in other biological areas. For example, picolinic acid could have acted as an anti-oxidant, stabilized blood brain barrier permeability or could have limited the production of inflammatory cytokines as picolinic acid does cross the BBB. All these are mechanisms by which tat protein could have

induced its toxic effects (Jones *et al*., 1998; Banks *et al*., 2005; Campbell and Loret, 2009). However further studies are required to confirm these mechanisms.

Chapter 5

Conclusion

Neurocognitive impairment in persons living with HIV has become the new concern in the fight against the HIV/AIDS pandemic, especially in lieu of the significant improvement in longevity brought about by antiretroviral treatment. In spite of the high prevalence rates of HIV associated neurocognitive disorders (HAND) its pathophysiology remains poorly understood and consequently the effective management thereof is also lacking. The present study was subsequently conducted to improve our current understanding of HAND and to investigate the potential of picolinic acid as an adjunct therapy for HAND.

The broad objectives of our study were therefore:

- To establish a rat model that displayed partial symptoms of HIV-associated neurocognitive disorder;
- To identify a molecular mechanism that may explain these symptoms; and
- To evaluate the potential of picolinic acid to be considered as an adjunct treatment in the management of HIV-associated neurocognitive disorders.

To achieve the first objective, bilateral injections of tat protein were made directly in the hippocampus of Sprague-Dawley rats. The cognitive behavior of these animals was assessed by testing their learning and memory ability in a Morris water maze and Light/Dark box. Our results showed that tat protein treated rats performed worse than their tat buffer treated controls, suggesting that learning and memory function was impaired in those animals that received tat protein.

We investigated whether the deleterious effects of tat protein on learning and memory could have been due to apoptotic cell death in the hippocampus. We focused on caspase 3 as our indicator of apoptosis. This was done by assessing caspase 3 gene transcription using PCR techniques, determining its expression with Western blots and measuring enzyme staining activity on a flow cytometer. These experiments led us to believe that tat protein may induce apoptosis, although additional measurements of apoptotic markers may be required to state this conclusively.

Pretreating rats with picolinic acid significantly decreased the damaging effects of tat protein with respect to cognitive behavior as well as caspase 3 activity. This result therefore suggested that picolinic acid may be considered as a therapeutic agent in the management of HAND. Further studies however, will be necessary to characterize its toxicological profile and pharmacokinetic properties.

A number of limitations of our study have been identified. Larger group sizes would have allowed us to increase the statistical power of experiments that could have led to more clear recommendations. At present we remain cautious about our findings. We also experienced difficulty in preparing a homogenous cell suspension for the flow cytometer experiments, and cellular damage during this preparation process could have clouded our data, resulting in less convincing observations. Another shortfall of our study was that we did not ascertain whether the toxic effects of tat protein were limited to neurons, glial cells or both. This however, may be the basis for a future study.

Additional studies that are considered for the future include investigating how

- exposure to stress would influence the toxic effects of tat protein,
- tat protein alters glutamate homeostasis in the hippocampus,
- neurotrophins in the hippocampus respond to tat protein toxicity, and
  further explore the beneficial effects of picolinic acid for tat protein-induced toxicity.

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Addendum A

- Fresh, frozen, or RNA*later* stabilized tissues can be used. Tissues can be stored at –70°C for several months. Flash-freeze tissues in liquid nitrogen, and immediately transfer to –70°C. Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates from step 4 can also be stored at –70°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 5. Avoid prolonged incubation, which may compromise RNA integrity.
- If desired, more than 30 mg tissue can be disrupted and homogenized at the start of the procedure (increase the volume of Buffer RLT proportionately). Use a portion of the homogenate corresponding to no more than 30 mg tissue for RNA purification, and store the rest at –80°C.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 8 for safety information.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.

  Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh or frozen in single-use aliquots. Buffer RLT containing DTT can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix D (page 69).

Procedure

1. **Excise the tissue sample from the animal or remove it from storage. Remove RNA*later* stabilized tissues from the reagent using forceps. Determine the amount of tissue. Do not use more than 30 mg.**

   Weighing tissue is the most accurate way to determine the amount.

   Note: If the tissues were stored in RNA*later* Reagent at –20°C, be sure to remove any crystals that may have formed.

2. **Follow either step 2a or 2b.**

2a. **For RNA*later* stabilized tissues:**
If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed to step 3.

If using only a portion of the tissue, cut it on a clean surface. Weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed to step 3.

RNA in RNA*later* stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNA*later* RNA Stabilization Reagent. Previously stabilized tissues can be stored at –80°C without the reagent.

2b. **For unstabilized fresh or frozen tissues:**
If using the entire tissue, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately to step 3.

If using only a portion of the tissue, weigh the piece to be used, and place it into a suitably sized vessel for disruption and homogenization. Proceed immediately to step 3.

RNA in harvested tissues is not protected until the tissues are treated with RNA*later* RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

Note: Remaining fresh tissues can be placed into RNA*later* RNA Stabilization Reagent to stabilize RNA (see protocol on page 36). However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.

3. **Disrupt the tissue and homogenize the lysate in Buffer RLT (do not use more than 30 mg tissue) according to step 3a, 3b, 3c, or 3d.**

   See "Disrupting and homogenizing starting material", pages 20–23, for more details on disruption and homogenization.

   Note: Ensure that β-ME is added to Buffer RLT before use (see "Things to do before starting").

After storage in RNA*later* RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem. For easier disruption and homogenization, we recommend using 600 µl Buffer RLT.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueLyser and rotor–stator homogenizers generally results in higher RNA yields than with other methods.

Table 8. Volumes of Buffer RLT for Tissue Disruption and Homogenization

Amount of starting material (mg)	Volume of Buffer RLT (µl)
<20	350 or 600*
20–30	600

* Use 600 µl Buffer RLT for tissues stabilized in RNA*later* RNA Stabilization Reagent or for difficult-to-lyse tissues.

3a. Disruption and homogenization using a rotor–stator homogenizer:
Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in a suitably sized vessel. Add the appropriate volume of Buffer RLT (see Table 8). Immediately disrupt and homogenize the tissue using a conventional rotor–stator homogenizer until it is uniformly homogeneous (usually 20–40 s). Proceed to step 4.

3b. Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer:
Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see Table 8). Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Proceed to step 4.

3c. Disruption using a mortar and pestle followed by homogenization using a needle and syringe:
Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see Table 8), and homogenize by passing the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. Proceed to step 4.

3d. **Disruption and homogenization using the TissueLyser:**
See the *TissueLyser Handbook*. Then proceed to step 4.

4. **Centrifuge the lysate for 3 min at full speed. Carefully remove the supernatant by pipetting, and transfer it to a new microcentrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps.**

   In some preparations, very small amounts of insoluble material will be present after the 3 min centrifugation, making the pellet invisible.

5. **Add 1 volume of 70% ethanol* to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.**

   Note: The volume of lysate may be less than 350 µl or 600 µl due to loss during homogenization and centrifugation in steps 3 and 4.

   Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. **Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.†**

   Reuse the collection tube in step 7.

   If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.†

   Optional: If performing optional on-column DNase digestion (see "Eliminating genomic DNA contamination", page 23), follow steps D1–D4 (page 69) after performing this step.

7. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.†**

   Reuse the collection tube in step 8.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

   Skip this step if performing optional on-column DNase digestion (page 69).

* Using 50% ethanol (instead of 70% ethanol) may increase RNA yields from liver samples.

† Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 8 for safety information.

8. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

   Reuse the collection tube in step 9.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

9. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane.**

   The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

12. **If the expected RNA yield is >30 µg, repeat step 11 using another 30–50 µl RNase-free water, or using the eluate from step 11 (if high RNA concentration is required). Reuse the collection tube from step 11.**

    If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Addendum B

BIO-RAD

iScript™cDNA Synthesis Kit

25 x 20 µl reactions	170-8890
100 x 20 µl reactions	170-8891

For Research purposes only
Store at -20 °C (not frost-free)

iScript cDNA Synthesis kit provides a sensitive and easy-to-use solution for two-step RT-PCR. This kit includes just three tubes - comprehensive of the reagents required for successful RT-PCR.

The iScript reverse transcriptase is RNAse H+, resulting in greater sensitivity than RNAse H- enzymes. iScript is a modified MMLV-derived reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided pre-blended with RNAse inhibitor. The unique blend of oligo (dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets < 1kb in length.

iScript cDNA Synthesis Kit produces excellent results in both real-time and conventional RT-PCR.

Storage and Stability

Store the iScript™ cDNA Synthesis Kit at -20 °C in a constant temperature freezer. When stored under these conditions the kit components are stable for a minimum of one year after ship date. Nuclease-free water can be stored at room temperature.

Kit Contents

Reagent	Volume
25 reaction kit	
5x iScript Reaction Mix	100µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	25µl
100 reaction kit	
5x iScript Reaction Mix	400µl
Nuclease-free water	1.5ml
iScript Reverse Transcriptase	100µl

Reaction Set Up

Component	Volume per reaction
5x iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	xµl
RNA template (100fg to 1µg Total RNA)*	xµl
Total Volume	20 µl

Reaction Protocol

Incubate complete reaction mix:
- 5 minutes at 25°C
- 30 minutes at 42°C
- 5 minutes at 85°C
- Hold at 4°C (optional)

Reagents and Materials Not Supplied

Reagents for PCR or real-time PCR
- Such as:
- iTaq™ DNA polymerase, 170-8870
- iQ™ Supermix, 170-8860 or
- iQ™ SYBR® Green Supermix, 170-8880

Pipette tips, aerosol barrier tips
- Such as:
- the Xcluda® Style B, 211-2006

Nuclease-free tubes
- Such as:
- 0.2ml Thin-Wall Tubes, 223-9473 or
- plates, 223-9441

RNA purification kit
- Such as the:
- Aurum™ Total RNA Mini Kit, 732-6820 or
- Aurum Total RNA Kit, 2 x 96 well, 732-6800

Recommendations for optimal results using the iScript cDNA Synthesis Kit:

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2µl.

*When using larger amounts of input RNA (>1µg) the reaction should be scaled up e.g. 40µl reaction for 2µg, 100µl reaction for 5µg to ensure optimum synthesis efficiency.

Bio-Rad Laboratories
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4106228 Rev A

Addendum C

BD Pharmingen™

Technical Data Sheet

PE Active Caspase-3 Apoptosis Kit

Product Information

Material Number:	**550914**
Reactivity:	QC Testing: Human Tested in Development: Mouse
Component:	**51-68655X**
Description:	PE Rabbit Anti- Active Caspase-3 (CPP32, Yama, Apopain)
Size:	100 tests (1 ea)
Vol. per Test:	20 µl
Clone Name:	C92-605
Immunogen:	Human Active Caspase-3 Fragment
Isotype:	Rabbit IgG
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.
Component:	**51-6896KC**
Description:	Cytofix/Cytoperm™ Fixation and Permeabilization Solution (1X)
Size:	65 ml (1 ea)
Storage Buffer:	Aqueous buffered solution containing paraformaldehyde and saponin.
Component:	**51-6897KC**
Description:	Perm/Wash™ Buffer (10X Solution)
Size:	65 ml (1 ea)
Storage Buffer:	Aqueous buffered solution containing saponin, fetal bovine serum and ≤ 0.09% sodium azide.

Description

The caspase family of cysteine proteases plays a key role in apoptosis and inflammation. Caspase-3 is a key protease that is activated during the early stages of apoptosis and, like other members of the caspase family, is synthesized as an inactive pro-enzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. The processed forms of caspases consist of large (17-22 kDa) and small (10-12 kDa) subunits which associate to form an active enzyme. Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits which is derived from the 32 kDa pro-enzyme. Active caspase-3 proteolytically cleaves and activates other caspases, as well as relevant targets in the cytoplasm, e.g., D4-GDI and Bcl-2, and in the nucleus (e.g. PARP). This antibody has been reported to specifically recognize the active form of caspase-3 in human and mouse cells. It has not been reported to recognize the pro-enzyme form of caspase-3.

Preparation and Storage

Store undiluted at 4°C and protected from prolonged exposure to light. Do not freeze.

Application Notes

Application

Intracellular staining (flow cytometry)	Routinely Tested

Recommended Assay Procedure:

Induction of Apoptosis by Camptothecin

Materials

1. Prepare a 1.0 mM stock solution of Camptothecin (Sigma-Aldrich Cat. No. C-9911) in DMSO. Camptothecin, an extract of the Chinese tree *Camptotheca acuminata*, is a potent inhibitor of topoisomerase I, a molecule required for DNA synthesis. Camptothecin has been reported to induce apoptosis in a dose dependent manner *in vitro*.
2. Jurkat cells (Human T-cell leukemia; ATCC TIB-152).

Procedure

1. Add camptothecin (4-6 µM final concentration) to 1x10e6 /ml proliferating Jurkat cells.
2. Incubate the cells for 4 hr at 37°C.

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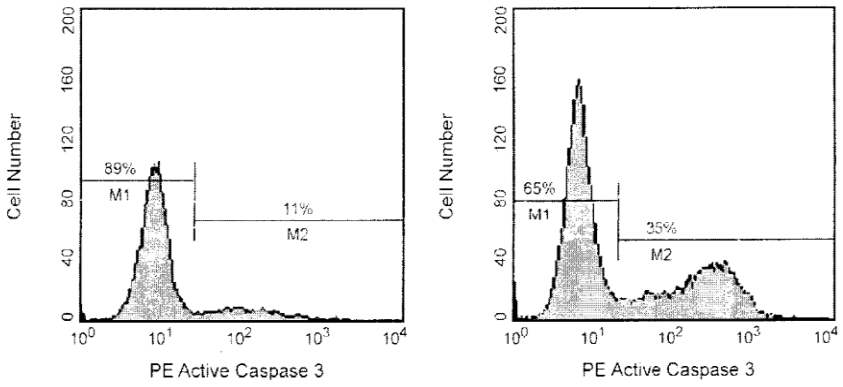
Active Caspase-3 Staining Protocol

Procedure

1. Determine total amount of experimental samples (tests) and calculate the amount of BD Perm/Wash™ buffer (1X) and antibody you will need so that each test will have 100 µl BD Perm/Wash™ buffer (1X) and 20 µl antibody (see chart).
2. Dilute the needed amount of BD Perm/Wash™ buffer (10X) 1:10 in distilled water prior to use. Note: Precipitate may be occassionally observable with the BD Perm/Wash™ buffer (10X) which will not effect performance of the buffer. The precipitate may be removed by filtering the 1X solution through a 0.45 µm filter.
3. Wash cells twice with cold 1X PBS, then resuspend cells in BD Cytofix/Cytoperm™ solution at a concentration of 1x10e6 cells/0.5 ml.
4. Incubate cells for 20 min on ice.
5. Pellet cells, aspirate, and discard BD Cytofix/Cytoperm™ solution; wash twice with BD Perm/Wash™ buffer (1X) at a volume of 0.5 ml buffer/1x10e6 cells at room temperature.
6. Resuspend cells in the above calculated BD Perm/Wash™ buffer (1X) plus antibody and incubate for 30 min at room temperature.
7. Wash each test in 1.0 ml BD Perm/Wash™ buffer (1X), then resuspend the test in 0.5 ml BD Perm/Wash™ buffer (1X) and analyze by flow cytometry.

Number of Tests	Number of cells	Perm/Wash™ Volume (ml)	Antibody Volume (µl)
1	$1x10^6$	0.10	20
5	$5x10^6$	0.50	100
10	$10x10^6$	1.00	200
20	$20x10^6$	2.00	400



Flow cytometric analysis of apoptotic and non-apoptotic populations for active caspase-3. *Jurkat cells (Human T-cell leukemia; ATCC TIB-152) were left untreated (left panel) or treated for 4 hr with camptothecin (right panel) to induce apoptosis. Cells were permeabilized, fixed, and stained for active caspase-3 as described in the accompanying Staining Protocol. Cells were then analyzed by flow cytometry. Untreated cells (left panel, M1) were primarily negative for the presence of active caspase-3, whereas greater than one third of the treated cells were positive for active caspase-3 staining (right panel, M2).*

Product Notices

1. This reagent has been pre-diluted for use at the recommended Volume per Test. We typically use 1 - 10e6 cells in a 100-µl experimental sample (a test).
2. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.
3. For fluorochrome spectra and suitable instrument settings, please refer to our Fluorochrome Web Page at www.bdbiosciences.com/pharmingen/colors.
4. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
5. Source of all serum proteins is from USDA inspected abattoirs located in the United States.

References

Alnemri ES, Livingston DJ, Nicholson DW, et al. Human ICE/CED-3 protease nomenclature. *Cell*. 1996; 87(2):171.(Biology)

Fujita N, Tsuruo T. Involvement of Bcl-2 cleavage in the acceleration of VP-16-induced U937 cell apoptosis. *Biochem Biophys Res Commun*. 1998; 246(2) 484-488.(Biology)

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