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The HIV-1 Tat Protein and Adverse Drug Reactions: A model system utilizing Jurkat T cells and sulphamethoxazole-hydroxylamine

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Graduate Program in Microbiology and Immunology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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THE HIV-1 Tat PROTEIN AND ADVERSE DRUG REACTIONS: A MODEL SYSTEM UTILIZING JURKAT T CELLS AND SULPHAMETHOXAZOLE-HYDROXYLAMINE

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by

Kaothar O. Adeyanju

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

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The HIV-1 Tat Protein and Adverse Drug Reactions:
A model system utilizing Jurkat T cells and sulphamethoxazole hydroxylamine

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of the requirements for the degree
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Chair of the Thesis Examination B

Abstract

In 2009 approximately 2.6 million people became inf

Immunodeficiency Virus (HIV). In addition to the estima

currently living with the virus, this makes HIV/ AIDS an

scale in modern times. Treatment of HIV infection require

as a number of other drugs such as antimicrobials. Hyp

reactions (ADRs) to a variety of drugs are common in HIV-infected individuals, but

antimicrobial Sulphamethoxazole remains a major culprit.

significant morbidity, with the skin and liver most common

top causes of death in the developed world. While the

hypersensitivity in general remains incompletely un

Sulphamethoxazole have been linked to one of its reac -HA.

Previous work from our lab has shown that the HIV-1 Tat protein plays a ro

SMX-induced hypersensitivity ADRs. We sought to determine

Tat would have an effect on cellular toxicity. We also want

toxicity and what region of the protein mediated those effects.

We created fusion proteins of Tat and its deletion mut

protein and placed them in an inducible vector which was

stably transfected Jurkat T cell lines. These were differentially induce

expression and then used in assays for cellular toxicity an

and presence of SMX-HA. We found that cellular toxicity was deper

of Tat used. In the preliminary tests, the first exon of the Tat protein

augment T cell death caused by SMX-HA, and in addition the cell death o

via apoptosis. This cell death took place without alteratio

later experiments using Tat variants differing in length, only the full-length protein affected death after SMX-HA treatment. Also, expression of the full-length protein was able to cause an increase in ROS generated after incubation with SMX-HA. None of the mutants had this effect.

To try to further elucidate the effects of the HIV-1 Tat on the redox set of experiments were carried out to detect the consequences of Tat expression in the presence and absence of SMX-HA. Following incubation of the Jurkat cells with SMX-HA, either cells were disrupted in the presence of iodoacetamide and the lysates analysed by two dimensional gel electrophoresis. In the absence of SMX-HA, the Tat-expressing cell lines were already under a fair amount of oxidative stress compared to the non-infected cell line. Also in untreated cells, a small number oxidized. Exposure of the Tat-expressing cells to 200 µM SMX-HA led to a dramatic increase in thiol protein oxidation.

Keywords: HIV-1 Tat, sulphamethoxazole, sulphamethoxazole hydroxylamine, hypersensitivity adverse drug reactions, Jurkat T-cells, redox, 2D gel electrophoresis.

Co-Authorship

Chapter 2: HIV Tat potentiates cell toxicity in a T ce
Sulphamethoxazole-induced adverse drug reactions

Drs. Dekaban and Rieder supervised the project and aide
manuscript. Adriana Krizova, a co-first author has contributed equally to this
manuscript. (Figures 2.1A, 2.1B, 2.2A, 2.2B, 2.3, 2.4A and

Chapter 3: Cytoplasmic distribution of HIV-1 Tat sensitizes Jurkat T ce
Sulphamethoxazole-hydroxylamine induced toxicity

Drs. Dekaban and Rieder supervised the project and aided in the p
manuscript. Dr. Bend also aided in preparation of the manuscript. I was responsible
for carrying out all the experiments in this Chapter with te
Chan and Lindsey Chow.

Chapter 4: Detection of oxidant sensitive thiol proteins in HIV-1 Tat expressing HI
cells by redox two-dimensional electrophoresis

Drs. Dekaban and Rieder supervised the project and aided in the p
manuscript. Dr. Bend also aided in preparation of the manuscript. I was responsible
for carrying out all the experiments in this Chapter with te
Mao.

All manuscripts in this thesis were written by K. Adeya
comments/contributions from the co-authors.

Dedication

This work is dedicated to my parents who always encourag

of my goals.

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I would like to extend my deepest gratitude to Dr. Greg for giving me the opportunity to pursue my graduate studies in his labor and encouragement have helped improve my aptitude for hypothesis-driven research develop my analytical and critical thinking skills. The training I have received wi serve me well in my future endeavours and the friendship enriching experience.

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LIST OF ABBREVIATIONS, SYMBOLS, NOMENCLATURE

2D	Two second dimension
ADR	Adverse Drug Reaction
ANT	adenine nucleotide translocase
AIDS	Acquired Immunodeficiency Syndrome
APC	antigen presenting cell
ART	antiretroviral therapy
ARV	anti-retrovirals
BSA	Bovine serum albumin
CBP	CREB-binding protein
CDK9	cyclin-dependent kinase 9
C_P	peroxidatic cysteine
C_R	resolving cysteine
CT	computed tomography
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
DTT	Diththiothretol
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
FI	fusion inhibitor
GSH	glutathione
H_2O_2	Hydrogen peroxide
HAD	HIV-associated dementia
HDAC	histone deacetylases
HNE	4-hydroxynonenal
HIV	Human Immunodeficiency Virus
iNOS	inducible nitric oxide synthase

IN	integrase
LC_{50}	lethal concentration, 50%
LTR	Long terminal repeat
MA	Matrix
MMP	matrix metalloproteinases
mtDNA	mitochondrial DNA
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
Ni	Nickel
NF-κB	Nuclear factor kappa B
NLS	nuclear localization sequence
NPC	nuclear pore complexes
NO	nitric oxide
NRTI	nucleoside reverse transcriptase inhibitors
NNRTI	non-nucleoside reverse transcriptase inhibitors
O_2^-	superoxide anion
$O_2^{\bullet}$	superoxide radical
PBMC	peripheral blood mononuclear cells
PCP	Pneumocystis pneumonia
PI	protease inhibitors
p-i	pharmacological interaction
PR	Protease
PSH	protein thiol
PSSP	protein disulphide
PSOH	sulphenic acid
PSO_2H	sulphinic acid
PSO_3H	sulphonic acid

Prx	Peroxiredoxins
PTD	protein transduction domain
P-TEFb	positive transcriptional elongation factor b
PTP	permeability transition pore
RCM	radio contrast media
RGD	arginine, glycine, asparagine
ROS	reactive oxygen species
RNS	reactive nitrogen species
RRE	Rev response element
RT	reverse transcriptase
SH	sulphydryl groups
SMX	Sulphamethoxazole
SMX-HA	Sulphamethoxazole-hydroxylamine
SMX-NO	sulphamethoxazole-nitroso
SOD	superoxide dismutase
SJS	Steven-Johnson Syndrome
SRX	sulphiredoxin
TAR	Tat activation region
TBP	Tat-binding protein
TCC	T cell clones
TCR	T cell receptor
TEN	Toxic epidermal necrolysis
TFIIH	transcription factor IIH
TMP	Trimethoprim
TNF	Tumour Necrosis Factor
Trx	thioredoxin
UNAIDS	United Nations Program on HIV/ AIDS

UTI	urinary tract infections
VDAC	voltage-dependent anion channel

CHAPTER 1: General Introduction

1.1 Introduction

The human immunodeficiency virus (HIV) is a lentivirus and this genus is a member of the family of viruses retroviridae. These are a group of RNA viruses that infect a wide range of animal species and are able to replicate in the host cell using the enzyme reverse transcriptase to co Lentivirus takes its name from the Latin word lenti meaning slow as the viruses are characterized by the ability to cause chronic illness with long incubation peri (Levy, 1998). They are transmitted as enveloped, single-stranded, positive-sense virus to a latent or inactive infection (Levy, 1998). HIV is the etiological agent of immune deficiency syndrome (AIDS), a disease characteriz and depletion of CD4+ T cells from the peripheral organs which leads to an immunocompromised state that in turn gives rise to life-threatening illnesses such as Pneumocystis pneumonia and tuberculosis (Morris et al., 2004; Sette et al., 2005).

There are two strains of HIV, HIV-1 and HIV-2 that share approximately amino acid sequence homology in their gag and pol genes with 30-40% homology in the env gene (Levy, 2009). HIV primarily infects CD4+ T cells, macroph cells. The infection eventually leads to a massive loss of these cells that results in the failure of the immune system and susceptibility of opportunistic illnesses and cancers. Infection with HIV-2 has been associated with transmissibility and pathogenic potential probably due to lower levels of circulat virus at all stages (Berry et al., 1998; Popper et al., 1999). Though HIV-2 is associated with a clinical spectrum similar to that of HIV-1, it is marked

reduced rate in the loss of CD4+ T cells and disease progression. The reasons for

not clear at present and [illegible] will be further defined

1.2 HIV Genome and Replication

The mature HIV-1 virion has a spherical morphology of 100-120 nm in diameter

and consists of a lipid membrane that surrounds a cone-shaped nucleocapsid

core (Sierra et al., 2005). The nucleocapsid contains the genomic RN

proteins protease (PR), reverse transcriptase (RT), Nef, integra

and some cellular factors (Sierra et al., 2005). The HIV-1 genome consists of t

identical single-stranded RNA, 9.2 kb in length with open reading frame

encoding nineteen proteins.

HIV infection begins with the attachment of the virion to the

by an interaction between the extracellular domain of gp120 and cellular

receptors. The major receptor for HIV-1 is the CD4 molecule; co-receptors are the

seven-transmembrane chemokine receptors, CCR5 and CXCR4 (Caplan & Knight,

2002). Binding to the receptors is followed by the fusion of th

membranes which leads to the injection of the viral nucleo

Viral uncoating is carried out by cellular factors as well as t

(MA), Nef and Vif. The viral RNA genome is then reverse tran

double-stranded DNA (dsDNA) by the viral RT (Harrich & Hooker, 2002). The viral

dsDNA associates with cellular and viral proteins to form a preintegration

in the cytosol of the infected cell [illegible]. Amongst the viral proteins in the

proteins Vpr and IN, play a significant role in PIC crossing the nuclear

membrane by taking advantage of the nuclear import machinery of the cell

is required for the integration of the viral DNA into host chromosome (Aono et al., Haffar & Bukrinsky, 2005; Popov et al., 1998). The initial round of proviral tra is carried out by cellular RNA polymerase II which leads t the HIV proteins Tat, Rev and Nef. Once a sufficient amou this trans-activator of transcription controls and enhances further t genes.

The HIV-1 genome can be transcribed to produce mRNA depending on the pattern of splicing (Schwartz, 1990). There is the 9kb transc the partially-spliced 4-5kb transcript and multiply-spliced 2kb transcripts (Watts & Edwards, 1999). Different transcripts predominate at different replication cycle. It has been demonstrated, in the early phase of the rep cycle, the multiply-spliced 2kb transcripts predominate producing high levels of the regulatory proteins Tat, Rev and Nef (Malim et al., 1988).

When the accumulation of Rev is sufficient, Rev binds t the Rev Response Element (RRE) present on unspliced and singly-spliced RNA o transcripts and facilitates their transport through the nuc they are translated (Pollard & Malim, 1998). The full-length transcript of HIV-1 is translated into the Gag-Pol polyprotein. The Env gene is translated from the 4-5kb transcript into the Env protein gp160. A -1 ribosomal frameshift during translatio to the expression of the Gag-Pol polyprotein that gives rise to the viral enzymes PR, RT, and IN. After translation the Env, Gag and Gag-Pol proteins move to the p membrane where the Env proteins insert into the membrane and vira takes place (Derdowski et al., 2004; Sefu et al., 2000). A complex consisting of

length genomic RNA, viral enzymes and cellular compounds
immature core and this complex buds through the plasm
immature virion. Budding triggers activation of PR which autocatalytical
the Gag and Gag-Pol polyproteins, releasing the mature structural proteins and enz
The individual proteins undergo further rearrangement to form mature HIV
virion that is able to infect another cell.

1.3 Transactivator of Transcription (Tat)

Once integrated, the HIV proviral genome is not only
regulation by the host cell, but is also able to control its
(Karn, 1999; Sodroski et al., 1985). This is in contrast to murine and avi
where transcription from the viral long terminal repeat
permissive cells and does not require viral regulators (Karn, 1999; Sodroski
al., 1985). Tat is synthesized at both early and late stages of v
length protein is encoded by two exons. The first exon of Ta
while the second codes for the remainder of the amino aci
protein with a molecular weight of 14-16 kDa that varies in length. HIV-1 Tat
exists predominantly in two different lengths, 86 or 101 residues. The 101
amino acid form of the protein is predominantly in clinical isolates from all HIV-1
subtypes except subtype D, which has a non-synonymous single nucleo
polymorphism creating a stop codon in the second exon and an
(Campbell & Loret, 2009). Several laboratory virus strains including
and NL4.3 contain a truncated variant of Tat that is only 86 amino acids long (Jeang et
al., 1999). It has been suggested that a premature termination

arose artificially during tissue culture passage of these (Nguyen & Jeang, 1996). It is this 86 amino acid form of Tat that has heretofore been most frequently used in research (Campbell & Loret, 2009). Tat is the product of a double sp of the viral transcript. Two forms of Tat are generated in t cycle, depending on the number of times splicing occurs. One fo from doubly spliced viral transcript that produces full-length 101 amino acid protein. The second form is evident after the onset of Rev expression, which transports unspliced or singly spli viral transcripts to the cytoplasm to be translated into st The latter of Rev means the shorter form of Tat encoded by the first exon and only 72 amino acids in length (Jones & Peterlin, 1994; Malim et al., 1989). In vitro, both variants are able to transactivate the LTR efficiently but handle HIV-1 replication differently.

Transcription of the integrated HIV-1 provirus is characterized by an independent and a late, Tat-dependent phase. HIV-specific transcription in the presence of Tat is increased several hundred-fold and essential for viral repl lentiviral LTR is composed of U3, R and U5 regions with divided into the modulatory and promoter region (Figure 1.1; Kilareski et al., 2009). The modulatory region is the binding site for a wide proteins that either enhance or repress transcription (Karn, 1999). The promoter regio has two NF-κB binding sites, while the promoter has three SpI b element that is recognized by TATA-binding protein (TBP). Downstream of th is the R region which contains the Tat activation region (TAR) (Brigati et al., 2003). The LTR contains several additional DNA binding doma

Figure 1-1. The structure of the HIV-1 genome. A detailed structure of th
the numerous regulatory sites that are involved in regulation of the HIV-1 up
transcription. Also in the LTR are two NF-κB binding sites, three
tandem repeats of Sp1-binding sites and a TATA box. The Tat prote
and encoded by two exons. The first exon codes for the f
sufficient for viral transactivation while the second exon give
101. Tat recruits transcription factors to the LTR to upre
HIV-1 genome. Image adapted from Romani et al 2010.

Figure 1

cellular transcription factors, including AP-1, NFAT, Ets (Williams & Greene, 2007). The NF-κB and Sp1 binding sites are required for HIV-1 replication. While other sites enhance transcription, they are nonetheless dispensable (Williams & Greene, 2007). Members of the NF-κB/Rel family can have both positive and on transcription depending on which members of NF-κB bind sites in the LTR (Bright et al., 2003; Williams & Greene, 2007). During latency, NF-κB p50 homodimers bind the LTR and promote recruitment of th histone deacetylase (HDAC) (Monte et al., 1996; Williams & Greene, 20 In response to cellular stimulation, including T-cell receptor ligation or cytokin IL-1β and TNF-α, Sp1 and NF-κB proteins bind the LTR cooperatively transcription synergistically (Perkins et al., 1993; Pereira et al., 1994; Popik & Pitha, 19 Prins et al., 1999; Sune & Garcia-Blanco, 1995). NF-κB heterodimers (RelA/p50) displace the p50 homodimers and also recruit the cellular CRE-binding protein (CBP) complex which includes histone acetylat addition, RelA directs recruitment of cellular RNA polym including P-TEFb (positive transcriptional elongation factor transcriptional elongation (Williams & Greene, 2007).

In the absence of Tat, RNA polymerase II in complex factors such as TFIIH (transcription factor IIH) initiates tr and is able to clear the viral promoter to transcribe TAR which characteristic TAR RNA stem loop structure that binds to RNA polymer RNA stem loop structure is located at the 5′ end of all na Tat, however, this transcription complex is defective for elongatio

production of abortive short transcripts (Karn, 1999; Rice, 2010). In the presence of Tat, the binding of RNA polymerase II to the Tat to the transcription complex where it also binds the TAR R with the P-TEFb (Dingwall et al., 1989). P-TEFb is composed of cyclin dependent kinase 9 (CDK9) which is the catalytic subunit and cyclin T1 the regulatory subunit. The binding of Tat complexed with P-TEFb to TAR RNA is believed to transcriptional elongation through the phosphorylation of CDK9 subunit of the P-TEFb complex (Parada & Roeder, 1996; Romano et al., 1999). This phosphorylation is thought to overcome the action of act to repress transcriptional elongation (Liou et al., 2004). Tat thus acts on the LTR as an anti-terminator of viral transcription.

There are six functional domains in the Tat protein (Figure 1 is the N-terminal or acidic domain (amino acids 1-21) that contains several acid acids and is predicted to form an α-helix. Single residue changes in this d tolerated (Jeang et al., 1999). The second domain (amino acids 22-37) is a cysteine-rich domain consisting of a highly conserved cluster of seve involved in intra-molecular disulphide bonds (Garcia et al., 1988; Ruben et al., 1989). Changes in six of the seven cysteine residues of Tat will a (Jeang et al., 1999). Next is the core domain (amino acids 38-47) with a conserved VC motif involved in tubulin binding and apoptosis and -1, the RK HIV-2 and SIV Tat, though it is still functional (Chen et al., 2002). Perhaps the best studied region of Tat is the fourth basic domain (48-60) which contains a basic RKKRRQRRR motif that is important for nu

Figure 1-2. Functional domains of the HIV-1 Tat protein. Six conserved functiona
domains have been identified in Tat. These are the (1) N-terminal, (2) cyst
rich, (3) core (4) basic-rich (5) glutamine and (6) C-terminal domains.

Figure 1.2

protein is the sequence that allows Tat to be excreted by any infected cell and taken up by other cells (Ensoli et al, 1989; Ensoli et al, 1993; Frankel & Pabo, 1988; Helland et al, 1991). The glutamine-rich domain (amino acids 72,) in combination with the basic domain, confers TAR RN (Dingwall et al, 1989; Roy et al, 1990; Weeks & Crothers, 1991). The second exon of Tat is the C-terminal domain and harbours the motif RGD (arg, gly, asp) se and the highly conserved ESKKKVE motif. The first is a integrins expressed by activated endothelial cells, macrop the significance of the latter has not been determined (Barillari et al, 1993; Brake et al, 1990)

In addition to its regulatory role in HIV-1 transcription, Tat has been i as a transactivator of numerous cellular genes. Tat has expression of cytokines such as IL-2 and IL-6 as well as the expression o adhesion molecules in HIV-infected cells (Ambrosino et al, 1997; Dhawan et al, 1997; Taylor et al, 1992; Vacca et al, 1994; Westendorp et al, 1994). The transactivating effects of Tat are not limited to HIV-infected cells due to a unique prope allows the protein to exit HIV-infected cells and enter other cells in the absenc signal peptide for secretion (Brigati et al, 2003). Tat is able to enter uninfec through interactions of its basic domain with heparan sulphate proteoglycans displayed o the surface of most mammalian cell types and translocate (Frankel & Pabo, 1988; Tyagi et al, 2001). In uninfected cells, Tat can tr cellular genes such as tumour necrosis factor (Sastry et al, 1990) and has also been shown to down regulate several genes involved in HIV path

l thereby providing a mechanism for the virus to invade the ... which in turn ... result in the establishment of persistent infection (Hewton et al, 1993; Verhofstede et al, 1998; Weissman et al, 1998)

1.4 HIV Infection and Pathogenesis

The Joint United Nations Program on HIV/AIDS (UNAIDS) estimates that HIV infection has killed more than 25 million people around the world since it was first described in 1981. There were approximately 33.3 million people living with HIV in 2009 with more than 50, 000 in Canada (UNAIDS, 2010). The course of HIV infection can be divided into three phases. The primary acute ... begins with symptomatic illness with flu-like symptoms within the first four weeks af... majority of individuals and is associated with a high viremia ... sharp drop in peripheral blood CD4+ T cells and the establishment of a res... latently infected CD4+ T cells (Clark et al, 1991; Daar et al, 1991; Henrard et al, 1995). This is followed by the onset of HIV-1 specific cellular and humoral responses that lead to the decline of plasma viral load to a point known as the set point (Borrow et al, 1994; Koup et al, 1994; Rosenberg et al, 1997). The chronic or asymptomatic phase is accompanied by persistent viral ... other viral reservoirs, a rapid turnover of plasma virions a... peripheral blood CD4+ T cell counts. This phase usually lasts several years ... eventually results in the CD4+ T cell count in peripheral blood falling to less than 200 cells/µl. At this stage the total number of CD4+ T cells in th... by at least ... (Douek et al, 2003). As a consequence the patient s imm...

incapable of controlling opportunistic pathogens and cancers that
final symptomatic phase of HIV infection (Douek et al., 2003)

The discovery of persistent viral replication and the correlation between a high viral
with an increase in the rate of disease progression has lead
more complete suppression of viral replication as an effec
infection. This lead to the introduction of antiretroviral th which is
currently conducted using a combination of at least three antiretrovira
at least two different classes. This has dramatically incr
lifespan of HIV patients. There are currently four classes of antiretrovira
drugs; nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse
transcriptase inhibitors (NNRTI), protease inhibitors (PI)
(Sierra et al., 2005). In the majority of HIV patients ART resulted in red
viremia to undetectable levels and increased CD4+ T cell
for years (Sierra et al., 2005). However, long-term success of ART in controlling viral
load is mitigated by resistance development, pharmacokineti
effects of the drugs

1.5 HIV and Adverse Drug Reactions

The use of antiretroviral therapy has had a significa
HIV infection and on the morbidity and mortality of HIV-infected patients. However
advantages of ART come with a marked increase in the num
(ADRs) in this patient population. These include both minor
reactions. In fact, within the first year of treatment ADRs wit

treatment failure that are the most common reasons fo
antiretroviral treatment (Borras-Blasco et al., 2008). Up to 80% of HIV-infected patients
experience an ADR at some point during the course of the
immune disregulation or altered drug metabolism and/or due
polypharmarcy used to treat the virus (Borras-Blasco et al., 2008; Mantz et al., 2007).

Adverse drug reactions are unintended reactions to a
standard doses for diagnoses, prophylaxis or treatment of (Pirmohamed
et al., 1998). They are classified pharmacologically into two maj
Type B (Rawlins & Thompson, 1977). Type A reactions can be predictable due t
known pharmacological or toxicological actions of the c
approximately 80% of ADRs (Rieder, 2009; Sanderson et al., 2005). For instance, the use
of minocycline can cause accumulation of drug-derived compounds in the skin l
hyperpigmentation. Such side effects are sometimes relatively
users of these drugs and are usually delayed by weeks or (Roujeau, 2006). Type
B reactions are unpredictable or idiosyncratic, beginning
introduction of the medication, while recurrence upon re-challenge usually within
two days (Rieder, 2009; Roujeau, 2006). This timing suggests sensitization
immunological memory rather than direct toxicity which sh
specific dose-related threshold. These idiosyncratic reactions also known as
hypersensitivity reactions, usually start with fever and, over the cours
cutaneous eruption develops and is sometimes accompani
and hypotension (Sullivan & Shear, 2001). Mild cutaneous reactions usua
shortly after the start of therapy while the most severe reactions often be

(Roujeau, 2006). Hypersensitivity reactions can be divided into two ma
immediate-type or delayed-type immune-mediated reactions. These hypersens
reactions can be mediated by drug-specific IgE or IgG antibodies (as exemplified by
penicillin) or drug-specific T-cells, respectively (Roychowdhury & Svensson, 200
factors that make an individual susceptible to these idiosyncratic r
speculative and the pathology of these reactions is probably multifactorial.

Though the use of ART often leads to good control of
HIV diagnosis often means that a subgroup of patients start ART with a very low C
count (<200 cells/µl) and a concomitant opportunistic infection. At the beginning of
antiretroviral treatment, these patients frequently show a variety of adve
drug effects such as rashes, hyperpigmentation, hypers
reactions such as erythema multiforme, Stevens-Johnson syndrome (SJS) or
epidermal necrolysis (TEN) (Borras-Blasco et al., 2008). Overall, HIV-infected patients
have a higher risk of developing cutaneous reactions to dr
(Hernandez-Salazar et al., 2006; Lazar et al., 1998). Cutaneous ADRs have been
reported to all antiretroviral medications used in the management of HIV in
well as to anti-convulsant and antimicrobial agents commonly used to treat complications
of infection in HIV positive individuals. The severity of cutaneou varies greatly
and patients with AIDS have up to a 1000-fold higher risk of developing
cutaneous reactions such as SJS and TEN than those in the (Rzany et
al., 1993). Exactly what predisposes a particular individual to be more or l
to developing cutaneous drug reactions is unknown, but is
as genetic background and concomitant medication use (Martin et al., 2006a)

One of the first opportunistic serious illnesses encountered by infected HIV persons is *Pneumocystis* pneumonia (PCP) which is caused by *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) (Morris *et al*., 2004). During the early years of th epidemic, PCP was the AIDS-defining illness for up to 60% of U.S. patient (Morris *et al*., 2004). Though prophylactic and ART therapy have re of *Pneumocystis* in HIV-infected population, trends over the last se that the incidence of *Pneumocystis* has plateaued rather than decreased, and PCP remains the most common serious opportunistic illness. One study in t showed that of those who died from an HIV-associated illness, the mortalit *Pneumocystis* pneumonia was 38% for those not receiving ART, and 3% in those receiving ART (Jain *et al*., 2003; Kaplan *et al*., 2000). The situation in th developing world is even worse with prevalence -infected of up to persons in Thailand and rates up to 51% in various age (Morris *et al*., 2004).

Sulphamethoxazole-Trimethoprim (SMX-TMP) is a combination of two drugs used for the first line treatment and prophylaxis of PCP. advantage of the fact that bacteria have an obligate requirement to synthesize fo while humans obtain folic acid through their diet. Folic acid is a cofactor synthesis of thymidine and other purines and bacterial D trimethoprim inhibit the bacterial synthesis of tetrahydrof active form of folic acid (Masters *et al*., 2003). Sulphamethoxazole is a st analogue of para-aminobenzoic acid and inhibits the synthesis of the int dihydrofolic acid. Trimethoprim is a structural analogue o

dihydrofolic acid and competitively inhibits dihydrofolate r synthesis of tetrahydrofolic acid (Figure 3). The sequential blockade of two a single pathway produces synergistic antibacterial action and helps prevent the development of bacterial resistance to either component al (Masters et al., 2003)

Sulphamethoxazole-Trimethoprim is active against many Enterobacteriaceae ac including *Escherichia coli* and *Klebsiella pneumonia* which is why it is widely us treat urinary tract infections (UTI). The combination dr community-acquired upper and lower respiratory tract infections, including fo prophylaxis and treatment of PCP in HIV-infected population. However, t SMX has been associated with very high incidence of hype infected patients, between 30-80% (Gordin et al., 1984; Hennessy et al., 1995; Jaffe et al., 1983; van der Ven et al., 1994). This is in comparison to 3% negative patients (Bigby et al., 1986; Koch-Weser et al., 1971). Clinical manifestations hypersensitivity to TMP-SMX similar in HIV-infected and uninfected patients the severe reactions such as SJS and TEN are much more common in HIV-infected patients (Vilar et al., 2003)

1.6 Proposed Mechanisms of Adverse Drug Reaction

Mechanism(s) to explain how drugs produce what clin immunologically-mediated reaction remain(s) unclear and to som Numerous lines of evidence suggest the involvement of the immune system in the pathogenesis of cu (Rieder, 2009) There are currently two major hypotheses that attempt to e

Figure 1-3. Folate synthesis and SMX-TMP site of action. Sulphamethoxa
(SMX) is a structural analogue of para-aminobenzoic acid and inhibits the
the intermediary dihydrofolic acid from its precursors. (TMP) is a
structural analogue of the pteridine section of dihydrofolic
dihydrofolate reductase and thus the production of tetrahy
dihydrofolic acid. Image adopted from Masters et al., 2003.

Figure 1.3

first is the Hapten Hypothesis whose premise is that chemically reactive drugs or their drug metabolites act as haptens when they covalently bind to proteins or peptides processed by an antigen presenting cell (APC) and presented in turn as hapten modified peptides on (Figure 4). These small molecular weight co by themselves are believed unable to elicit an immune response. However, v drug compounds are themselves chemically reactive drugs. SMX must be metaboliz a reactive species prior to hapten formation (Roychowdhury & Svensson, 2005). A survey of drugs associated with cutaneous ADRs re either metabolized or degraded to reactive species (Roychowdhury & Svensson, 200 For SMX the major route of metabolism in the liver is N-acetylation (Figure 5), a detoxification. A small fraction of a given SMX dose undergoes cytochrome P450 2C9 enzyme bioactivation to a reactive hydroxylamine (SMX-HA) metabolite sufficiently stable to circulate and be excre (Naisbitt et al., 2001). Further autooxidation gives rise to nitroso sulfamethoxa (SMX-NO), believed by some to be the ultimate (Gerber & Pichler, 2006). While the liver is the primary site of bioactivation, N-acetyltransferase, cytochrome P4 other drug metabolizing enzymes have been found to be other cells and organs (Roychowdhury & Svensson, 2005). The nitroso metabolite c detoxified by the antioxidant glutathione (GSH), converting it to the subsequently, the parent drug SMX (Posadas & Pichler, 2007). This becomes importa in the context of HIV infection as the virus exerts a degree cellular glutathione content, leading to a decrease in the c nitroso metabolite to its parent compound and thus increas

Figure 1.4. Proposed mechanism of the hapten-induced hypothesis in drug reactions. Metabolism of SMX produces chemically reac drug metabolites, SMX-HA and SMX-NO. These haptens then bind cov peptides or proteins thus producing adduct which are su antigen presenting cell and present modified peptides to T cells as ba neoantigens. Image adapted from Sanderson et al. 2006.

Figure 1.4

Figure 1-5. The metabolism of Sulphamethoxazole. The majority acetylated. The rest is bioactivated by cytochrome P450 e sulphamethoxazole-hydroxylamine that is subsequently bio sulphamethoxazole-nitroso. Both metabolites can be reduced back compound by glutathione. Image adapted from Sanderson et al 2006.

Figure 1.5

hypersensitivity to SMX in HIV-infected patients (Posadas & Pichler, 2007). In extra-
hepatic tissues, many of the enzymes responsible for generating reactive dru
metabolites are less active than in the liver.

Another set of factors that may determine individ
hypersensitivity ADRs to SMX are the known polymorphisms i
enzymes. A deficiency of N-acetylation at both the genotypic and phenotyp has
been reported to be a risk factor for ADRs in HIV-negative patients (Pirmohamed &
Park, 2001; Rieder et al., 1991; Zielinska et al., 1998). In contrast, neither the N-acetyl
transferase genotype nor phenotype were shown to be major predispos and do
not predict individual susceptibility in HIV-positive patients (Pirmohamed et al., 2000).
Functionally significant polymorphisms in the other genes
in SMX metabolism were also investigated using polymerase chain reaction (
PCR-Restriction fragment length polymorphism analysis. Polymorphisms in cytochr
P450 2C9 (the CYP2C9*2 and CYP2C9*3 genotypes) were over-represented in the
hypersensitivity group, though not reaching statistical significance. Furthermore,
polymorphisms in the glutathione transferase genes (GSTM
not associated with SMX hypersensitivity (Pirmohamed et al., 2000; Wolkenstein et al.,
2005).

There have been a number of observations that support this hypothesis.
These include the finding that SMX metabolites haptenate
mononuclear cells (PBMC) in vitro (Cribb et al., 1996; Naisbitt et al., 1999).
SMX-haptenated proteins have also been detected in patien anti-SMX
antibodies in patients ([illegible] et al., 2000; Gruchalla et al., 1998; Naisbitt et al.,

2003). Reactive SMX metabolites can stimulate T cell prolife
or cytotoxicity in an MHC-restricted manner (Schnyder et al., 1997a; Schnyder et al.,
1997b). In the last decade the key role of specific T cells in drug hypers
has been supported by the establishment of drug-specific T cell clones (TCC)
from the blood lymphocytes or skin lesions of a variety of patients with
reactions (Mauri-Hellweg et al., 1995; Schnyder et al., 2000; Schnyder et al., 1997a). T
cell clones could be isolated from patients with a drug ingestion h
of cutaneous drug reactions (Roychowdhury & Svensson, 2005). As these TCC were
obtained from already sensitized patients in vitro after stimulation with the
drug, they may not be relevant in reflecting the pathogenesis underlying
sensitization.

Despite this evidence, there are questions about the validity of t
hypothesis. For example, for several drugs, the kinetics of T cell activation
appears to be too fast for any involvement of processing (Schnyder et al., 1997a).
SMX, in the presence of APC, activates T cells almost imm
and sustained intracellular calcium increase (Schnyder et al., 1997a). This timing cannot
be reconciled with an intermediate metabolism and processing that re
at least an hour (Schnyder et al., 1997a). In addition, the bioactivation
a key requirement for the initiation of an immune response in the hapten hypothe
many studies have provided evidence against processing and covalent bi
Many different, chemically inert drugs were found to be able to bind to the T
cell receptor (TCR) in an MHC-dependent manner including SMX, lid
carbamazepine (Schnyder et al., 1997a). Specifically, TCC reacted to the parent d

even when the APC were fixed by glutaraldehyde which excludes metabolism and processing.

In contrast, unlike typical haptens, certain drugs do n After pulsing APC with the drug for an hour then incubating with T cells, there was no stimulation of the T cells by drugs lidocaine, carbamazepine, SMX (Pichler et al., 2006; Zanni et al., 1998). However, the reactive-metabol NO was able to stimulate the T cells as well, capable of covalently modif MHC-peptide complex (Schnyder et al., 1997). Many studies of cutaneous ADRs using immunolabelling of skin biopsies have found infiltration of a predominance of CD4+ T cells in the dermis and CD8+ (Miyauchi et al., 1991; Villada et al., 1992).

In an effort to account for the above observations, P proposed a second model to explain drug-induced cuta, termed the pharmacological interaction with immune receptors (p-i) concept. According to authors, this hypothesis is not intended to replace or cont rather to complement it. The p-i concept states that certain drugs can bi reversibly to the highly-variable, antigen-specific TCR, directly binding the MHC-peptide complex (Figure 6), leading to an immune response dire drug alone (Pichler et al., 2006). Such a TCR-drug interaction would be metabolism- and processing-independent, but the MHC-peptide complex would still be nece cell activation and would in fact mimic drug-immunological interactions w receptors. This model was elucidated for TCRs, but the authors suggest it is possible that B-cell receptors are also activated in a similar manner. This mechanism

situation seen in the case of divalent nickel ions (Ni), w
haptens even though they do not bind covalently to proteins but rather fo
coordination complexes. Thierse et al. (2005) identified and characterized a
promiscuous, Ni-specific TCR where Ni interacts simultaneously with
TCR by making contact with conserved His81 in the HLA-DR as well as
Tyr29 and Tyr94 in CDR1± of the TCR. Thus, much like a
bridge between both receptors though requiring idiotypic re

The p-i hypothesis would explain one of the confounding aspects o
cutaneous drug reactions, namely the timing. T-cell mediated drug
reactions can occur within a few hours after administrat
exposure to the drug. This is the case with radiocontrast media (RCM), a
contrast agent used to improve the visibility of internal b
X-ray based imaging techniques such as CT (computed tomography) (Christiansen, o
2002; Christiansen et al., 2000). The kinetics of the RCM-induced reaction is much
fast to be explained by the induction of a classical, primary, or a
how else could RCM or other inert drugs stimulate the in
usually required to induce an immune response?

To explain these phenomena, the authors proposed t
bypassing the innate immune system as they stimulate specific memory T cell
Memory T cells have a lower threshold of reactivity compa
threshold might be further decreased if a generalized imm
addition, a secondary, memory immune response is generally faster ar
immune reaction within the time frame observed for some A

Figure 1.6. A comparison of the mechanism behind the p-i hapten concept in stimulating T-cells. The solid lines indicate covalent bo antigens and the MHC while the dashed lines depict nonc cases, haptens may also bind covalently to the TCR. Image adapted from Pichler 2006.

Figure 1.6

would explain the higher incidence of ADRs during infections
The p-i concept implies that ADRs are actually due to pre-existing cross-spe
memory T cells to certain drugs. In line with this notion, HIV, i
EBV or CMV infection, where there is a general stimulation of T ce
risk factor for ADRs. The incidence of ADRs to SMX is 2-5% in the general popula
but increases to 50% in the HIV population, while ADRs to amoxi from 4-
5% in the general population to 90% during an active EBV

While some clinical and laboratory evidence exists in
and p-i hypothesis, I believe that the preponderance of evid
the primary pathway for the development of hypersensitiv
associated with ADRs have known reactive metabolites, bu
note that the absence of known reactive metabolites does
drug is not bioactivated, but absence of detection of reactive metabo could be a
function of the limits of currently available analytical met, notably with
our ability to detect very reactive and thus very unstable metabolites (Lavergne et al.
2008). As well, although the quantitative majority of drug metabolism occurs in th
these liver generated metabolites are unlikely to stimulate T cells in oth
organs, unless the metabolite is able to avoid detoxification
stable to circulate (Lavergne et al. 2008). Studies over the past decade hav
presence of drug metabolic CYP enzymes in immune cells as well as s
skin cells including keratinocytes, melanocytes, fibroblasts and Langerhans dendritic
cells (Roychowdhury & Svensson, 2005; Sanderson et al. 2006). These skin cells
express less of drug-metabolizing enzymes than hepatocytes

counterbalanced by the larger weight and surface area of t
explain why the skin is a primary target for ADRs, mainly hypersen
2008). In addition, while most important hepatic CYPs are exp
several CYPs that are much more abundant in the skin and
the liver (Sanderson et al., 2006). As well, as noted above, the liver has high
concentrations of many detoxifying enzymes that have evolved to deal with th
products of drug metabolism, systems which are often muc
Also, the liver is an immunologically privileged organ, such that T cell activati
is more likely to result in tolerance rather than a pathogenic immune response (Sanderson
et al., 2006). Thus, immunologically relevant metabolism may be tis
than hepatic.

Recently, the generation of T cell clones has added to the wealth of knowle
the ongoing attempts to decipher the mechanisms behind
Using this methodology, Castrejon et al. (2010) determined the relative fr
drug- and drug metabolite-specific T cells in the peripheral circulatio
a history of hypersensitivity ADRs. The authors found that
(78%) displayed proliferative responses against SMX metabolites (both
SMX-NO). They also showed that there were 3 specific T cell
stimulation; first, clones that were metabolite specific; second, clones that were
stimulated by the parent drug, and third, clones stimulated by SMX an
metabolites. The authors also showed data that concluded
responsive T cells were stimulated through a hapten mech
APC.

The strongest evidence against the p-i concept is the finding of asso
between certain drug hypersensitivity reactions and HLA alleles. The majo
patients with ADRs to abacavir, approximately 94%, carried the HLA-B*5701 allele, carri
compared to only 1.7% of controls. The association of carbam
treatment with the appearance of Stevens-Johnson syndrome in Han Chinese carrying the
HLA-B*1502 allele (Chung et al., 2004). Such strong associations with HLA
support to the important role for HLA molecules in ADRs,
hapten hypothesis for at least these drugs. Furthermore,
shown evidence that suggest polymorphisms in detoxicatio
play a role in an individual's susceptibility to ADRs. This
al (2007) discovered a polymorphism in cytochrome b5 of an HIV patient wi
of ADRs to SMX, a polymorphism that caused the enzyme to

1.7 Oxidative Stress and Redox Proteomics

It is generally accepted that a central pathologic featu
oxidative stress, leading to apoptosis and depletion of CD
be defined as a situation where cellular homeostasis is altered by production
of reactive oxygen/nitrogen species (ROS/RNS respectiv
cellular anti-oxidant defences, leading to a disruption of redox c
or molecular damage (James, 2006). These reactive species target a la
biological molecules such as carbohydrates, nucleic acid, unsaturated lip
with the latter being the most common target making up
oxidized molecules (Rinalducci et al., 2008). Reactive oxygen/nitrogen s
can cause specific, reversible and/or irreversible oxidati

proteins that can lead to a change in function or activity (Dalle-Donne et al., 2007).

Reactive oxygen/nitrogen species can target lipid me peroxidation and the formation of multiple aldehydes, including hydroxynonenal (HNE) a highly reactive molecule capable of inhibiting DNA (Sacktor et al., 2004). Sphingolipids are a major class of membrane cells and are particularly abundant in the nervous system, most abundant sphingolipid in the brain (Sacktor et al., 2004). Sphingomyelin and HN levels have been used as markers of oxidative stress and i these two markers are significantly increased in the fluid of brai patients with HIV-associated dementia (HAD). It is estimated that one-third of adults infected with HIV develop dementia. HAD is now the leading cause of dementia in adults younger than 60 in the United States (Pocernich et al., 2005).

Antioxidant systems have evolved to counter the potentially detrimental consequences of living in an oxygen-rich atmosphere related to reactive oxygen nitrogen species, including enzymes like superoxide dis reductase, peroxiredoxin and catalase (Hale et al., 2009; Pocernich et al., 2005). Small, non-protein molecules such as GSH, thioredoxin, vitamin C and E flavanoids also act as antioxidants and it is the decrease in the levels of these antioxidants that lead to added oxidative stress in HIV-infected patients. The concentratio decreased in the serum, liver and CNS of HIV-infected patients while adminis the GSH precursor, N-acetyl-cysteine (NAC) to patients in a pre-ART study

shown to decrease mortality (Herzenberg et al. 1997; Pocernich et al. 2005). The micronutrients selenium, vitamin C and vitamin E are also HIV-infected subjects (Olmsted et al. 1989; Pocernich et al. 2005). Selenium supplementation increases glutathione peroxidise activity and HIV replication while supplementation with vitamins C red infection and produced a downward trend in HIV load in a study (Allard et al., 1998; Ho et al. 1997). Supplementation with vitamins A, C and decreased the levels of oxidized DNA bases and lipid pe activity of antioxidant enzymes superoxide dismutase (Jaruga et al. 2002) and c

Oxidative stress induced by Tat through ROS is a major cause of ch neurodegenerative diseases. Specifically, some studies suggest that contributor to HIV-1 dementia. In the brain the most commonly HIV-1 infected cell type are microglia, macrophages and monocytes, though extensive viral replica limited in astrocytes (Kaul & Lipton, 2006; Kure et al. 1990; Saito et al. 1994). Tat is actively released by these infected cells and is taken up b such as neurons (Mattson et al. 2005). In patients infected with HIV-1, significant dysfunction and neuronal loss occur despite the fact tha (Nath, 2002). Tat is thought to be a mediator of neurotoxicity with brain patients with HIV-1 dementia having elevated levels of Tat (Li et al. 2009; Pocernich et al., 2005). The Tat sequences from these brain extracts are substitutions in the cationic region (Bratanich et al. 1998; Li et al. 2009). These mutations may decrease the ability of Tat to be taken up by cells, thus increasing concentrations and possibly producing neurotoxic (Hayman et al. 1993). Tat

induced neurotoxicity is believed to be mediated through two distinct excito
mechanisms involving calcium (Mattson et al., 2005). Tat is capable of depolari
hippocampal neurons and human neurons, increasing intracellular Ca^{2+}
followed by mitochondrial calcium uptake, generation of RO
apoptosis (King et al., 2006). In neurons, Tat also activates kinase, phosphatid
increases levels of IP$_3$, releases calcium from IP$_3$-sensitive endoplasmic reticulu
internal stores, increases the activity of protein kinase C, all precursors o
stress (Haughey et al., 1999; Kruman et al., 1998; Sabatier et al., 1991). Tat activates
astrocytes and induces the expression of inducible nitric o
to the overproduction of nitric oxide (NO) that can react with superoxide (O_2^-) to
form neurotoxic peroxynitrite (ONOO$^-$) (Kruman et al., 1998; Liu et al., 2002). Tat also
induces TNF-α, which in turn induces iNOS, leading to the incr
in HIV-infected macrophages (Bukrinsky et al., 1995; Li et al., 2009).

TNF is known to stimulate HIV replication in infected c
of the transcription factor NF-κB. Based on this knowledge Westendorp et al. (1995)
examined the ability of Tat to modulate TNF-induced activation of NF-κB. They found
Tat expression was able to enhance NF-κB activation in response to TNF in
stably transfected with the Tat gene (Westendorp et al., 1995). Similarly, when synthetic
Tat protein was added to Jurkat T cells or HeLa cells, it p TNF-induced NF-κB
activation and TNF-mediated cell death. As this was inhibited b
suggested to the authors that the activity of Tat was linked to redox state. The
further observed that Tat, either produced intracellularly
significantly reduced RNA and protein levels of manganese-dependent superoxid

dismutase (MnSOD), an enzyme which serves as the primary de
derived free radicals in cells. In another study looking at
redox state of T cells, Gullo et al. (2005) showed that Jurkat T cells in
recombinant Tat revealed an increase in intracellular H_2O_2. To clarify that the incre
H_2O_2 was due to Tat, the authors treated the cells with anti-Tat antibodies neu
that resulted in the inhibition of the production of H_2O_2.

The reaction of oxidants with biomolecules is the molecular
changes in the cellular redox state (Eaton, 2006). Und
nitrosative stress, the sulphur-containing residues, cysteine and methio
susceptible to oxidation and can undergo various reversible and
alterations in response to ROS and/or RNS (Dalle-Donne et al., 2007). Thiols are a class
of organosulphur derivatives characterized by the presence (-SH)
at their active centre (Dalle-Donne et al., 2007; Rinaldi et al., 2008). Biological
thiols can be classified as large molecular mass-proteins and non
proteins, free thiols such as cysteine and GSH. Not all thiols are important
sensors, as most protein thiols do not react with oxidants
found in cells (Eaton, 2006). The majority of cytoplasmic protein cysteine co
sulphydryl with a pKa greater than 8.0 and in the reducing
remain protonated at physiological pH (Dalle-Donne et al., 2007). However, thiol pKa
values can be lowered by their surrounding environment as
with neighbouring, positively charged residues making so
sensitive (Sheehan, 2006).

Cysteinyl thiols can undergo a diverse array of redox reactions whi
dependent on the species and concentration of the oxidant
the presence of increasing ROS concentration and an ox
reactive protein thiols (those ionized at physiological pH) can be oxidized to a disul
(PSSP), sulphenic (PSOH), sulphinic (PSO_2H) or sulphonic (PSO_3H) acids. The first
three of these modifications are reversible (Dalle-Donne et al., 2009). Disulphide
bonds may form within a protein, intramolecularly, or betw
In the presence of oxidants, protein thiols may also form
molecular mass thiols such as GSH (Dalle-Donne et al., 2007). It is widely thought that
the formation of protein disulphides can protect the protei
damaging, oxidation (Rinalducci et al., 2008).

A number of methods have been developed to study protein disulp
including diagonal gel electrophoresis which is a two dimensional form of analysis f
investigating redox-based modifications since disulphide bridged v
highly resolved (Figure 7). Proteins are electrophoresed in the firs
under non-reducing conditions then reduced in the gel and thi
onto a second gel and electrophoresed at 90° to the original direction. In the
proteins migrate at right angles to their original 1D migration. As the majority of cellul
proteins are not disulphide-linked they will fall on the diagonal in th
migrated equidistance in both directions during electropho
the diagonal line connecting opposite corners of the gel.
subunits of proteins connected by intermolecular disulphid
diagonal because the individual units migrate faster than the linked complex than t

Figure 1-7. Redox two-dimensional gel electrophoresis. Electroph
dimension is carried out under non-reducing conditions. The relevant st
placed in a reducing solution and then layered onto the second gel and elect
90° to the original direction. In the second gel, proteins migrate at rig
original migration. The majority of cellular proteins are not linked and will n
fall on the diagonal, having migrated equidistance in
electrophoresis. Upon reduction, component subunits of proteins c
intermolecular disulphide bonds will resolve below the d
intramolecular disulphide bonds will migrating more slowly
diagonal. Image adapted from Leichert et al. 2006.

Figure 1.7

the second dimension (2D). Proteins with intramolecular dis
more compact structure that will be unfolded following re
slowly in 2D and resolving above the diagonal line of redox-regulated protei
(Rinalducci et al., 2008)

1.8 Research Hypothesis and Objectives

HIV currently infects 33 million people around the world, 70 000 people in Canada. Infection with HIV usually leads to
to opportunistic diseases and AIDS-defining diseases such as
Twenty years after the identification of HIV as the etiological
no cure for HIV though there is an effective regimen o
antimicrobials that can sustain an infected individual for 15 years. However,
hypersensitivity adverse drug reactions (ADRs) to many drugs,
used to treat PCP, SMX, TMP, are significantly more common in pati
infection than in uninfected people. Hypersensitivity ADR
antiretroviral drugs. These ADRs can be fatal; ADRs are one o
death in Canada. Despite this, the mechanism(s) leading
understanding of the mechanism(s) of ADRs in the context
us to better predict the population at risk. It will also offer so
design safer and more effective therapeutic agents or tr
clinical complications that would mean a higher quality o
HIV.

<u>Hypothesis</u>: That the HIV-1 Tat protein alters the intracellular envir
cells to increase cellular sensitivity to reactive metabolite

<u>Specific Ob</u>jectives:

1.a) Establish T cell lines in which the HIV-1 Tat protein (Tat101) and
mutants (Tat86, Tat72, Tat(C22G), Tat(45-48)) are differentially induced and b)
characterize the cell lines for the expression of the Tat protein.

2. Determine if the differential expression of Tat101 or th
differential cellular sensitivity in the presence of SMX-HA, a reactive metabolite of SMX.

3. Determine the ability of intracellular Tat to be a pro-oxidant, and to evaluate the effect of Tat and the deletion mutant on the generation of ROS in T cell lines in the presence and absence of the reactive metabolite, SMX-HA.

4. Determine what effect Tat or its deletion mutant have on the disulfide proteome with or without SMX-HA in the Jurkat T cell lines.

1.9 References

Allard, J. P., Aghdassi, E., Chau, J., Tam, C., Kovacs, C. M., Salit, I
Walmsley, S. L. (1998). Effects of vitamin E and C supplementa
oxidative stress and viral load in HIV-infected subjects. AIDS (London,
England) 12, 1653-1659.

Ambrosino, C., Ruocco, M. R., Chen, X., Mallardo, M., Baudi, F., Tremate
S., Quinto, I., Venuta, S. & Scala, G. (1997). HIV-1 Tat induces the
expression of the interleukin-6 (IL6) gene by binding to the IL6 le
and by interacting with CAAT enhancer-binding protein beta (NF-IL6)
transcription factors. The Journal of biological chemistry 272, 14883-
14892.

Ao, Z., Danappa Jayappa, K., Wang, B., Zheng, Y., Kung, S
Depping, R., Kohler, M., Cohen, E. A. & Yao, X. (2010). Importin alpha3
interacts with HIV-1 integrase and contributes to HIV-1 nuclear import and
replication. Journal of virology 84, 8650-8663.

Barillari, G., Gendelman, R., Gallo, R. C. & Ensoli, B. (199
The Tat protein of human immunodeficiency virus type 1, a growth fact
sarcoma and cytokine-activated vascular cells, induces adhesio
same cell types by using integrin receptors recognizin
acid sequence. Proceedings of the National Academy of Sciences
United States of America 90, 7941-7945.

Bedard, K., Smith, S. & Cribb, A. (2000). Sequential assessment of an ant
antibody response in a patient with a systemic delayed
-onset sulphonamide hypersensitivity syndrome reaction. The British journal of
dermatology 142, 253-258.

Berry, N., Ariyoshi, K., Jaffar, S., Sabally, S., Corrah, T.,
Whittle, H. (1998). Low peripheral blood viral HIV-2 RNA in individuals
with high CD4 percentage differentiates HIV-2 from HIV-1 infection.
Journal of human virology 1, 457-468.

Bigby, M., Jick, S., Jick, H. & Arndt, K. (1986). Drug-induced cutaneous
reactions. A report from the Boston Collaborative Drug
Program on 15,438 consecutive inpatients, 1975 to 19 JAMA 256,
3358-3363.

Borras-Blasco, J., Navarro-Ruiz, A., Borras, C. & Casterad, E. (2008). Adverse
cutaneous reactions associated with the newest antiret
patients with human immunodeficiency virus infection. The Journal of
antimicrobial chemotherapy 62, 879-888.

Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M. & Oldsto
Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with
of viremia in primary human immunodeficiency virus ty
Journal of virology 68, 6103-6110.

Brake, D. A., Debouck, C. & Biesecker, G. (1990). Identification of an Arg-Gly-
Asp (RGD) cell adhesion site in human immunodeficien
transactivation protein, tat. The Journal of cell biology 111, 1275-1281.

Bratanich, A. C., Liu, C., McArthur, J. C., Fudyk, T., Glass, J
Klassen, G. A. & Power, C. (1998). Brain-derived HIV-1 tat sequences

from AIDS patients with dementia show increased mole
heterogeneity. Journal of neurovirology 4, 370-383.

Brigati, C., Giacca, M., Noonan, D. M. & Albini, A. (2003). HIV Tat, its TARgets and the control of viral gene expression. FEMS microbiology letters 220, 57-65.

Bukrinsky, M. I., Nottet, H. S., Schmidtmayerova, H., Dubr
Flanagan, C. R., Mullins, M. E., Lipton, S. A. & Gendelman, H.
(1995). Regulation of nitric oxide synthase activity in hum
immunodeficiency virus type 1 (HIV-1)-infected monocytes: implicatio
for HIV-associated neurological disease. The Journal of experimental medicine 181, 735-745.

Burkhart, C., von Greyerz, S., Depta, J. P., Naisbitt, D. J.
K. B. & Pichler, W. J. (2001). Influence of reduced glutathione on
proliferative response of sulfamethoxazole-specific and sulfamethoxazo
metabolite-specific human CD4+ T-cells. British journal of pharmacolo
132, 623-630.

Campbell, G. R. & Loret, E. P. (2009). What does the structure-function relationship of the HIV-1 Tat protein teach us about developing
vaccine? Retrovirology 6, 50.

Castrejon, J. L., Berry, N., El-Ghaiesh, S., Gerber, B., Pichler, W. J.,
K. & Naisbitt, D. J. (2010). Stimulation of human T cells with sulfonamides and sulfonamide metabolites. The Journal of allergy and clinical immunology 125, 411-418 e414.

Chen, D., Wang, M., Zhou, S. & Zhou, Q. (2002). HIV-1 Tat targets microtubules to induce apoptosis, a process promoted
apoptotic Bcl-2 relative Bim. The EMBO journal 21, 6801-6810.

Christiansen, C. (2002). Late-onset allergy-like reactions to X-ray contrast media. Current opinion in allergy and clinical immunology 2, 333-339.

Christiansen, C., Pichler, W. J. & Skotland, T. (2000). Delayed allergy-like reactions to X-ray contrast media: mechanistic consideration
radiology 10, 1965-1975.

Chung, W. H., Hung, S. I., Hong, H. S., Hsih, M. S., Yang, L. C.,
J. Y. & Chen, Y. T. (2004). Medical genetics: a marker for Steven
Johnson syndrome. Nature 428, 486.

Clark, S. J., Saag, M. S., Decker, W. D., Campbell-Hill, S., Roberson, J. L.,
Veldkamp, P. J., Kappes, J. C., Hahn, B. H. & Shaw, G. M. (1991). High
titers of cytopathic virus in plasma of patients with sy
HIV-1 infection. The New England journal of medicine 324, 954-960.

Cribb, A. E., Nuss, C. E., Alberts, D. W., Lamphere, D. B
Grossman, S. J. & Spielberg, S. P. (1996). Covalent binding of
sulfamethoxazole reactive metabolites to human and ra
fractions assessed by immunochemical detection. Chemical research in toxicology 9, 500-507.

Daar, E. S., Moudgil, T., Meyer, R. D. & Ho, D. D. (1991). Transient high levels
of viremia in patients with primary human immunodefic
infection. The New England journal of medicine 324, 961-964.

Dall-Donne, I., Rossi, R., Colombo, G., Giustarini, D. & Milzani, A. (2009). Protein S-glutathionylation: a regulatory device from bacte
Trends in biochemical sciences, 34, 85-96.

Dall-Donne, I., Rossi, R., Giustarini, D., Colombo, R. & Milz S-glutathionylation in protein redox regulation. Free radical biology & medicine, 43, 883-898.

Derdowski, A., Ding, L. & Spearman, P. (2004). A novel fluorescence resonance energy transfer assay demonstrates that the
immunodeficiency virus type 1 Pr55Gag I domain media Gag-Gag interactions. Journal of virology, 78, 1230-1242.

Dhawan, S., Puri, R. K., Kumar, A., Duplan, H., Masson, J.
B. (1997). Human immunodeficiency virus-1-tat protein induces the cel
surface expression of endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in human endothelial cells. Blood, 90, 1535-1544.

Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Heaph
Lowe, A. D., Singh, M., Skinner, M. A. & Valerio, R. (1
immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA in vitro. Proceedings of the National Academy
Sciences of the United States of America, 86, 6925-6929.

Douek, D. C., Picker, L. J. & Koup, R. A. (2003). T cell dynamics in HIV infection. Annual review of immunology, 21, 265-304.

Eaton, P. (2006). Protein thiol oxidation in health and disease: te
measuring disulfides and related modifications in comp
mixtures. Free radical biology & medicine, 40, 1889-1899.

Endo, S., Kubota, S., Siomi, H., Adachi, A., Oroszlan, S.,
Hatanaka, M. (1989). A region of basic amino-acid cluster in HIV-1 Tat protein is essential for trans-acting activity and nucleolar localiza
Virus genes, 3, 99-110.

Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gende
R. A., Wingfield, P. & Gallo, R. C. (1993). Release, uptake, and effect
extracellular human immunodeficiency virus type 1 Tat
growth and viral transactivation. Journal of virology, 67, 277-287.

Frankel, A. D. & Pabo, C. O. (1988). Cellular uptake of the tat protein fr
human immunodeficiency virus. Cell, 55, 1189-1193.

Garcia, J. A., Harrich, D., Pearson, L., Mitsuyasu, R. & Ga
Functional domains required for tat-induced transcriptional activatio
the HIV-1 long terminal repeat. The EMBO journal, 7, 3143-3147.

Gerber, B. O. & Pichler, W. J. (2006). Noncovalent interactions of drugs
immune receptors may mediate drug-induced hypersensitivity reactions
The AAPS journal, 8, E160-165.

Gill, H. J., Tjia, J. F., Kitteringham, N. R., Pirmohamed, M
Park, B. K. (1999). The effect of genetic polymorphisms in CY
sulphamethoxazole N-hydroxylation. Pharmacogenetics, 9, 43-53.

Gordin, F. M., Simon, G. L., Wofsy, C. B. & Mills, J. (1984). Adverse reactions to trimethoprim-sulfamethoxazole in patients with the acquire immunodeficiency syndrome. Annals of internal medicine, 100, 495-499.

Gruchalla, R. S., Pesenko, R. D., Do, T. T. & Skiest, D. J. (1998). Sulfonamide-induced reactions in desensitized patients with AIDS--the role of covalent protein haptenation by sulfamethoxazole. The Journal of allergy and clinical immunology, 101, 371-378.

Gulow, K., Kaminski, M., Darvas, K., Suss, D., Li-Weber, M. & Krammer, P. H. (2005). HIV-1 trans-activator of transcription substitutes for signaling in activation-induced T cell death. J Immunol, 174, 5249-5260.

Haffar, O. & Bukrinsky, M. (2005). Nuclear translocation as a novel target for anti-HIV drugs. Expert review of anti-infective therapy, 3, 41-50.

Harrich, D. & Hooker, B. (2002). Mechanistic aspects of HIV-1 reverse transcription initiation. Reviews in medical virology, 12, 31-45.

Hauber, J., Malim, M. H. & Cullen, B. R. (1989). Mutational analysis of the conserved basic domain of human immunodeficiency vir Journal of virology, 63, 1181-1187.

Haughey, N. J., Holden, C. P., Nath, A. & Geiger, J. D. (19 Involvement of inositol 1,4,5-trisphosphate-regulated stores of intracellular calci calcium dysregulation and neuron cell death caused by HIV-1 protein tat. Journal of neurochemistry, 73, 1363-1374.

Hayman, M., Arbuthnott, G., Harkiss, G., Brace, H., Filippi Thomson, D., Vigne, R. & Wright, A. (1993). Neurotoxicity of peptide analogues of the transactivating protein tat from Maedi-Visna virus and human immunodeficiency virus. Neuroscience, 53, 1-6.

Hennessy, S., Strom, B. L., Berlin, J. A. & Brennan, P. J. (Predicting cutaneous hypersensitivity reactions to cotrimoxazole in HIV-infected individuals receiving primary Pneumocystis carinii pne Journal of general internal medicine, 10, 380-386.

Henrard, D. R., Daar, E., Farzadegan, H., Clark, S. J., Phillips, J., Shaw, G. & Busch, M. P. (1995). Virologic and immunologic characterizat symptomatic and asymptomatic primary HIV-1 infection. J Acquir Immune Defic Syndr Hum Retrovirol, 9, 305-310.

Hernandez-Salazar, A., Rosales, S. P., Rangel-Frausto, S., Criollo, E., Archer-Dubon, C. & Orozco-Topete, R. (2006). Epidemiology of adverse cutaneous drug reactions. A prospective study in hosp Archives of medical research, 37, 899-902.

Herzenberg, L. A., De Rosa, S. C., Dubs, J. G., Roederer, M., Anderson, M. T., Ela, S. W., Deresinski, S. C. & Herzenberg, L. A. (Glutathione deficiency is associated with impaired survival in HIV Proceedings of the National Academy of Sciences of th America, 94, 1967-1972.

Hori, K., Hatfield, D., Maldarelli, F., Lee, B. J. & Clouse, Selenium supplementation suppresses tumor necrosis f induced human immunodeficiency virus type 1 replicati AIDS research and human retroviruses, 13, 1325-1332.

Howcroft, T. K., Strebel, K., Martin, M. A. & Singer, D. S. Repression of MHC class I gene promoter activity by exon Tat of HIV. Science (New York, N.Y 260, 1320-1322.

Jaffe, H. S., Abrams, D. I., Ammann, A. J., Lewis, B. J. & (1983). Complications of co-trimoxazole in treatment of AIDS-associated Pneumocystis carinii pneumonia in homosexual men. Lancet 2, 1109-1111.

Jain, M. K., Skiest, D. J., Cloud, J. W., Jain, C. L., Burns, E. (2003). Changes in mortality related to human immunodeficienc infection: comparative analysis of inpatient-deaths in 2000. Clin Infect Dis 36, 1030-1038.

Jaruga, P., Jaruga, B., Gackowski, D., Olczak, A., Halota, & Olinski, R. (2002). Supplementation with antioxidant vitamin oxidative modification of DNA in lymphocytes of HIV-infected patients. Free radical biology & medicine 32, 414-420.

Jeang, K. T., Xiao, H. & Rich, E. A. (1999). Multifaceted activities of the H transactivator of transcription, Tat. The Journal of biological chemist 274, 28837-28840.

Jones, D. P. (2006). Redefining oxidative stress. Antioxidants & redox signali 8, 1865-1879.

Jones, K. A. & Peterlin, B. M. (1994). Control of RNA initiation and elong the HIV-1 promoter. Annual review of biochemistry 63, 717-743.

Kaplan, J. E., Hanson, D., Dworkin, M. S., Frederick, T., B Lindegren, M. L., Holmberg, S. & Jones, J. L. (2000). Epidemiology of human immunodeficiency virus-associated opportunistic infections in th United States in the era of highly active antiretroviral Clin Infect Dis 30 Suppl 1, S5-14.

Karn, J. (1999). Tackling Tat. Journal of molecular biology 293, 235-254.

Kaul, M. & Lipton, S. A. (2006). Mechanisms of neuronal injury and dea HIV-1 associated dementia. Current HIV research 4, 307-318.

Kilareski, E. M., Shah, S., Nonnemacher, M. R. & Wigdahl, Regulation of HIV-1 transcription in cells of the monocyte-macrophage lineage. Retrovirology 6, 118.

King, J. E., Eugenin, E. A., Buckner, C. M. & Berman, J. W HIV tat and neurotoxicity. Microbes and infection / Institut Pasteur 8, 1347-1357.

Koch-Weser, J., Sidel, V. W., Dexter, M., Parish, C., Finer, P. (1971). Adverse reactions to sulfosoxazole, sulfamethox nitrofurantoin. Manifestations and specific reaction rat courses of therapy. Archives of internal medicine 128, 399-404.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C. & Ho, D. D. (1994). Temporal association of cellula immune responses with the initial control of viremia in immunodeficiency virus type 1 syndrome. Journal of virology 68, 4650-4655.

Kruman, II, Nath, A. & Mattson, M. P. (1998). HIV-1 protein Tat induces apoptosis of hippocampal neurons by a mechanism invo

activation, calcium overload, and oxidative stress. Experimental neurology, 154, 276-288.

Kure, K., Weidenheim, K. M., Lyman, W. D. & Dickson, D. W. (1990). Morphology and distribution of HIV-1 gp41-positive microglia in subacu AIDS encephalitis. Pattern of involvement resembling a degeneration. Acta neuropathol, 80, 393-400.

Kurian, J. R., Longlais, B. J. & Trepanier, L. A. (2007). Discovery and characterization of a cytochrome b5 variant in humans hydroxylamine reduction capacity. Pharmacogenetics and genomics, 17, 597-603.

Lavergne, S. N., Park, B. K. & Naisbitt, D. J. (2008). The role of metabolism in the pathogenesis of T-cell-mediated drug hypersensitivit Current opinion in allergy and clinical immunology, 8, 299-307.

Lazarou, J., Pomeranz, B. H. & Corey, P. N. (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. Jama, 279, 1200-1205.

Levy, J. A. (1998). HIV and the Pathogenesis of AIDS. Washington, DC: ASM (American Society for Microbiology) Press.

Levy, J. A. (2009). HIV pathogenesis: 25 years of progress and pe challenges. AIDS (London, England), 23, 147-160.

Li, W., Li, G., Steiner, J. & Nath, A. (2009). Role of Tat protein in HIV neuropathogenesis. Neurotoxicity research, 16, 205-220.

Liou, L. Y., Herrmann, C. H. & Rice, A. P. (2004). HIV-1 infection and regulatio of Tat function in macrophages. The international journal of bioche cell biology, 36, 1767-1775.

Liu, X., Jana, M., Dasgupta, S., Koka, S., He, J., Wood, C. Human immunodeficiency virus type 1 (HIV-1) tat induces nitric synthase in human astroglia. The Journal of biological chemistry, 277, 39312-39319.

Malim, M. H., Hauber, J., Fenrick, R. & Cullen, B. R. (1988). Immunodeficiency virus rev trans-activator modulates the expression of the vira genes. Nature, 335, 181-183.

Manzardo, C., Zaccarelli, M., Aguero, F., Antinori, A. & Miro, J. Optimal timing and best antiretroviral regimen in treatment-naive HIV infected individuals with advanced disease. Journal of acquired immune deficiency syndromes, 46 Suppl 1, S9-18.

Masters, P. A., O'Bryan, T. A., Zurlo, J., Miller, D. Q. & Jos Trimethoprim-sulfamethoxazole revisited. Archives of internal medicine 163, 402-410.

Mattson, M. P., Haughey, N. J. & Nath, A. (2005). Cell death in HIV dementia. Cell death and differentiation, 12 Suppl 1, 893-904.

Mauri-Hellweg, D., Bettens, F., Mauri, D., Brander, C., Hunzi W. J. (1995). Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and ca J Immunol, 155, 462-472.

Miyauchi, H., Hosokawa, H., Akaeda, T., Iba, H. & Asada, Y T-cell subsets in drug-induced toxic epidermal necrolysis. Possible mechanism induced by CD8-positive T cells. Archives of dermatology 127, 851-855.

Montano, M. A., Kripke, K., Norina, C. D., Achacoso, P., Herzen Roy, A. L. & Nolan, G. P. (1996) NF-kappa B homodimer binding wit the HIV-1 initiator region and interactions with TFII-I. Proceedings of the National Academy of Sciences of the United States 93, 12376-12381.

Morris, A., Lundgren, J. D., Masur, H., Walzer, P. D., Hans Frederick, T., Huang, L., Beard, C. B. & Kaplan, J. E. Current epidemiology of Pneumocystis pneumonia. Emerging infectious disease 10, 1713-1720.

Naisbitt, D. J., Farrell, J., Wong, G., Depta, J. P., Dodd, C Gibney, C. A., Chadwick, D. W., Pichler, W. J., Pirmoh Park, B. K. (2003) Characterization of drug-specific T cells in lamotrig hypersensitivity. The Journal of allergy and clinical immunology 111, 1393-1403.

Naisbitt, D. J., Hough, S. J., Gill, H. J., Pirmohamed, M., & Park, B. K. (1999) Cellular disposition of sulphamethoxazol metabolites: implications for hypersensitivity. British journal of pharmacology 126, 1393-1407.

Naisbitt, D. J., Williams, D. P., Pirmohamed, M., Kitteringh B. K. (2001) Reactive metabolites and their role in drug rea Current opinion in allergy and clinical immunology 1, 317-325.

Nath, A. (2002) Human immunodeficiency virus (HIV) proteins in neuropathogenesis of HIV dementia. The Journal of infectious diseas 186 Suppl 2, S193-198.

Neuveut, C. & Jeang, K. T. (1996) Recombinant human immunodeficienc type 1 genomes with tat unconstrained by overlapping reading fra reveal residues in Tat important for replication in tissu Journal of virology 70, 5572-5581.

Olmsted, L., Schrauzer, G. N., Flores-Arce, M. & Dowd, J. (1989) Selenium supplementation of symptomatic human immunodeficiency virus infect patients. Biological trace element research 20, 59-65.

Parada, C. A. & Roeder, R. G. (1996) Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carb terminal domain. Nature 384, 375-378.

Perkins, N. D., Edwards, N. L., Duckett, C. S., Agranoff, A & Nabel, G. J. (1993) A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. The EMBO journal 12, 3551-3558.

Pichler, W. J., Beeler, A., Keller, M., Lerch, M., Posadas, S., Sch Spanou, Z., Zawodniak, A. & Gerber, B. (2006) Pharmacological interaction of drugs with immune receptors: the p-i concept. Allergology international 55, 17-25.

Pirmohamed, M., Alfirevic, A., Vilar, J., Stalford, A., Wilkins, E. G., Sim, E.
Park, B. K. (2000). Association analysis of drug metabolizing
gene polymorphisms in HIV-positive patients with co-trimoxazole
hypersensitivity. *Pharmacogenetics, 10*, 705-713.

Pirmohamed, M., Breckenridge, A. M., Kitteringham, N. R. & Park, B. K.
(1998). Adverse drug reactions. *BMJ (Clinical research ed), 316*, 1295-
1298.

Pirmohamed, M. & Park, B. K. (2001). HIV and drug allergy. *Current opinion in
allergy and clinical immunology, 1*, 311-316.

Pocernich, C. B., Sultana, R., Mohmmad-Abdul, H., Nath, A. & Butterfiel
A. (2005). HIV-dementia, Tat-induced oxidative stress, and antiox
therapeutic considerations. *Brain research, 50*, 14-26.

Poli, G., Kinter, A. L. & Fauci, A. S. (1994). Interleukin 1 induces expressio
the human immunodeficiency virus alone and in synerg
in chronically infected U1 cells: inhibition of inductive
interleukin 1 receptor antagonist. *Proceedings of the National Acade
Sciences of the United States of America, 91*, 108-112.

Pollard, V. W. & Malim, M. H. (1998). The HIV-1 Rev protein. *Annual review of
microbiology, 52*, 491-532.

Popik, W. & Pitha, P. M. (1993). Role of tumor necrosis factor alpha in
and replication of the tat-defective human immunodeficiency viru
Journal of virology, 67, 1094-1099.

Popov, S., Rexach, M., Zybarth, G., Reiling, N., Lee, M. A.
C. M., Moore, M. S., Blobel, G. & Bukrinsky, M. (1998). Viral protein R
regulates nuclear import of the HIV-1 pre-integration complex. *The EMBO
journal, 17*, 909-917.

Popper, S. J., Sarr, A. D., Travers, K. U., Gueye-Ndiaye, A., Mboup, S.,
Essex, M. E. & Kanki, P. J. (1999). Lower human immunodeficiency
(HIV) type 2 viral load reflects the difference in pathogenicity of
HIV-1 and HIV-2. *The Journal of infectious diseases, 180*, 1116-1121.

Posadas, S. J. & Pichler, W. J. (2007). Delayed drug hypersensitivity rea
new concepts. *Clin Exp Allergy, 37*, 989-999.

Prins, J. M., Jurriaans, S., van Praag, R. M., Blaak, H., van Rij, R
Schellekens, P. T., ten Berge, I. J., Yong, S. L., Fox,
de Wolf, F., Goudsmit, J., Schuitemaker, H. & Lange, J
Immuno-activation with anti-CD3 and recombinant human IL-2 in HIV-1-
infected patients on potent antiretroviral therapy. *AIDS (London, England),
13*, 2405-2410.

Rawlins, M. D. & Thompson, J. W. (1977). *Pathogenesis of adverse drug
reactions*. Oxford: Oxford University Press.

Rieder, M. J. (2009). Immune mediation of hypersensitivity adverse drug
reactions: implications for therapy. *Expert opinion on drug safety, 8*, 331-
343.

Rieder, M. J., Shear, N. H., Kanee, A., Tang, B. K. & Spiel
Prominence of slow acetylator phenotype among patien

sulfonamide hypersensitivity reactions. Clinical pharmacology and therapeutics, 49, 13-17.
Rinaldicci, S., Murgiano, L. & Zolla, L. (2008). Redox proteomics: basic principles and future perspectives for the detection of plant. Journal of experimental botany, 59, 3781-3801.
Romani, B., Engelbrecht, S. & Glashoff, R. H. (2010). Functions of Tat: the versatile protein of human immunodeficiency virus type general virology, 91, 1-12. The Journal of
Romano, G., Kasten, M., De Falco, G., Micheli, P., Khalili, K. & Giordano, (1999). Regulatory functions of Cdk9 and of cyclin T1 in H transactivation pathway gene expression. Journal of cellular biochemis 75, 357-368.
Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Bosw Sax, P. E., Kalams, S. A. & Walker, B. D. (1997). Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. Science (New York, N.Y.), 278, 1447-1450.
Roujeau, J. C. (2006). Immune mechanisms in drug allergy. Allergol Int, 55, 27-33.
Roy, S., Delling, U., Chen, C. H., Rosen, C. A. & Sonenberg, A bulge structure in HIV-1 TAR RNA is required for Tat binding ar mediated trans-activation. Genes & development, 4, 1365-1373.
Roychowdhury, S. & Svensson, C. K. (2005). Mechanisms of drug-induced delayed-type hypersensitivity reactions in the skin. The AAPS journal, 7, E834-846.
Ruben, S., Perkins, A., Purcell, R., Joung, K., Sia, R., Bur Haseltine, W. A. & Rosen, C. A. (1989). Structural and functional characterization of human immunodeficiency virus tat protein. Journal of virology, 63, 1-8.
Rzany, B., Mockenhaupt, M., Stocker, U., Hamouda, O. & S Incidence of Stevens-Johnson syndrome and toxic epidermal ne patients with the acquired immunodeficiency syndrome in Germany. Archives of dermatology, 129, 1059.
Sabatier, J. M., Vives, E., Mabrouk, K., Benjouad, A., Roch Hue, B. & Bahraoui, E. (1991). Evidence for neurotoxic activity of human immunodeficiency virus type 1. Journal of virology, 65, 961-967.
Sacktor, N., Haughey, N., Cutler, R., Tamara, A., Turchan, Vargas, D. & Nath, A. (2004). Markers of oxidative stress in actively progressive HIV dementia. Journal of neuroimmunology, 157, 176-184.
Saito, Y., Sharer, L. R., Epstein, L. G., Michaels, J., Mintz, M. Golding, K., Cvetkovich, T. A. & Blumberg, B. M. (1994) Overexpression of nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous tiss Neurology, 44, 474-481.
Sandefur, S., Smith, R. M., Varthakavi, V. & Spearman, P. Mapping and characterization of the N-terminal I domain of human

immunodeficiency virus type 1 Pr55(Gag). Journal of virology 74, 7238-7249.

Sanderson, J. P., Naisbitt, D. J. & Park, B. K. (2006). Role of bioactivation in drug-induced hypersensitivity reactions. The AAPS journal 8, E55-64.

Sastry, K. J., Reddy, H. R., Pandita, R., Totpal, K. & Agga HIV-1 tat gene induces tumor necrosis factor-beta (lymphotoxin) in a human B-lymphoblastoid T-cell line. The Journal of biological chemist 265, 20091-20093.

Schnyder, B., Burkhart, C., Schnyder-Frutig, K., von Greyerz, S., Naisb J., Pirmohamed, M., Park, B. K. & Pichler, W. J. (2000 Recognition of suflamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. J Immunol 164, 6647-6654.

Schnyder, B., Mauri-Hellweg, D., Zanni, M., Bettens, F. & Pichler (1997). Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. The Journal of clinical investigat 100, 136-141.

Schnyder, B., Zanni, M. P. & Pichler, W. J. (1997). [Drug-induced hypersensitivity syndrome. A review and presentation o cases]. Schweizerische medizinische Wochenschrift 127, 1355-1359.

Schwartz, S., Felber, B. K., Fenyo, E. M. & Pavlakis, G. N. Env and Vpu proteins of human immunodeficiency virus type 1 a multiple bicistronic mRNAs. Journal of virology 64, 5448-5456.

Sheehan, D. (2006). Detection of redox-based modification in two-dimensional electrophoresis proteomic separations. Biochemical and biophysical research communications 349, 455-462.

Sierra, S., Kupfer, B. & Kaiser, R. (2005). Basics of the virology of HIV-1 and its replication. J Clin Virol 34, 233-244.

Sodroski, J., Rosen, C., Wong-Staal, F., Salahuddin, S. Z., Popovic, Arya, S., Gallo, R. C. & Haseltine, W. A. (1985). Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science (New York, N.Y 227, 171-173.

Steele, C., Shellito, J. E. & Kolls, J. K. (2005). Immunity against the opportunistic fungal pathogen Pneumocystis. Med Mycol 43, 1-19.

Sullivan, J. R. & Shear, N. H. (2001). The drug hypersensitivity syndrome: what is the pathogenesis? Archives of dermatology 137, 357-364.

Sune, C. & Garcia-Blanco, M. A. (1995). Sp1 transcription factor is requi in vitro basal and Tat-activated transcription from the human immunodeficiency virus type 1 long terminal repeat. Journal of virology 69, 6572-6576.

Taylor, J. P., Cupp, C., Diaz, A., Chowdhury, M., Khalili, K Amini, S. (1992). Activation of expression of genes coding fo matrix proteins in Tat-producing glioblastoma cells. Proceedings of the National Academy of Sciences of the United States of 89, 9617-9621.

Thierse, H. J., Gamerdinger, K., Junkes, C., Guerreiro, N. ... (2005). T cell receptor (TCR) interaction with haptens: me... classical haptens. *Toxicology*, *209*, 101-107.

Tyagi, M., Rusnati, M., Presta, M. & Giacca, M. (2001). Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *The Journal of biological chemistry*, *276*, 3254-3261.

UNAIDS (2010). UNAIDS Report on the Global AIDS Epidemic 2010.

Vacca, A., Farina, M., Maroder, M., Alesse, E., Screpanti, ... Gulino, A. (1994). Human immunodeficiency virus type-1 tat enhances interleukin-2 promoter activity through synergism with phor... calcium-mediated activation of the NF-AT cis-regulatory motif. *Biochemical and biophysical research communications*, *205*, 467-474.

van der Ven, A. J., Koopmans, P. P., Vree, T. B. & van der ... Drug intolerance in HIV disease. *The Journal of antimicrobial chemotherapy*, *34*, 1-5.

Verhoef, K., Bauer, M., Meyerhans, A. & Berkhout, B. (199... On the role of the second coding exon of the HIV-1 Tat protein in virus replication ... MHC class I downregulation. *AIDS research and human retroviruses*, *14*, 1553-1559.

Vilar, F. J., Naisbitt, D. J., Park, B. K. & Pirmohamed, M. ... Mechanisms of drug hypersensitivity in HIV-infected patients: the role of the im... system. *Journal of HIV therapy*, *8*, 42-47.

Villada, G., Roujeau, J. C., Clerici, T., Bourgault, I. & Rev... (1992). Immunopathology of toxic epidermal necrolysis. Keratin... HLA-DR expression, Langerhans cells, and mononuclear cells: ... immunopathologic study of five cases. *Archives of dermatology*, *128*, 50-53.

Watson, K. & Edwards, R. J. (1999). HIV-1-trans-activating (Tat) protein: bo... target and a tool in therapeutic approaches. *Biochemical pharmacology*, *58*, 1521-1528.

Weeks, K. M. & Crothers, D. M. (1991). RNA recognition by Tat-derived peptides: interaction in the major groove? *Cell*, *66*, 577-588.

Weissman, J. D., Brown, J. A., Howcroft, T. K., Hwang, J., ... Roche, P. A., Schiltz, L., Nakatani, Y. & Singer, D. S. ... HIV-1 tat binds TAFII250 and represses TAFII250-dependent transcription of ma... histocompatibility class I genes. *Proceedings of the National Acade... Sciences of the United States of America*, *95*, 11601-11606.

Westendorp, M. O., Li-Weber, M., Frank, R. W. & Krammer, P. H. (... Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. *Journal of virology*, *68*, 4177-4185.

Westendorp, M. O., Shatrov, V. A., Schulze-Osthoff, K., Frank, R., Kraft, ... Los, M., Krammer, P. H., Droge, W. & Lehmann, V. (19... HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity b... altering the cellular redox state. *The EMBO journal*, *14*, 546-554.

Williams, S. A. & Greene, W. C. (2007). Regulation of HIV-1 latency by T-cell activation. *Cytokine*, *39*, 63-74.

Wolkenstein, P., Loriot, M. A., Flahault, A., Cadilhac, M., Eliaszewicz, M., Beaune, P., Roujeau, J. C. & Chosidow, Association analysis of drug metabolizing enzyme gene AIDS patients with cutaneous reactions to sulfonamide *The journal of investigative dermatology, 125*, 1080-1082.

Zanni, M. P., von Greyerz, S., Schnyder, B., Brander, K. A., Fru Y., Valitutti, S. & Pichler, W. J. (1998). HLA-restricted, processing- and metabolism-independent pathway of drug recognition by hu beta T lymphocytes. *The Journal of clinical investigation, 102*, 1591-1598.

Zielinska, E., Niewiarowski, W., Bodalski, J., Rebowski, G. Mianowska, K. & Sekulska, M. (1998). Genotyping of the arylamine N acetyltransferase polymorphism in the prediction of idi to trimethoprim-sulfamethoxazole in infants. *Pharm World Sci, 20*, 123-130.

CHAPTER 2: HIV Tat potentiates cell toxicity in model for Sulphamethoxazole-induced adverse drug reac

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2.1 Introduction

Human Immunodeficiency Virus (HIV) is the etiologica
acquired immune deficiency syndrome (AIDS), a disease
immune dysfunction resulting in opportunistic infections
(Huigen et al. 2004). Though the mechanism(s) underlying the
pathogenesis of AIDS is still not completely understood, st
between the loss of CD4+ T cells and the appearance of the
manifestations (Douek et al. 2003). The link between the disease an
suggests there is a contribution by HIV-1 regulatory proteins. The
trans-activator (Tat) protein is believed to be one of those pr

Tat is a 14-15 kDa protein essential for efficient HIV transc
(Huigen et al. 2004; Pugliese et al. 2005). The Tat protein is a transcription
factor generated by translation of multiple spliced viral transcript (Bridge et al. 2003;
Huigen et al. 2004). Very early after viral entry into the infected ce
its two-exon form as a 101 amino acid protein. Subsequently,
results in the predominant translation of singly spliced viral transcript, the produci
one-exon form of the 72 amino acid protein (Huigen et al. 2004; Malim et al.
1988).

In addition to its role in the HIV-1 life cycle, Tat has the ability to influen
cell s phenotype by modulating the expression of cellular (de la Fuente et al.
2002a; Weber et al. 2000). The protein has been found to stimulate
immuno-regulatory cytokines, including the production of IL-6, dysregulation
which is implicated in the pathogenesis of several AIDS-associated su

psoriasis, B cell lymphoma and Kaposi's sarcoma (Albini et al., 1997). In another study, Tat was able to increase tumor necrosis factor-beta (TNF-β) expression b activating the TNF promoter (Sastry et al., 1990). Tat also inhibits the transcri tumor suppressor gene, p53, possibly contributing to the HIV-related cancers (Li et al., 1995b). Tat suppresses the expression of mang dismutase (Mn-SOD), contributing to oxidative stress, a condition HIV-1 infection (Choi et al., 2000; Flores et al., 1993). Additionally, this situati exacerbated by a systemic decrease in glutathione content in HIV-infected individuals (Buhl et al., 1989; Staal et al., 1992b; Walmsley et al., 1997). Tat is also proposed to have a major, but indirect, role in apoptosis mechanism thought to contribute to the progressive loss o HIV-1 infection (Huigen et al., 2004). This is thought to occur by mechanisms ability to up-regulate the expression of caspase-8 and CD95L in T cells the increasing their susceptibility to extrinsic apoptotic signals (Bartz & Emerman, 199 Westendorp et al., 1995a).

The steady decline of CD4+ T cells during the course of HIV-1 infection progresses to a stage where the patient develops multiple the most common of these diseases is *Pneumocystis jirovecii*, a fungus responsible for the lung infection known as pneumocystis pneumonia (PCP) (Morris et al., 2004). Without treatment, over 75% of people with AIDS d this infection (Morris et al., 2004). The drug combination trimet- sulphamethaxazole (TMP-SMX) is the treatment of choice for the prophylaxis of PCP (Martin et al., 2003). SMX is a sulphonamide drug c

associated with hypersensitivity adverse drug reactions (ADRs). ADRs (A
in patients with AIDS usually present as a high fever
cutaneous eruption (Roujeau & Stern, 1994). The risk for patients with A
approximately 50%, significantly more prevalent than seronegative
population 2-5% (Carr et al., 1993). There is a marked increase in ri
among people with HIV infection and AIDS compared to bo
people with other causes of immunodeficiency such as chemical or primary
immunodeficiency (Davis & Shearer, 2008). While the reasons for this are
increase in risk for ADRs appears to be very specific for
broad range of drugs (Lin, 2006).

Previous data indicates that hypersensitivity ADRs to SMX are mediated by the
reactive metabolites of SMX, hydroxylamine (SMX-HA) (Rieder et
al., 1988). Given the increased risk of adverse drug events
infection, alterations in the ability of HIV-infected cells to handle react
metabolites may be an important element in the pathophysiology of adverse drug
reactions among people with HIV and AIDS.

In a study by Carr et al. (1993), the degree of in vivo hypersensitivity
to SMX-HA was linked to in vitro toxicity by showing that primary ly
from hypersensitive, HIV-infected patients are significantly more
metabolite than lymphocytes from non-hypersensitive controls. We further
this study by showing that CD4+ HIV-infected cell line is more susce
toxicity by SMX-HA than the uninfected parent cell line (Rieder et al., 1995). The
metabolite produced concentration-dependent toxicity in uninfected and HIV

human T lymphoblasts, a result significantly greater than -infected then
cells. HIV-infected cells also had substantially lower glutathion
infected cells. There was a further concentration-dependent decline in g
content when both cell types were incubated with SMX-HA. Subsequent studies us
lymphocyte cell lines transfected with the HIV regulato
analogous results ([illegible] et al., 2005). These in vitro cell models of SMX-HA ADRs
suggested that there was a relationship between SMX-HA and the cellular s
Tat protein.

The relationship between Tat expression and HIV infection dynamics.
significant increase in the viral load of a patient at the la
indicative of a similarly considerable overall increase in t
extracellularly. This increase in Tat might have a profound effect on the hypersensit
and its metabolites (Calista et al., 2002). In this study we sought to deter
differential expression of Tat protein is associated with differential
SMX-HA. Thus, we conducted this study to investigate ho
expression changes the ability of cells to detoxify reactive

2.2 Materials and Methods

2.2.1 Cell lines

The human T lymphocyte cell line Jurkat E6-1 (ATCC TIB-152), was obtained
from the American Type Culture Collection. The Jurkat and
HIV$_{IIIB}$ obtained though the AIDS Research and Reference Rea
AIDS, National Institute of Allergy and Infectious Diseases
The cell lines were maintained in complete RPMI (Roswell Park Memorial Inst
1640 medium (Invitrogen) supplemented with 20% fetal calf s 1% L-glutamine,
1mM sodium pyruvate and 100units/ml of penicillin and stre

Construction of plasmids and cell lines

The Tat gene was PCR-amplified from the plasmid pSV2tat72.
encodes the first exon (residues 1-72) of the HIV-1 Tat protein and was obtained thr
AIDS Research and Reference Program, Division of AIDS
from Dr. Alan Frankel (**Frankel** & Pabo, 1988). The Tat fragment was cloned
plasmid pEGFP-N1 (Clontech Inc.), resulting in a TatGFP fusion gene.
was excised from pEGFP-N1 as an Nhe I/Not I fragment and inserted i
digested plasmid pBIG2i, an inducible doxycycline-responsive expression ve
(Strathdee et al., 1999). The result was pBIG TatGFP. As a control, GFP from
pEGFP-N1 was also cloned into pBIG2i to create pBIG GFP.

pBIG TatGFP or pBIG GFP were transfected into Jurkat E6-1 T cells by
electroporation (BioRad Gene Pulser II) according to the m
generate stable cell lines of Jurkat pBIG TatGFP and Jurkat pBIG GFP

expression of both TatGFP and GFP was induced by incub transfected
cells with doxycycline (Sigma).

Selective media

Post transfection, positive cells were selected using B (Invitrogen) at
a concentration of 0.8mg/ml. Stable clones were derived
limiting dilution in 96 well plates and the resulting cell lines we
complete RPMI 1640 medium (Invitrogen) supplemented w -glutamine, 10%
fetal calf serum, 100units/ml of penicillin and streptomycin
B.

2.2.2 Confocal microscopy

Confocal microscopy was performed with a BioRad R
scanning microscope. Jurkat pBIG TatGFP and Jurkat pBIG GFP cells were incubated
with 500ng/ml of doxycycline for 72hrs, fixed with 1% par
4°C, then centrifuged onto a microscope slide using a cyto
were cover slipped with Vectashield Mounting Medium containing Dapi
(Vector Laboratories). The images were processed using Image-Pro 5.0 (Media
Cybernetics) and Adobe Photoshop 6.0 (Adobe Systems, In

2.2.3 Real time PCR analyses of Tat/GAPDH mRNA levels

RNA was isolated from HIV-infected Jurkat T cells as well as cell
cell line following doxycycline induction. Total RNA was
Mini Kit (Qiagen, Valencia, CA) by following the manufact
total RNA was reverse transcribed using the High Capacity c

Biosystems, Foster City, CA) according to the manufacture

time-PCR reactions were performed in the absence of cDNA t

used as a housekeeping gene. RT-PCR analysis was performed using

7900HT Sequence Detection System (Applied Biosystems

Relative expression levels were determined by the compar

(Wong & Medrano, 2005).

2.2.14 Immunoblot analysis

To characterize the expression of TatGFP and GFP pro

lines, Jurkat pBIG TatGFP and Jurkat pBIG GFP cells wer

doxycycline for 38 hours, solubilized and the proteins -PAGE gel reso

containing 6M Urea (Arp et al., 2005). After transferring to a PVDF (pol

difluoride) membrane, the proteins were probed with -Tat and mouse anti

monoclonal antibodies (Advanced Biotechnologies Inc. a

Another membrane was probed with GFP rabbit polyclonal anti (Chemicon

Bound antibody was detected using goat anti-mouse or goat anti horseradish

peroxidase-conjugated secondary antibody and enhanced

(Amersham). β-actin was used as a control for protein loading.

Apoptosis and HAS-MX toxicity was assessed by Western b

cytochrome-c and caspase-3 in Jurkat pBIG TatGFP cells cultured with 0, 31 or 500

of doxycycline and then treated with 0, 50 or 200 µM HAS-MX for 2 hours. The dru

were removed and the cells left to recover in an incubator

the cells by centrifugation, the cell pellets were resuspended in lysis buffer [

$NaPO_4$, 100mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1%

EDTA pH 8, with the addition of a protease inhibitor

Diagnostics)]. The cytosolic fractions of the lysates were d on 15% SDS-

PAGE gels. The proteins were transferred to PVDF membra

monoclonal anti-cytochrome c antibody (Santa Cruz Biotechnology) or anti-caspase 3

rabbit monoclonal antibody (Cell Signaling Technology). The anti-caspase 3 antibody

detects endogenous levels of activated/cleaved caspase-3 (17 kDa). These bound

antibodies were subsequently detected using goat anti-mouse horseradish peroxi

(HRP)-conjugated secondary antibody or HRP-conjugated anti-rabbit secondar

antibody respectively (Jackson ImmunoResearch Laborato

chemiluminescence (Amersham). The consistency of protei

β-actin monoclonal antibody.

2.2.5 MTT cell viability assay

Drug toxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide; Sigma-Aldrich) cell proliferation assay. Cel

was quantified by colorimetric conversion of MTT, a meth

(Mosmann, 1983). Briefly, Jurkat E6.1 (control), maximally induc

(control) and differentially induced Jurkat pBIG-wtTLGFP c

plates and incubated with 100 µM SMX (parent drug) (Sigma-Aldrich) or

25-800 µM of SMX-HA (active drug metabolite) for 2 hours. Aft

removed, the cells were resuspended in media and allowed at 37°C for 18 hrs.

Next, 1mg/ml MTT was added to the cells and incubated at 37°C for another 4 hours

The reaction product was solubilized overnight at room te

dimethylformamide/ 20% SDS solution and the percentage o
by an ELISA plate reader at 590nm.

2.26 Flow Cytometry analysis of GFP expression and apoptos

Dose-response experiments were carried out after incuba
and Jurkat pBIG GFP-1 cells with 0-1000ng/ml of doxycycline for 38hrs
analyzing them using a FACS (fluorescence activated cell sorting) calibur
(Becton Dickinson). Time course experiments were conduct
TatGFP cells with 500ng/ml of doxycycline for up to 72hrs, pr
samples through the flow cytometer. At least 10,000 events were measure
each sample. Flow cytometry data was analyzed using Flo
appropriate, camptothecin-induced apoptosis (6µM) was used as a positive con
each sample, events collected were gated for GFP fluorescence

Alternatively, caspase-3 staining and GFP fluorescence was use
HA toxicity and Tat expression. Jurkat pBIG TatGFP cells
concentrations of doxycycline for 24hrs then treated with HA at concentrations of
0, 100 and 200 nM for 8hrs. The cells were then washed in PBS,
formaldehyde, permeabilized with cold 70% ethanol and th
4°, in the dark. After removing the ethanol, the cells were
with phycoerythrin (PE)-conjugated rabbit active caspase-3 monoclonal antibod
(BD Biosciences) for 1hr. GFP and Caspase-3-PE fluorescence was then ana
flow cytometry.

2.2.7 Statistics

MTT cell viability data were analyzed in SPSS Version
deviations (SD) were used to report continuous variables. One-way ANOVA with
Tukey's post hoc procedure was used to compare group means for di
different concentration levels. To account for multiple com
considered statistically significant. GraphPad InStat progr
San Diego, CA) were used to calculate the mean and standard ... real-time PCR
data. The EC_{50} values were calculated using GraphPad Prism 5 where the X values
(concentration of HA-SMX) were log transformed and the Y values (% ce
were normalized to define 0% and 100% as the smallest and ... respectively.
This was followed by sigmoidal dose-response (variable slope) curve fit.

2.3 Results

2.3.1 Characterization of Doxycycline-Inducible cell lines

Our goal was to test whether the HIV Tat protein cont
in a Jurkat T cell model for sulphonamide metabolite (SMX-HA)-induced ADR. To this
end, the first exon of the Tat coding region was amplified by
fragment was inserted into an EGFP plasmid to create the fusi
product was then inserted into the doxycycline-inducible vector pBIG2i (Strathdee et al.
1999). The control vector consisted of the GFP fragment of
into the pBIG2i vector. After transfection, selected resistant Jurkat
E6.1 cells, single cell clones were isolated by limiting dil
confocal microscopy and western blotting were utilized to
fusion protein and GFP in their corresponding cell lines.
pBIG TatGFP transfected Jurkat cells with 500ng/ml of dox 2 hrs
resulted in the respective induction of GFP and TatGFP
microscopy images showed GFP fluorescence throughout
pBIG GFP and pBIG TatGFP (Figure 21A and Figure 21B, respectively).
Immunoblotting after 38 h of induction with 1000ng/ml doxycy anti-GFP
(Figure 21C) and anti-Tat (Figure 21D) antibodies showed that Jurkat pB
cells not induced with doxycycline did not produce the Ta
Figure 21C and 21D).

Next, we used flow cytometry to further assess the n
inducible expression. Analysis of the Jurkat pBIG TatGFP
doses of doxycycline showed differential induction. In order to of GFP

Figure 2-1 Expression of GFP and the TatGFP fusion protein. Confocal images o
Jurkat pBIG GFP (A) and Jurkat pBIG TatGFP (B) cells,
500ng/ml of doxycycline. The images were captured with a Bio-Rad Radiance 2000 Ma
laser scanning confocal microscope with a 40x oil objective. Left panels show
nuclear staining with Dapi, while the right panels show GFP staining.
lysates from Jurkat pBIG GFP cells (lane 1) and Jurkat pB
1000ng/ml doxycycline (lane 2) or uninduced (lane 3) were assessed by SDS-PAGE
and western blot analysis. The membranes were probed with anti-GFP
antibody (C) or a mouse anti-Tat monoclonal antibody (D).

Figure 2.1

determine the time course of induction, Jurkat pBIG TatGFP

varying concentrations of doxycycline for 0-72 hrs and then analyzed. As exp

level of induction is time dependent and the effect plate

(Figure 22A). The viability of the cell population in these time-course experiments

was consistently over 95% throughout the duration of the e

To determine the ability of TatGFP to be differential

TatGFP and Jurkat pBIG GFP cells were treated with increasin

doxycycline (0-1000 ng/ml) and analyzed after 38 hrs. Western b

Tat antibodies showed the gradual increase in the level

concentration of doxycycline increased (Figure 22B). Densitometric examinatio

these blots revealed that TatGFP expression was maximal

of doxycycline (data not shown). These results not only indicate that

expressed in the absence of doxycycline, but also that the level of

directly correlates to the amount of GFP fluorescence whe

population is treated with doxycycline. In parallel, similar

GFP cells (data not shown).

We have previously demonstrated that H9 and Jurkat

show increased cellular sensitivity to SMX-HA compared with the uninfected

cell lines (Arp et al., 2005; Reilly et al., 1995). It is, therefore, important to de

doxycycline concentration that induces physiologically rel

chose to do this by Real Time PCR since it was not possible to quantify the

Tat protein expressed in the HIV-infected H9 cell line. The amount of Tat

concentration of doxycyline was measured by real time PCR as the amount of T

Figure 2-12 Increases in the concentration of doxycycline resu

expression.

(A) Time course of TatGFP induction upon exposure of Ju

doxycycline. Jurkat pBIG TatGFP cells ($5 x 10^5$) were treated with 0, 15, 31, 62

500 or 1000ng/ml of doxycycline for 72hrs. Flow cytometry was used to d

level of induction at 0, 12, 24, 36, 48 and 72hrs. Data

independent experiments carried out in triplicate.

(B) Differential expression of TatGFP protein. Cell lysates obtained fro

TatGFP cells treated with 0-1000ng/ml of doxycycline were probed with anti-Tat

or anti-β-actin antibodies. Data are representative of three ind

(C) Total RNA was isolated and transcribed into cDNA. PCR was performe

using Tat-specific primers and mRNA expression of Tat was a

copies of GAPDH in each sample. The assay was perfor

separate occasions.

Figure 2.2

mRNA in HIV-infected Jurkat and H9 cells. The results showed t

250ng/ml) of doxycycline induction are well within the ran

HIV-infected Jurkat cell line (Figure 22C).

2.3.2 Cell viability is further decreased in the presence of Tat following t with SMX-HA

Earlier studies demonstrated that the toxic effects of SMX-HA were amplified in the presence of Tat (Arp et al, 2005). To determine if there was a relatio increasing levels of Tat protein and toxicity of SMX-HA exposed cell popu Jurkat pBIG TatGFP cells expressing different levels o concentrations of SMX-HA or 100µM of SMX then assessed cell viabili MTT assay. The assay confirmed the lack of toxic effect of the parent drug (SMX, 100µM; data not shown) in all cell lines. There was, with respec induced TatGFP expression, a dose-dependent decrease in cell viability w concentrations of SMX-HA (Figure 23). The assays confirmed that Jurkat cells differentially induced for TatGFP expression were s the effects of SMX-HA than either of the control cell lines, Jurkat GFP cells and also significantly from the uninduced Jurkat pBI (Jurkat pBIG-TatGFP 0ng/ml) (Figure 23). Furthermore, MTT assays were out with Jurkat E6.1 cells pretreated with 500ng/ml of do with various concentrations of SMX-HA. There was no significant differe Jurkat E6.1 cells pretreated with doxycycline and cells tha

Figure 2.3 TatGFP expression decreases cell viability upon ex -HA.
The MTT cell viability assay shows the viability profile of cell population
doxycycline (ng/ml Dox) to express various levels of Tat
exposed to increasing concentrations of SMX-HA ([illegible]00 μM). Data are
representative of three independent experiments with each performed in tr
*P<0.01, Jurkat E6.1 vs. all other groups; &P<0.01, Jurkat E6.1 vs. TatGFP:
and 500ng/ml dox groups; #P<0.01, GFP vs. TatGFP: 31, 62, 125 and
P<0.01, TatGFP: 0ng/ml dox vs. all other groups; P<0.01, TatGFP: 31ng/
dox vs. all other groups; §P<0.01, TatGFP: 31ng/ml dox vs. TatGFP: 1
dox.

Figure 2.3

EC$_{50}$, the median effective concentration, or the SMX-HA concentration at which 50% of the cell population is dead, was determined by curve-fitting program GraphPad Prism, v4.0 (GraphPad Software, Inc., San Dieg EC$_{50}$ values, shown in Table 2.1, confirm our interpretation of the results o Namely, that in our model TatGFP expression and cell viab higher the TatGFP expression, the lower the cellular viabi SMX-HA (p<0.05). The induction of GFP expression with 500ng/ml cell line did not have the same effect (Table 2.1).

2.3.3 Apoptosis is a mechanism of SMX-HA toxicity

We have previously shown, using flow cytometric anal of apoptosis-induced cell death as detected by Annexin V stain treatment of cells with SMX-HA in a dose dependent fashion, followi TatGFP expression (Asrani et al., 2005). Cells not treated with drugs (SMX-HA 0 μM) were highly viable and the initial stages of apoptosis were not SMX 100 μM, again confirming the non-toxic effect of the parent compound (Asrani et al., 2005). These previous experiments demonstrated dose-related SMX-HA toxicity at concentrations of 50 μM and 200 μM in all cell populations that was ma pronounced in cell populations expressing higher levels of

The relationship between apoptosis, SMX-HA treatment, and Tat express further extended here by examining whether key participant pathway are activated as assessed by a cytochrome c release western blot assay. Cytochrome c, is a protein normally confined to the intermem

Table 1-2 Expression of Tat-GFP decreases EC_{50}. EC_{50} values were obtained after a MTT data using the GrapdPad Prism 4 software. Data are mean of three inde experiments. dox: doxycycline. *$P<0.01$ vs. pBIG; #$P<0.01$ vs. Jurkat E6.1; GFP: 500ng/ml dox and $P<0.01$ vs. pBIG TatGFP: 0ng/ml dox; &$P<0.05$ vs. pBIG TatGF 31ng/ml dox.

Table12

Cell Populatio	EC_{50}(uM)	95% Confid Intervals
Jurkat E6.1	175.3	16.00 19.20
pBIG GFP: 500ng/	146.0*	13.62 15.64
pBIG TatGFP: 0n	143.0*	13.5 15.10
pBIG TatGFP: 31	68.02 ð*#	63.20 73.22
pBIG TatGFP: 62	58.02 ð*# &	53.74 62.64
pBIG TatGFP: 12	51.51 ð*# &	47.16 56.27
pBIG TatGFP: 50	52.50 ð*# &	48.46 56.89

mitochondria (Green, 2005). In the event of apoptotic cell death, it i
cytosol where it functions to activate the caspase cascade (Green, 2005). The result
of this cascade is the activation of the executioner caspase-3 and apoptosis
induction of Tat expression and drug treatment, cytosolic
from each of the various cell lines and loaded onto SDS-PAGE gels.

None of the Jurkat pBIG TatGFP cell populations exposed to 0 µM SMX-HA
contained cytosolic cytochrome c (Figure 24A). In non-induced Jurkat pBIG TatG
cells, 200 µM of SMX-HA was required to cause the translocation of cytochrome c to the
cytoplasm (Figure 24A). As higher levels of TatGFP are induced with
presence of increasing SMX-HA concentrations, cytosolic cytochrome c increases
(Figure 24A). The level of cytochrome c expression doubles in the Jurkat p
cells as the concentration of doxycycline increases from 3
exposure to 50 µM of SMX-HA as assessed by densitometry (data not shown). The results
demonstrate that there is a direct connection between the
the amount of cytochrome c released from the mitochondria, with
expression associated with a more pronounced release of cytochrome c at 50 and
200 µM of SMX-HA.

Western blot analysis of active caspase-3 expression also confir
our previous annexin V flow cytometry data and SMX-HA-in TatG
treated cells. Jurkat pBIG TatGFP cells were induced and treated wit
SMX-HA for 2h then left to recover for 18h, after which the
lysates analyzed by SDS-PAGE. Figure 24B shows that caspase-3 is not activated in t
absence of TatGFP expression (0 µg/ml Dox). In the Jurkat pBIG TatGFP population

with low TatGFP expression (Jurkat pBIG TatGFP-1 ng/ml Dox), exposure to 50 µM SMX-HA resulted in the activation of caspase-3 with more pronounced activa 200 µM SMX-HA. An analogous effect was observed in the cell high TatGFP concentration (Jurkat pBIG TatGFP-500 ng/ml Dox). Densitometric analy showed that as doxycycline was increased from 1 ng/ml to increase in caspase-3 activation of cells in the presence of 200 µM SMX-HA. Also, there is no activation of caspase-3 in the absence of SMX-HA (0 µM) despite the expression of high levels of TatGFP (Jurkat pBIG TatGFP-500 ng/ml Dox).

Additional flow cytometry experiments were conducted to dire TatGFP and activated caspase-3 expression so as to further examine th TatGFP expression, SMX-HA and apoptosis. After inducing TatGFP ex the Jurkat pBIG TatGFP cells were treated with SMX-HA for 8h, fixed, permeabi and stained for caspase-3 using an anti-active caspase-3 monoclonal antibody, t analyzed for activated caspase-3 and TatGFP fluorescence. In agreemen annexin V flow cytometry experiments (Asgari et al., 2005) and the western blo experiments described above, the results showed a dose-dependent increase in toxicity in all cell populations. This was further enhanced with inc expression as demonstrated by the increased caspase-3 fluorescence coinciding with higher levels of TatGFP fluorescence (Figure 24C). This was not seen in similar experiments carried out with Jurkat cel pBIG GFP (Figure 24C, inset). Taken together, the data indicates t metabolite, SMX-HA, was able to induce apoptosis and that this e

Figure 2.4 - TatGFP expression in combination with SMX-HA results in the inductio
apoptosis.

(A) TatGFP expression increases cytochrome c translocation to the cytosol i
pBIG TatGFP cells upon treatment with SMX-HA. Uninduced cells (0ng/ml d
cells induced with 100 and 500ng/ml of doxycycline were each exp
200μM of SMX-HA for 2hrs, followed by a recovery period o
populations were harvested and the cytosolic fractions is
blotting. β-actin was analyzed to monitor consistency of protein loading.

(B) Increases in TatGFP expression result in higher caspase-3 activation. Uninduced
cells and cells induced to express two different levels of T
50 or 200μM of SMX-HA for 2hrs, followed by a recovery period of 18hrs.
populations were harvested and total protein was analyzed
blot data are representative of three independent experime

(C) The rise in TatGFP expression is concurrent with caspase-3 activation incr
upon treatment of Jurkat pBIG TatGFP cells with SMX-HA. Jurkat pBIG TatGFP c
were induced with 0, 100 and 500ng/ml of doxycycline th
200μM of SMX-HA for 8hrs. The cells were fixed, stained with active caspase-
3 antibody and then run through the flow cytometer. Data
independent experiments.

Inset is the same experiment carried out with Jurkat pBI
induced with 500ng/ml of doxycycline, treated with 200μM of SMX-HA for 8hrs and
processed as above.

Figure 2.4

cells expressing increasing levels of TatGFP.

2.4 Discussion

The advent of effective treatments for HIV infection
challenges. The treatment regime requires a complex co
combat the primary viremia as well as the many opportunis
the disease. However, HIV infection is associated with an
hypersensitivity ADRs to many medications, with SMX rank
et al., 1992; Davis & Shear, 2008; Lin et al., 2003). There are several factors t
influence the susceptibility to these ADRs. These include HIV-induced
immune hyperactivation, perturbation of drug metabolism a
in cells. These ADRs usually manifest as asymptomatic HIV
AIDS (Rieder & Dekaban, 2000). This suggests that risk for the develop
linked with expression of a factor unique to HIV. We hav
expression of HIV Tat correlates with sensitivity to SMX-HA to the r
(Arp et al., 2005). Though it is unclear if the expression level of
progression from HIV infection to AIDS and its effect on the incid
of ADRs, there is evidence that maintaining control of vir
appears to lower the risk for drug hypersensitivity ADRs
study, we tested whether a differential level of Tat expre
cellular sensitivity to SMX-HA in a dose-dependent manner in a T-cell mode
HA induced ADR. To do this, we used a system to differentially induce t
Tat and then assess cell death upon exposure to SMX-HA.

The inducible pBIG vector developed by Strathdee et al. (1999)
enabled us to differentially express a TatGFP fusion pro

directly by fluorescence-based methods. The TatGFP fusion protein doxycycline induction as can be seen in the confocal ima using flow cytometry and western blots illustrated the indu system.

Our laboratory has previously demonstrated that cons produces a cellular sensitivity to the reactive drug metabolite SMX-HA that is equivalen to that seen among HIV-infected cells (et al, 2005). Cell viability experiments the MTT assay demonstrated no toxicity to the parental d significant decrease in cell viability with increasing concentration the active metabolite, SMX-HA. Concentration-dependent cell death associa incubation with SMX-HA was seen in all cell populations, even th TatGFP. In the case of cells expressing TatGFP, an additional decrea in cell viability was evident as the expression of the physiologically relevant levels of Tat seen in HIV infecte TatGFP had a cooperative effect on SMX-HA toxicity, the effect was dependent the level of Tat expression. Of importance, cell death occ levels of Tat but also in the range of SMX-HA concentrations likely to be s tissues of patients undergoing SMX treatment (10^{-8} M) (Rieder et al, 1995)

There is limited data on the relative contribution of a death in the cells of patients who have sustained hyper Keratinocytes isolated from lesions of patients with sulphonamide-induced toxic epidermal necrolysis (TEN) and Stevens-Johnson Syndrome, two-potentia threatening diseases resulting from ADRs, have been sho

apoptosis (Abe et al., 2003; Paquet & Pierard, 1996). The present study suggested a potential mechanism for the sensitivity of Jur -HA. Though SMX-HA was able to induce apoptosis in cells lacking TatG greatly potentiated as TatGFP expression increased. The role of a data showing the release of cytochrome c and the presence of the activat caspase-3, in a manner dependent on increasing TatGFP e mechanism for this may involve Tat's known ability to supp In models of traumatic brain injury, reductions in MnSOD g of cytochrome c (Lewen et al., 2001; Sullivan et al., 2002). However, further experiments are needed to establish this mechanism. It is that there is a s synergistic effect of SMX-HA and Tat protein that potentiates apoptot cells. The apparent pro-apoptotic effects of Tat, which have been fou such as those by Li (Li et al., 1995a) and Park (Park et al., 2001c) could contribute to the increased incidence of ADRs in HIV in This is potentially an example of apoptosis deregulation, which h other conditions such as neurodegeneration and autoimmunity (Jan et al., 2004; Liu et al., 2004; Rego & Oliveira, 2003).

Tat protein expression has been linked to an increase and mitochondrial damage (Chen et al., 2000; Reid et al., 2002). Specifically, in th association between Tat and SMX-HA-induced toxicity established by Arp (Arp et al al., 2005c), cells stably transfected with full-length Tat were significantly mor to SMX-HA and this sensitivity correlated with decreased total GSH these observations, we hypothesized that Tat-expressing cells increased

to SMX-HA would be related to a perturbation in the GSH red
GSSG/GSH ratio as Tat expression increased. However, the concen
oxidized forms of GSH in the cells not expressing TatGFP
from those expressing increasing levels of TatGFP (data no
to the use of the truncated, 72-amino acid (single-exon) form of Tat in o
model. Westendorp (Westendorp et al., 1995b) showed that total GSH conte
cells exposed to soluble 86-amino acid Tat protein was significantly lo
the control cells. This same study also demonstrated that
exposed to the same cells was unable to change the GSH content of those
et al. (Choi et al., 2000) showed that the expression of full-length
transgenic mice was associated with decreased GSH synt (Choi et al.,
2000). The fact that the GSH content of the cell populations
irrespective of the level of the 72 amino acid form of Tat
are other factors involved in the pathogenesis of hypersen
Tat expression and HIV infection. Additional studies simila
full-length and 86-amino acid forms of Tat will help to clarify this

Furthermore, the role of other HIV proteins such as
mitochondria needs to be addressed (Muthumani et al., 2003; Yedavalli et al., 2005). Our
data suggests that Tat, in addition to conferring increa
mediators of apoptosis (Huigen et al., 2004; Yang et al., 2003), also confers an increa
sensitivity to the intrinsic activation of apoptosis involvin
at least in T cells. The data presented here constitutes an importan
the mechanism of hypersensitivity ADRs during HIV infecti

in Tat expression during the progression of the HIV infection to AIDS
the infected individual to hypersensitivity ADRs related
important in the care of patients living with HIV infection.

2.5 References

Abe, R., Shimizu, T., Shibaki, A., Nakamura, H., Watanabe, H. & Shimizu, H. (2003) Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. Am J Pathol, 162, 1515-1520.

Ambrosino, C., Ruocco, M. R., Chen, X., Mallardo, M., Bau S., Quinto, I., Venuta, S. & Scala, G. (1997) HIV-1 Tat induces the expression of the interleukin-6 (IL6) gene by binding to the IL6 le and by interacting with CAAT enhancer-binding protein beta (NF-IL6) transcription factors. J Biol Chem, 272, 14883-14892.

Arp, J., Rieder, M. J., Urquhart, B., Freeman, D., Tucker, M. J., Kriz Lehmann, D. & Dekaban, G. A. (2005) Hypersensitivity of HIV-1-infected cells to reactive sulfonamide metabolites correlated to HIV-1 viral protein Tat. J Pharmacol Exp Ther, 314, 1218-1225.

Bartz, S. R. & Emerman, M. (1999) Human immunodeficiency virus type induces apoptosis and increases sensitivity to apoptot regulating FLICE-8 caspase. J Virol, 73, 1956-1963.

Blum, R. N., Miller, L. A., Gaggini, L. C. & Cohn, D. L. (1992) Comparative trial of dapsone versus trimethoprim/sulfamethoxazole prophylaxis of Pneumocystis carinii pneumonia. J Acquir Immune Defic Syndr, 5, 341-347.

Brigati, C., Giacca, M., Noonan, D. M. & Albini, A. (2003) HIV Tat, its TAR gets and the control of viral gene expression. FEMS Microbiol Lett, 220, 57-65.

Buhl, R., Jaffe, H. A., Holroyd, K. J., Wells, F. B., Mastran Cantin, A. M. & Crystal, R. G. (1989) Systemic glutathione deficienc symptom-free HIV-seropositive individuals. Lancet, 2, 1294-1298.

Calista, D., Morri, M., Stagno, A. & Boschini, A. (2002) Changing morbidity of cutaneous diseases in patients with HIV after the intro active antiretroviral therapy including a protease inhib Am J Clin Dermatol, 3, 59-62.

Carr, A., Tindall, B., Penny, R. & Cooper, D. A. (1993) In vitro cytotoxicity as marker of hypersensitivity to sulphamethoxazole in pat Clin Exp Immunol, 94, 21-25.

Carr, A., Tindall, B., Penny, R. & Cooper, D. A. (1993) Patterns of multiple drug hypersensitivities in HIV-infected patients. Aids, 7, 1532-1533.

Choi, J., Liu, R. M., Kundu, R. K., Sangiorgi, F., Wu, W., M Forman, H. J. (2000) Molecular mechanism of decreased gluta content in human immunodeficiency virus type 1 Tat-transgenic mice. J Biol Chem, 275, 3693-3698.

Davis, C. M. & Shearer, W. T. (2008) Diagnosis and management of HIV hypersensitivity. The Journal of allergy and clinical immunolo 121, 826-832 e825.

de la Fuente, C., Santiago, F., Deng, L., Eadie, C., Zilberman, I. Maddukuri, A., Baylor, S., Wu, K., Lee, C. G., Pumfery Kashanchi, F. (2002) Gene expression profile of HIV-1 Tat expressing

cells: a close interplay between proliferation and diffe... signals. BMC Biochem 3, 14.
Douek, D. C., Picker, L. J. & Koup, R. A. (2003). T cell dynamics in HIV infection. Annu Rev Immunol 21, 265-304.
Flores, S. C., Marecki, J. C., Harper, K. P., Bose, S. K., N
McCord, J. M. (1993). Tat protein of human immunodeficiency virus
1 represses expression of manganese superoxide dismu
cells. Proc Natl Acad Sci U S A 90, 7632-7636.
Frankel, A. D. & Pabo, C. O. (1988). Cellular uptake of the tat protein fr
human immunodeficiency virus. Cell 55, 1189-1193.
Green, D. R. (2005). Apoptotic pathways: ten minutes to dead. Cell 121, 671-674.
Hess, D. A., Sisson, M. E., Suria, H., Wijsman, J., Puvane
Madrenas, J. & Rieder, M. J. (1999). Cytotoxicity of sulfonamide rea
metabolites: apoptosis and selective toxicity of CD8(+) ce
hydroxylamine of sulfamethoxazole. Faseb J 13, 1688-1698.
Huigen, M. C., Kamp, W. & Nottet, H. S. (2004). Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection. Eur J Clin Invest 34, 57-66.
Jaattela, M. (2004). Multiple cell death pathways as regulators of
initiation and progression. Oncogene 23, 2746-2756.
Lewen, A., Fujimura, M., Sugawara, T., Matz, P., Copin, J.
(2001). Oxidative stress-dependent release of mitochondrial cyto
after traumatic brain injury. J Cereb Blood Flow Metab 21, 914-920.
Li, C. J., Friedman, D. J., Wang, C., Metelev, V. & Pardee,
Induction of apoptosis in uninfected lymphocytes by HI
Science 268, 429-431.
Li, C. J., Wang, C., Friedman, D. J. & Pardee, A. B. (1995). Reciprocal
modulations between p53 and Tat of human immunodefi
1. Proc Natl Acad Sci U S A 92, 5461-5464.
Lin, D., Tucker, M. J. & Rieder, M. J. (2006). Increased adverse drug reacti
to antimicrobials and anticonvulsants in patients with
The Annals of pharmacotherapy 40, 1594-1601.
Liu, C. C., Navratil, J. S., Sabatine, J. M. & Ahearn, J. M. (2004). Apoptosis,
complement and systemic lupus erythematosus: a mechanistic v
Curr Dir Autoimmun 7, 49-86.
Li-Weber, M., Laur, O., Dern, K. & Krammer, P. H. (2000). T cell activation-induced and HIV tat-enhanced CD95(APO-1/Fas) ligand transcription involves NF-kappaB. Eur J Immunol 30, 661-670.
Malim, M. H., Hauber, J., Fenrick, R. & Cullen, B. R. (1988). Immunodeficiency
virus rev trans-activator modulates the expression of the vira
genes. Nature 335, 181-183.
Mansoor, M. A., Svardal, A. M. & Ueland, P. M. (1992). Determination of the in
vivo redox status of cysteine, cysteinylglycine, homocy
glutathione in human plasma. Anal Biochem 200, 218-229.

Masters, P. A., O'Bryan, T. A., Zurlo, J., Miller, D. Q. & Jo
Trimethoprim-sulfamethoxazole revisited. Arch Intern Med, 163, 402-410.

Morris, A., Lundgren, J. D., Masur, H., Walzer, P. D., Hans
Frederick, T., Huang, L., Beard, C. B. & Kaplan, J. E. Current
epidemiology of Pneumocystis pneumonia. Emerg Infect Dis, 10, 1713-1720.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth a
application to proliferation and cytotoxicity assays. J Immunol Methods, 65, 55-63.

Muthumani, K., Choo, A. Y., Hwang, D. S., Chattergoon, M.
Zhang, D., Lee, M. D., Duvvuri, D. & Weiner, D. B. (2003). Mechanism of
HIV-1 viral protein R-induced apoptosis. Biochem Biophys Res Commun, 304, 583-592.

Neuveut, C. & Jeang, K. T. (1996). Recombinant human immunodeficienc
type 1 genomes with tat unconstrained by overlapping
reveal residues in Tat important for replication in tiss
J Virol, 70, 5572-5581.

Paquet, P. & Pierard, G. E. (2002). Keratinocyte injury in drug-induced toxic
epidermal necrolysis: Simultaneous but distinct topogr
CD95R and calprotectin. Int J Mol Med, 10, 541-547.

Park, I. W., Ullrich, C. K., Schoenberger, E., Ganju, R. K.
(2001). HIV-1 Tat induces microvascular endothelial apoptosis thr
caspase activation. J Immunol, 167, 2766-2771.

Paul, C., Wolkenstein, P., Adle, H., Wechsler, J., Garchon
Roujeau, J. C. (1996). Apoptosis as a mechanism of keratinocy
toxic epidermal necrolysis. Br J Dermatol, 134, 710-714.

Pugliese, A., Vidotto, V., Beltramo, T., Petrini, S. & Torre,
of HIV-1 Tat protein biological effects. Cell Biochem Funct, 23, 223-227.

Raidel, S. M., Haase, C., Jansen, N. R., Russ, R. B., Sutli
W., Day, B. J., Hoit, B. D., Samarel, A. M. & Lewis, W. Targeted
myocardial transgenic expression of HIV Tat causes ca
mitochondrial damage. Am J Physiol Heart Circ Physiol, 282, H1673-1678.

Rego, A. C. & Oliveira, C. R. (2003). Mitochondrial dysfunction and react
oxygen species in excitotoxicity and apoptosis: implica
pathogenesis of neurodegenerative diseases. Neurochem Res, 28, 1563-1574.

Rieder, M. J. & Dekaban, G. A. (2000). Adverse reactions
to nonnucleoside reverse transcriptase inhibitors. The Annals of
pharmacotherapy, 34, 940-942.

Rieder, M. J., Krause, R., Bird, I. A. & Dekaban, G. A. (19
Toxicity of sulfonamide-reactive metabolites in HIV-infected, HTLV-infected, and
noninfected cells. J Acquir Immune Defic Syndr Hum Retrovirol, 8, 134-140.

Rieder, M. J., Uetrecht, J., Shear, N. H. & Spielberg, S. P. Synthesis
and in vitro toxicity of hydroxylamine metabolites of su
J Pharmacol Exp Ther, 244, 724-728.

Roujeau, J. C. & Stern, R. S. (1994). Severe adverse cutaneous reaction
drugs. N Engl J Med 331, 1272-1285.

Sastry, K. J., Reddy, H. R., Pandita, R., Totpal, K. & Agga
HIV-1 tat gene induces tumor necrosis factor-beta (lymphotoxin) in a
human B-lymphoblastoid cell line. J Biol Chem 265, 20091-20093.

Staal, F. J., Roederer, M., Israelski, D. M., Bubp, J., Mole
Deresinski, S. C., Ross, W., Sussman, H., Raju, P. A.
Intracellular glutathione levels in T cell subsets decrease in HIV
individuals. AIDS Res Hum Retroviruses 8, 305-311.

Strathdee, C. A., McLeod, M. R. & Hall, J. R. (1999). Efficient control of
tetracycline-responsive gene expression from an autoregula
directional expression vector. Gene 229, 21-29.

Sullivan, P. G., Keller, J. N., Bussen, W. L. & Scheff, S. W
Cytochrome c release and caspase activation after trau
Brain Res 949, 88-96.

Tasciotti, E., Zoppe, M. & Giacca, M. (2003). Transcellular transfer of active
HSV-1 thymidine kinase mediated by an 11-amino-acid peptide from HIV-
1 Tat. Cancer Gene Ther 10, 64-74.

Walmsley, S. L., Winn, L. M., Harrison, M. L., Uetrecht, J.
(1997). Oxidative stress and thiol depletion in plasma and peripheral bloo
lymphocytes from HIV-infected patients: toxicological and path
implications. Aids 11, 1689-1697.

Westendorp, M. O., Frank, R., Ochsenbauer, C., Stricker, K
Walczak, H., Debatin, K. M. & Krammer, P. H. (1995). Sensitization of T
cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature 375,
497-500.

Westendorp, M. O., Shatrov, V. A., Schulze-Osthoff, K., Frank, R., Kraft,
Los, M., Krammer, P. H., Droge, W. & Lehmann, V. (19
potentiates TNF-induced NF-kappa B activation and cytotoxicity b
altering the cellular redox state. Embo J 14, 546-554.

Wong, M. L. & Medrano, J. F. (2005). Real-time PCR for mRNA quantitation
BioTechniques 39, 75-85.

Yang, Y., Tikhonov, I., Ruckwardt, T. J., Djavani, M., Zapata, J. C., Pauz
D. & Salvato, M. S. (2003). Monocytes treated with human
immunodeficiency virus Tat kill uninfected CD4(+) cell
necrosis factor-related apoptosis-inducing ligand-mediated mechanism. J
Virol 77, 6700-6708.

Yedavalli, V. S., Shih, H. M., Chiang, Y. P., Lu, C. Y., Char
Y., Chuang, C. Y., Dayton, A. I., Jeang, K. T. & Huang
Human immunodeficiency virus type 1 Vpr interacts wit
mitochondrial protein HAX-1. J Virol 79, 13735-13746.

CHAPTER 3: Cytoplasmic distribution of HIV-1 Tat sensitizes Jurkat T cells to Sulphamethoxazole hydroxylamine induced toxicity.

3.1 Introduction

One consequence of HIV-infection is the gradual decline of t immune system which allows for vulnerability to opportun Pneumocystis jirovecii takes advantage of this immunodeficiency to pneumonia, a lung infection that is commonly treated sulphamethoxazole-trimethoprim (SMX-TMP). This combination antimicrobial used to treat urinary tract infections in the HIV-negative population. The SMX moiety of the drug is associated with an incidence of 50% or more of adverse drug reactio (ADRs) in HIV-1 infected patients, but only 2-5% in patients not infected with Adverse drug reactions can be defined as a response to unintended, occurring at doses normally used for (Rieder, 2009). The most serious of these is the hypersensitivity ADR which usually presents as delayed reaction characterized by the onset of fever followed by the development of a rash such as Stevens-Johnson syndrome or toxic ep necrolysis. The liver and kidney can also become involved, presenting cli hepatitis or interstitial nephritis (Rieder, 2009).

The mechanism behind hypersensitivity ADRs in general and those stimulated by SMX in particular remains unclear, however the hapten hypothesis bioactivation and haptenation of the drug to cellular proteins is critical in initiating an immune response. Sulphamethoxazole undergoes bioactivation by cytochrome P450 enzyme CYP2C9 to a hydroxylamine metabolite, which is oxidized to SMX-NO. Both metabolites are known to be toxic to cells in vitro, however this cytotoxicity is mitigated by co-incubation with glutathione

(GSH), an antioxidant, its precursor N-acetylcysteine (Rieder et al., 1989). Indeed, the in vitro toxicity was the basis of the diagnosis of hypersensitivity A
peripheral blood lymphocytes from patients with a histor
showed significantly increased toxicity across a range of concentrations of SMX-HA compared to those from controls and patients without a history of this reaction (Rieder et al., 1989). A similar finding was reported in HIV-positive patients where SMX-HA cytotoxicity was greater in patients with hypersensitivity ADRs (Carr et al., 1993). Enhanced lymphocyte sensitivity to SMX-HA was also correlated with expression of the HIV-1 Trans-Activator of Transcription (Tat) protein, which was associated with reduced intracellular GSH concentrations (Asher et al., 2005).

Tat is a small, multifunctional transcriptional regulator essent
replication of human immunodeficiency virus type 1 (HIV-1). Tat binds to the spe
sequences of TAR (Tat activation region), a stable RNA ha
all nascent viral transcripts. This binding recruits and a
potentiate RNA polymerase II to synthesize full-length HIV transcripts (Romani et al., 2010).

The coding sequence of the Tat gene is located on two exons and
protein has a molecular weight of 14-16 kDa. Tat is made up of 101 amino
residues; 1-72 encoded by the first exon and residues 73-101 by the second exon. The 101 amino acid form of Tat is found predominantly in clinical is
all HIV-1 subtypes except subtype D, which has a non-synonymous single nucle
polymorphism creating a stop codon in the second exon a
(Campbell & Loret, 2009). Though there exist a few laboratory strains that exp

amino acid form of Tat, this version is thought to represent an artificially tru
protein. However, this 86 amino acid Tat has frequently b
properties of the protein. The full length, 101 amino acid Tat protein, is divided into six
domains. They are the N-terminal domain (1-21 aa), cysteine rich domain (22-37 aa),
core domain (38-47 aa), basic domain (48-60), the glutamine rich domain (61-72)
and the C-terminal domain (73-101) (Campbell & Loret, 2009; Jones et al. 2009). The first
three domains (1-48) together circumscribe the minimum domain of the activa
protein which mediates its interactions with cellular machine (Carroll et al., 1991;
Derse et al., 1991; Romani et al., 2010). The basic domain, also known as
transduction domain, contains the nuclear localization s
confers on the protein the ability to exit HIV-1 infected cells and enter uninfected tar
cells. Ultimately, extracellular Tat facilitates the spread of HIV-1
infection. The basic domain in conjunction with the glutamine rich domain confers TAR
RNA binding properties to Tat. The product of exon 1 (1-72) which contains the first five domains
represents the combination of the activation and binding domains that i
required for functional HIV-1 transactivating activity.

In this study we sought to determine which region of th
cellular toxicity and SMA. Furthermore to determine if the same region of the
protein was responsible for modulating the cellular

3.2 Materials and Methods

3.2.1 Cell line

The human T lymphocyte cell line Jurkat E6.1 (ATCC TIB-152) was obtained from the American Type Culture Collection (ATCC). The cell line was maintai
Complete RPMI 1640 medium (Invitrogen) supplemented w L-glutamine, 10%
fetal calf serum and 100 units/ml of penicillin and streptomycin. The Cos 7 cell line
(ATCC CRL 1651), a monkey kidney fibroblast-like cell line maintained in Dulbecco's
Modified Eagle's Medium (DMEM) with 10% fetal calf serum and 1
penicillin and streptomycin, was obtained from ATCC.

Construction of plasmids and cell lines

The Tat gene was PCR-amplified from the plasmid pSVTat. T
encodes the full-length Tat gene and was a kind gift from Dr. KT J
of Molecular Microbiology, the Molecular Virology Section
length Tat gene (Tat101), with or without stop codons, and
Tat72, Tat48 and Tat(D45-57) were cloned into the plasmid pEGFP-N1 (Clontech Inc.),
resulting in Tat101, Tat101GFP, Tat86GFP, Tat72GFP, Tat
genes, respectively. The fusion genes were excised from pE as Nhe I/Not I
fragments and inserted into the Nhe I/Not I digested pla
doxycycline-responsive expression vector (Strathdee et al. 1999). The results were th
following plasmid constructs; pBIG Tat101, pBIG Tat101GFP, pBIG Tat86GFP,
Tat72GFP, pBIG Tat48GFP and pBIG Tat(D45-57)GFP. As a control, the GFP ge
pEGFP-N1 was also cloned into pBIG2i to create pBIG GF

constructs or pBIG GFP were transfected into Jurkat E6.1 T cells by nucleofec
(Lonza, Amaxa Nucleofector) according to the manufacture
stable cell lines of Jurkat pBIG TatGFP and Jurkat pBIG G, respectively. Cos 7 cells
were also transfected with the pBIG TatGFP constructs for studies.
The expression of TatGFP fusion protein or GFP in either cell type induced
by incubating the pBIG2i-transfected cells with doxycycline (Sigma).

Selective media

Post nucleofection, constructs positive Cos 7 and Jurkat cells were selected using
hygromycin B (Invitrogen) at a concentration of 0.8mg/ml. Jurkat clones were
derived from single cells following limiting dilution cultures in 96 well plates. Bulk cultures of
stably selected transfected cells were also maintained and us. The
resulting cell lines were maintained in complete RPMI 1
supplemented with 1% L-glutamine, 10% fetal calf serum, 100units/m
streptomycin and 0.5mg/ml of hygromycin B.

3.2.2 Confocal microscopy

Cos 7 pBIG TatGFP cells were incubated with 500ng/ml o, h
incubated with 6µM MitoTracker Far Red (Invitrogen) for °C and 30 M
Hoechst 33258 stain. The images were taken with Zeiss LSM 510 META confo
microscope. The images were processed using Image Pro 5.0 (Media Cybernetics
Adobe Photoshop CS2 (Adobe Systems, Inc.).

3.2.3 Dose response and time course of doxycycline induction

The concentration of doxycycline required to induce e

fusion proteins was determined in dose-response experiments where cells

different cell lines were incubated with 0-1000ng/ml of doxycycline for 40hr

the cells were analyzed on a FACS-Calibur (Fluorescence Activated Cell Sorting)

instrument (Becton Dickinson). Time course experiments were c

cells with 1000ng/ml of doxycycline for 72hrs, with aliquots ta

analyzed by flow cytometry on a FACS Calibur. In both the dose respons

course experiments at least 10,000 gated events were measured for each sample. Flow

cytometry data were analyzed and the percentage of GFP+ cells d

FlowJo program (Tree Star, Inc.).

3.2.4 Real time PCR analyses of Tat/GAPDH mRNA levels

To compare the level of Tat mRNA present in the sta

HIV-infected cells, cells from each of the stable pBIG TatGF

treatment with 0, 200, 400, 600, 800 and 1000ng/ml doxycycline for

was extracted using the RNeasy Mini Kit (Qiagen) by fol

instructions. RNA was also isolated from HIV_{IIIB} infected Jurkat T cells. Tota

(10µg) was reverse transcribed using the High Capacity cDNA Arch

Biosystems) according to the manufacturer's instructions. RT-PCR

reactions were performed in the absence of cDNA template

control housekeeping gene. PCR analysis was performed using the ABI

PRISM 7900HT Sequence Detection System (Applied Biosys

Relative expression levels were determined by the compar

(Wong & Medrano, 2005)

3.2.15 Immunoblot analysis

To characterize the expression of the TatGFP fusion lines, cells from each cell line were differentially induced which the cells were washed twice in PBS then solubilized with RIPA buffer 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycho 0.1% SDS, 1 mM EDTA and complete mini protease inhibito lysates were sonicated for 2 x 20 sec then centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were collected and the protein concentratio BioRad DC Protein Assay that is a modification of the Lo albumin (BSA) was used as the standard in the protein assay.

The proteins were resolved on 15% SDS-PAGE gel then transferred to (polyvinylidene difluoride) membrane. The membranes were anti-Tat antibody (Advanced Biotechnologies Inc.). Bound antibody mouse horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Amersham). GAPDH antibody (Sigma) was used as a co for protein loading.

3.2.16 MTT cell viability assay

Drug toxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) cell proliferation assay. Cel was quantified by colorimetric conversion of MTT, a meth (1983). Briefly, JurkatTatGFP cells were differentially induced with doxycycl

for 40h, washed twice with PBS then $7.5x10^4$ cells/well were seeded in 96 well plates and incubated with 200µM SMX (Sigma-Aldrich) or 25µM of SMX-HA for 2 h. After the drug was removed, the medium was added and incubated at 37°C for 18hrs. Next, 1mg/ml MTT was added to the 37°C for another 4 h. The reaction product that precipitated was so room temperature by the addition of N-N-dimethylformamide (50/ 20% SDS) to the wells. The following morning the abso determined by an ELISA plate reader at 590nm and equate cells with the help of a standard curve. This assay was performed on thr occasions for each cell line.

3.2.7 Detection of reactive oxygen species (ROS)

Following the 40h doxycycline induction, the cells were washed twice in PB and resuspended in HBSS (Hank s buffered saline solut $1x10^6$ cells/ml. Cell suspension (100µl) was placed in each well of a 96 well plate, the centrifuged at 1500rpm for 5min and the supernatant deca 20µM DCFH-DA (2',7'-dichlorfluorescein-diacetate, Sigma) in HBSS was added an the plate was incubated at 37°C for 1h after which the plate was centrifuged at 1500r for 5min and the supernatant decanted. The wells were filled with 100µl HBS background fluorescence read at 485nm excitation and 527nm emission. SMX-HA was added to the wells in quadruplicates and the plate was incubated at 37°C with fluoresce readings at 30min intervals for 2 h. This assay was performed on three sep for each cell line.

3.28 Statistics

MTT cell viability data were analyzed in GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA). One-way ANOVA with Bonferroni post hoc procedure was used to compare mean differences between concentration levels. The IC_{50} values were calculated using GraphPad Prism, where X values (concentration of SAHA) were transformed and the Y values viability) were normalized to define 0% and 100% as the respectively. This was followed by nonlinear dose response (variable slope) curve fit. GraphPad Prism (GraphPad Software Inc., San Diego, CA) was also used to calculate mean and standard error for the real time-PCR data

3.3 Results

3.3.1 TatGFP constructs

The full-length HIV Tat protein (Tat101) was PCR amplifie
the N-terminal end of EGFP plasmid to create the fusion protei
number of deletion mutants of the Tat protein were simila
other Tat-EGFP fusion protein constructs; Tat72GFP, which encodes the
Tat protein; Tat86GFP, which encodes the first exon of T
terminal domain; Tat48GFP expresses the first three dom
TatΔGFP expresses the full-length protein without the protein trans
(domain 4, Figure 12). The fusion constructs were inserted into a -inducible
vector pBIG2i and the different plasmids were subsequentl
T cells. After a period of Hygromycin B selection, limiting dil
single cell clones that were used to establish stable cell li

3.3.2 Intracellular localization of TatGFP constructs

Confocal microscopy was used to visualize the intracellular localization of the
different Tat constructs following transient transfection of Cos 7 cells. The intracellular
localization study was carried out in Cos 7 cells as they are more conducive to this type
of imaging analysis than the small Jurkat T cells that have minimal cytopla
Studies have previously shown that the HIV-1 Tat protein is able to transport
mitochondria (Del Gaizo & Payne, 2003). In an effort to confirm this, the
treated with 500ng/ml doxycycline for 48 h, incubated with 200nM of mito
specific MitoTracker Deep Red 633 for 30 min and Hoechst 33258 which stains for D

The confocal images showed that the GFP fluorescence of the Tat101GFP, Tat86GFP
Tat72GFP constructs colocalized with the Hoechst stain in the nuc
C). The mito-tracker stained mitochondria were found to be evenly distributed throughout th
the cytoplasm. In contrast, both the Tat"GFP and Tat48GFP constructs showed GFP
fluorescence in the entire cell. The distribution of the mitochondria in the
cytoplasm of the Tat48GFP-expressing cells was similar to that of the other co
(Figure 3.1D). In contrast, the mitochondria appeared to be aggregated around th
nucleus in the cells expressing the Tat"GFP construct (Figure 3.1E). The non-
punctate/diffuse nature of the GFP fluorescence in the Tat"GFP and Tat48GFP
expressing cells suggests a non-colocalization between the GFP and the relatively
structured mitochondria (Figure 3.1D and E). It is commonly accepted w
visualizing colocalization, to present results as a simple o
channels. For example, an overlay of green and red images will give
spots where the two molecules of interest are present in t
are limits to this rather elementary method as the simple presence c
mean, in a physical definition of colocalization, two or more types of fluo
molecules occupy the same pixel in the image (Zinchuk & Grossenbacher,
2009). However, biological colocalization means the attachment of two or more diffe
molecules to the same structure. The method of choice to quan
colocalization on subcellular structures relies on their flu
(Bolte & Cordelières, 2006). An examination of line profiles for the images
Tat"GFP and Tat48GFP-expressing cells showed there was no GFP colo
fluorescence with the mitochondria.

Figure 3-1. Confocal images showing localization of the variou
from (A) Tat101GFP, (B) Tat86GFP, (C) Tat72GFP, (D) Ta
lines were treated with doxycycline for 40 h, stained with
33258 stain, and analyzed under a Zeiss LSM 510 META confocal microscope. The
scale bar represents 20μm.

Figure 3.1

3.3.3 Characterization of the TatGFP fusion constructs

The next set of experiments involved the characterization of the Jurkat Tat cell lines. Time course experiments were carried out to det induction of protein expression by different doxycycline concentrations as well as the time point at which the cell lines expressed the maximal level of their TatGFP fusion protein. The different cell lines were incubated with 0-1000ng/ml of doxycycline and taken at various time points and analyzed by flow cytom doxycycline incubation, the Tat101GFP cells were substantially free of detectable GFP expression (Figure 3.2A). At the 12h time point analyzed, the inc concentration of doxycycline compound produced an increase in the percentage expressing GFP. For example, at 12h post induction, incubation with 1000ng/ml of doxycycline had produced approximately 80% of cells exp concentrations of doxycycline produced fewer GFP-positive cells. This trend co at the 24h and 36h time points by which point the percentage of GFP expressing fac 500ng/ml and 1000ng/ml doxycycline reached a maximum of approximat 90% of the cells analyzed. This value held steady to the 72h time point of the experiment and there was little evidence of cells expressing GFP at the 0ng/ml doxycycline concentration over the 72h the cells were analyzed

The other cell lines expressing Tat deletion mutants demonstrated ve kinetics of GFP induction to the full-length Tat101GFP cell line as asses cytometry. In all the cell lines, increasing the concentratio increase in GFP-expressing cells at every time point after the sta

Figure 3-2. Time course of TatGFP expression following diff
induction. Cells from (A) Tat101GFP, (B) Tat86GFP, (C) Ta
(E) TatGFP lines were treated with various concentration
incubated for 72 h. Aliquots of cells were analyzed by flow
expression at six different time points. Data are means of three independent e

Figure 3.2

incubation (Figure 3.2B-E). The exact percent of GFP-expressing cells at ea concentration of doxycycline tested differed slightly for instance, at 24 h and 250ng/ml doxycycline, the Tat101GF 50% GFP-expressing cells compared to 55% and 57% in the Tat72GFP and T cell lines respectively. Furthermore, the maximal percent expressing cells in the Tat101GFP and Tat86GFP cell lines is maximal at 500 Dox for the other cell lines. In addition, GFP expression generally b 36 h time point and is maintained for at least the 72 h pe in these experiments. In four of the five cell lines, there cells expressing GFP in the absence of doxycycline (0ng cytometry. The exception was the Tat48GFP cell line, wh (Figure 3.2D, 0 h) approximately 6% of cells expressed de absence of doxycycline (0ng/ml). Approximately 85% of the GFP at the maximal level of induction by 1000ng/ml doxy Tat GFP cell line (Figure 3.2E), but somewhat lower than th for the other cell lines.

Each of the stably transfected Jurkat T cell lines wa western blotting to determine that the correct fusion prote data in Figure 3.2 detected GFP fluorescence derived from the TatGFP fusion protein it does not explicitly confirm that the Tat portio present. To this end, Jurkat pBIG Tat cells were incubated doxycycline identical to the time course experiment (1000ng/ml) then the cells were lysed and protein extracts prepared.

The use of specific anti-Tat antibodies showed the presence of t
each of the cell lines. The western blots showed that the fusion protein is expressed
even in the absence of doxycycline in the Tat101GFP cell line (Figure 3.3A) and the
expression of the protein increased with increasing concen
Significant expression of the protein was not detected by
doxycycline was used although trace of the protein could be
The other cell lines showed a similar trend, with significan
doxycycline for Tat86GFP (Figure 3.3B) and Tat72GFP (F
250ng/ml doxycycline for the Tat"GFP cell line (Figure 3.3E), respe
exception to the general trend is the Tat48GFP cell line
Tat48GFP expression even in the absence of doxycycline t
of protein is comparable in the lower range of doxycycline
only shows significant increases when concentrations of 2
were used. The western blots were confirmed the expecte
constructs in each case (Figure 3.3).

3.3.4 Quantitative RT-PCR of HIV Tat and TatGFP specific mRNA

In an effort to determine the expression level of the engineered
is comparable to that in HIV-infected cells, quantitative RT-PCR was performed
This was necessary as the Tat protein in the infected Jurkat T-cells (Jurkat-HIV) was
undetectable via western blots. Using primers specific
quantitative PCR detected Tat mRNA in all the cell lines expressing TatGFP fusion
proteins as well as in the Jurkat-HIV cells.

Figure 3.8 Expression of the proteins from the various Tat co
induction. Cells from the (A) Tat101GFP, (B) Tat86GFP, (C
and (E) GFP-Tat lines were treated with different concentrati
1000ng/ml) for 40 h and then the cells were lysed and protein
were analyzed on SDS-PAGE and membranes blotted with anti-Tat and GAPDH
was used as a loading control. Data representative of two independen

Figure 3.3

The Tat101 cell line showed Tat mRNA in the absence of induction (0ng/ml Dox, Figure 3.4) that is already more than half of the amou… HIV cells. There was an increase in the amount of Tat mRNA as the concentratio… doxycycline was increased and it was not until an exposure concentration of approximately 400ng/ml Dox that the Tat101 cell line reached the level of Tat mRNA in the Jurkat-HIV cells. The Tat101GFP cell line showed a 4-fold lower amount of mRNA at 0ng/ml Dox compared to the Jurkat-HIV line, but showed a much steeper inclin… increase of Tat mRNA in response to increasing doxycycline… Furthermore, the Tat101GFP cell line had a much higher amount of Tat mRNA overall compared to the Tat101 cell line, 200ng/ml Dox was sufficient to reach… Tat mRNA seen in the Jurkat-HIV cells.

In the Tat86GFP cell line there was a small, but detectable amount of Tat mRNA amou… at 0ng/ml Dox, approximately 10-fold lower than the amount seen in Jurkat-HIV cells and a concentration of 200ng/ml Dox was enough to induce Tat86GFP mRNA at similar amounts to those found in Jurkat-HIV cells (Figure 3.4). The Tat86GFP cell line also showed a general increase in Tat mRNA with the increase in doxycycline with the maximal amount of Tat mRNA occurring with 800ng/ml Dox, there was a subsequent decrease of the mRNA levels at 1000ng/ml Do… cell line also showed a gradual increase in Tat mRNA as the doxycycline was increased with a maximal amount at the highest concentration of dox… (Figure 3.4). Like the previous cell line, the Tat72GFP cell line produced approximately… 200ng/ml Dox which is comparable to that seen in Jurkat-HIV cells.

Figure 3.4 Quantification of Tat mRNA in the various cell lines. Cel
Tat101, (B) Tat101GFP, (C) Tat86GFP, (D) Tat72GFP (E)
lines were treated with doxycycline for 40 h then RNA was
was reverse transcribed and used to generate a standard curve with GAPDH se
the housekeeping gene. Tat mRNA was detected using Tat-specific primers. Data are
mean of three independent experiments.

Figure 3.4

The Tat48GFP cell line expressed a lower amount of Tat mRNA at 0ng/ml Dox (Figure 3.4E) in comparison to HIV-Tat cells. Again, there increase in Tat mRNA levels as doxycycline concentration is inc cell line also had considerably less Tat mRNA than all descr lines, needing 1000ng/ml Dox to reach the levels of HIV-Tat mRNA TatGFP cell line follows the general trend as Tat mRNA with tracking with increases in doxycycline concentration (Figur mRNA levels coincide with the HIV-Tat Jurkat cells between 200 and 40 Dox. RT-PCR analysis was also carried out using RNA from un well as Jurkat T cells expressing only GFP and neither ce amount of Tat mRNA (data not shown).

3.3.5 Cell Viability of Tat-expressing cell lines after treatment with S

Earlier studies have consistently shown concentration dependent toxicity upo incubation of SMX-HA with cells from patients with a history of hypersensi to SMX. Studies have also shown that this effect on viabil of the Tat protein and is also influenced by the amount o 2005). To determine which domain of the Tat protein is involv toxicity, the full-length protein and deletion mutants fused to GFP described above were evaluated. These cell lines were employed in MTT assays t activity to test the effect of SMX and its reactive metabolite SMX-HA on cell viability. The assays were carried out using untreated cells (control concentrations of doxycycline to induce different amounts fusion protein; or induced to yield the amount of Tat-equivale

infected Jurkat cells (Figure 3.4) or that highest Tat mRNA levels mRNA in the cell line.

Previous studies have shown that SMX-HA has significant cellular viability of Jurkat T cells. The cell lines confirmed from each of the Tat constru assayed by the MTT assay. The MTT assay was then used to assess cell viability of differentially induced Tat101 Jurkat E6.1 T cells in the presence of various concentrations of the reactive metabolite SMX-HA. Indeed, cell viability re largely unchanged for all of the cell populations until the addition of SMX-HA. At 200µM SMX-HA stably transfected Jurkat cells treated with 1000ng/ml Dox showed a trend towards lower cell viability compared to Tat101 cells 0ng/ml Dox and the untransfected Jurkat E6.1 T cells. The difference be viability of the Tat101-expressing cell line and E6.1 cells was significant at 400µM of SMX-HA. At this concentration there was no significant di the differentially induced Tat101 cells.

For the Tat101GFP cell line doxycycline concentrations of 100 and 500ng/ml were tested. The vehicle DMSO (0µM SMX-HA) had no discernable effect on viability of the aforementioned cells. However, there was a concentration-dependent decrease in cell viability with the increase in SMX-HA concentration (Figure 3.5) in the Jurkat E6.1 cells as well as the doxycycline treated cell populations. Cell toxicity of the Jurkat E6.1 cells was significantly different with 100ng/ml Dox at 200-400µM SMX-HA. At 50µM of SMX-HA, the decrease in cell viability of induced cells with 100ng/ml doxycycline began to b compared to both the Jurkat E6.1 cells and cells not induced for Tat10

expression (0ng/ml Dox) (Figure 3.5B). At the higher concentration of do
however, this decline was abrogated by 200μM of SMX-HA, there is a
significant difference between the 0ng/ml Dox and 100ng/ml doxycycline cell
populations and this was also at 400μM SMX-HA. While cells induced w
500ng/ml doxycycline followed the general trend of decr
increasing concentrations of SMX-HA, these cells did not demonstrate changes
viability that were significantly different from doxycycline
0ng/ml Dox).

In the Tat86GFP cell line, the effect of increasing con -HA was
determined using three cell populations induced at 0, 20
(Figure 3.5C). Between 25 and 100μM SMX-HA there was a slight increas
proliferation of cells treated with 200ng/ml Dox in comparis
experiment. In fact cells induced with 200ng/ml Dox hav
increase in cell proliferation compared to Jurkat E6.1 cel -HA. There
was also a significant decrease in viability of the Tat86GFP c
cells at 400 and 800μM SMX-HA. As well there was a significant decre
200ng/ml Dox and the 800ng/ml Dox cell populations at 1 -HA. Similar
results were seen in the Tat72GFP cell line (Figure 3.5D), that there was a concen-
dependent decrease in cell viability due to the increasing -HA and
a significant difference between Jurkat E6.1 cells and the
cells of the Tat72GFP cell line at 400μM SMX-HA. However, the differential in
of Tat72GFP had no significant effect on cell viability at a -HA.

Figure 3.5 The effect of Tat constructs on cell toxicity HA-Tat in the p
expression was induced for 40 h from (A) Tat101, (B) Tat
Tat72GFP, (E) Tat48GFP or (F) 5"GFP cell lines by treatment with various c
of doxycycline (0-1000ng/ml) then incubated with 200μM SM
concentrations of HA-SMX (0-800μM) for 2 h after which the drug was
media and the cells incubated at 37°C. The next day the cells were inc
5mg/ml of MTT at 37°C for a period of 4 h. The percent cell viab
determined the following day via a plate reader at 590nm.
mean of three independent experiments, with the Jurkat E6.1. The
from the Jurkat E6.1 cell line are the average of 25 different
carried out in conjunction with different Tat-expressing cell lines over a cours
months. *P<0.001 vs. Jurkat E6.1.

Figure 3.5

The viability of the Jurkat E6.1 cells and the Tat48GFP cell line was unchanged when treated with SMX (Figure 3.5). There was, however, a significant decrease in viability of the Tat48GFP cells when compared to Jurkat E6.1 cells at 200 and 400μM SMX-HA. Furthermore, a significant decrease in viability of doxycycline induced cell populations became evident at 50μM SMX-HA, a lower exposure than seen with the cell lines above. At 100μM SMX-HA, the viability of the 0ng/ml population dropped to approximately 40%. At this concentration, the cells induced with 200ng/ml Dox showed 30%. When the cells were induced with 100ng/ml Dox, cell viability dropped but was not significantly different from control (0ng/ml Dox). Cell viability continued to decrease at the higher concentrations of SMX-HA, but there was no significant difference in the differentially induced cells. Similarly there was no significant difference between the differentially induced GFP-Tat expressing cell line (Figure 3.5). The main difference between this cell line and above is the general sensitivity of these cells to SMX-HA. These show approximately 70% and 30% cell viability with 100μM and 50μM SMX-HA respectively, concentrations at which the cell lines expressing Tat101, Tat101GFP, Tat72 and Tat72GFP displayed cell viabilities of 90% and above.

Comparing the LC_{50} (lethal concentration, 50%) of each of these, that both the Tat48GFP and "GFP-Tat" cell lines are particularly sensitive to SMX-HA. As shown in Table 3.1, the LC_{50} values for the Jurkat E6.1, Tat101, Tat101GFP and Tat72GFP cell lines were in the 300μM SMX-HA concentration range while that of Tat48GFP and "GFP-Tat" was 2-fold to six-fold lower (106μM and 59μM)

Table 3-1 LC_{50} values of the various cell lines used in the MTT cel

the cells used in the experiments were stably transfected, -specific cell line

with the exception of the two cell lines given the pooled mo

lines were generated by hygromycin B selection post trans

multiple clones. LC_{50} values were calculated using GraphPad Prism v.5

the methods.

Table 13.

Cell Line	0ng/ml Dox	400ng/ml	1000ng/ml Dox
Jurkat E6.1	300	n/a	n/a
Tat101	264	250	243
Tat101GFP	302	156	265
Tat86GFP	328	310	280
Tat72GFP	262	197	228
Tat48GFP	77	67	106
TatΔGFP	59	52	57
Pooled Tat48	161	162	137
Pooled TatΔGFP	149	146	138

SMX-HA respectively. In an effort to determine if this phenomenon was specific,
the pBIG Tat48GFP"a and pBIG GFP DNA was used to transfect Jurkat
the pool of stably selected transfectants rather than sing
MTT assay. The resulting LC_{50} was of 3160µM and 4193µM respectively. Wh
in both cases this is higher than the LC_{50} found in the assay using specific cl
much lower than the demonstrated selective toxicity of the cell lines to SM
two cell lines.

3.3.6 Generation of ROS by Tat-expressing cell lines

To evaluate one possible cause for the cell death obse
pro-oxidant effects of the HIV-1 Tat protein and its deletion mutants w
next series of experiments. The cells from the various cell
same doxycycline concentrations as in the MTT assays
presence of SMX-HA or SMX and DCFH, a cell permeable dye whose
fluoresces in the presence of ROS. Incubation with SMX genera
amounts similar to the fluorescence seen when the cells a
DMSO used to facilitate dissolution of SMX or SMX-HA in aqueous buffer. Signi
production of ROS is only evident upon the addition of SMX-HA to the Tat101 cell l
(Figure 3.6A) and resulted in a 4- and 8-fold increase in ROS produced by HA
concentrations of 50 and 200µM, respectively. Furthermore
whereby increased doxycycline concentration lead to increas
Cells induced with 400ng/ml Dox, produced a small, but
compared to the control (doxycycline untreated) cell popul
SMX-HA. Similarly, cells induced with 1000ng/ml Dox, generated

Figure 3.6 Reactive oxygen species generated in the presence of HA ... various Tat constructs. After induction with doxycycline, c... (B) Tat86GFP, (C) Tat72GFP, (D) Tat48GFP, (E) Tet cell lines were seeded ... concentration of 1×10^6 cells/ml and incubated with 10 μM DCFH at 37°C ... followed an incubation with different concentration of HA for 2 h which ... SD ... the fluorescence was determined. *P<0.01 vs. parental (control cells), #P<0.05 vs 200 mg/l HA ... D. Data are mean of three independent experi...

Figure 3.6

increase in ROS compared with the uninduced cells and when this was significant at 50 and 200µM SMX-HA. However, the higher concent doxycycline did not result in a production of more ROS and from the amount of ROS produced by the cells induced a SMX-HA.

The relationship between the Tat101-GFP expressing cell line not dissimilar to that of the Tat101 cell line with SMX prod and the addition of SMX-HA resulting in 4- to 8-fold increases in the am of ROS produced. However, ROS production from Tat101-GFP expressing cells sho a biphasic response to the doxycycline concentration as initially, then decreases and is subsequently followed by a (Figure 3.6B). The initial increase in ROS occurs with cells induced with 100ng/ml Dox produced significantly more ROS than was seen from the treated with 50µM SMX-HA. This is followed by a decrease in ROS producti 200ng/ml Dox, a decrease that is significant at 200µM SMX-HA. Finally, cells indu with 500ng/ml Dox generated significantly more ROS than from uninduced cells, b significantly lower than ROS from cells induced with 100 and 200ng/ml Dox at 50µM SMX-HA, respectively.

Production of ROS from the rest of the cell lines confirms that SMX-HA furth produced significantly more ROS than the parent drug, SM -induced expression of Tat86-GFP (Figure 3.6C) and Tat72-GFP (Figure 3.6D) lead to a slight, b non-significant increase in ROS production compared to the the fusion proteins. While the generation of ROS from SMX-HA was still evident in

Tat48GFP (Figure 3.6) and TatGFP (Figure 3.6) cells, the expression of t

constructs had no significant effect on ROS production.

3.4 Discussion

HIV-1 currently infects 33 million people worldwide. The use of antiretrovi therapy (ART) has dramatically improved the quality of l individuals infected with the disease. There are several classes of antir based on the life-cycle of the virus that the drug inhibits, but the primary function anti-retrovirals (ARVs) is the suppression of HIV replication. This is carried out mo effectively when combination ARVs are used. However, these drugs can cause serious adverse effects. HIV-1 primarily targets CD4+ T cells and one of t HIV-1 infection is the rapid and massive depletion of CD4+ T cells. This in turn le to a state of immune deficiency that allows the propagati that also require treatment. HIV-1 infection is known to be associated with an incre risk of ADRs to ARV that vary by individual, drug, ethnicity, and interaction with othe drugs. One of the most common drugs producing adverse effects in HIV-infected individuals is the antimicrobial agent SMX. ADR to SMX usually manifest as HIV-1 infection progresses to AIDS which suggests that the virus is a key contributing factor. Previous research has shown that the Tat protein can be one such factor as the expression of the protein correlates w metabolite of SMX (Asensio et al 2005). We have also demonstrated that the differentia expression of this protein alters cellular sensitivity to SMX-HA (Adeyanju et al. 2009). In this study we sought to determine the region of the Tat this effect. To this end, deletion mutants were created that wer upon treatment with SMX-HA.

The initial experiments conducted were to establish the cellular distribution of th full-length Tat-GFP fusion protein as well as the deletion mutants in COS-7 cells. As expected, the full-length Tat protein localized to the nucleus to its nuclear localization sequence (NLS). There was minimal GFP fluorescence detected in cytoplasm with the Tat101GFP cell line. The deletion mutants Tat72GFP and Tat86GFP and showed the same cellular distribution. In contrast, the TatGFP, Tat48GFP and Tat"GFP fusion proteins were distributed throughout the cell, this pattern is likely due to the loss of the NLS, allowing diffusion of the proteins into This overall distribution however did not include colocaliz evidenced by the difference in the structural pattern of the TatGFP fusion proteins ar the mitochondria. The lack of colocalization of the GFP fluor mitochondria in the Tat"GFP and Tat48GFP cells was expected as both proteins a missing the NLS which also functions as a protein transduction domain that allows the proteins to pass through the cell membrane. The increase in GFP flu in the nucleus of the Tat"GFP and Tat48GFP cells is most likely due t Tat proteins to members of the transcriptional complex such as TFIIE and p300/CREB binding protein. Similar observations were obtained in the co transfected Jurkat cell lines (data not shown).

The apparent mitochondrial staining in the Tat"GFP-expressing cell could be indicative of an early step towards apoptosis. Mitochondria play an impo role in apoptotic signal transduction by regulating Bcl-2 family members and cytoc c release and caspase activation. Studies have also shown that mitochondrial dynamics have a significant role in regulating apoptosis (2003). I

showed quantitatively by laser scanning cytometry that mitochon
precedes cytochrome c release during apoptosis and that cytochrome c rele
dependent on mitochondrial aggregation". The GFP expression, sugge
in the absence of any other stimuli, is able to prime the ce

The characterization of the cell lines with time course experiments as
well as western blot and Q-PCR experiments. The time course experiments were
necessary to demonstrate that doxycycline dependent induction of Tat-GFP mRNA
expression showed a dose response. Also, the time course experiments were
instrumental in determining the time point at which the induction of the
protein was maximal. The Q-PCR experiments proved a relatively easy
the difference in Tat mRNA produced in each of the cell lines required. This wa
particularly for both the HIV-infected cell line and the Tat101 cell line as western
blotting protocols were unable to detect Tat protein from the former, only
able to detect very faint amounts of Tat101 protein in the latter. The lack of sensitivity of
the western blot in this instance was most likely because the Tat protein leaves
Thus, the full-length Tat101 protein (14-16 kDa) is able to pass through the nuclear pore
complex by passive diffusion despite the presence of NLS. Further, the exist
of the protein transduction domain as well as in Tat101. In addition
the use of Q-PCR allowed the comparison of the Tat mRNA produced at
different doxycycline concentrations to the single Tat mRNA in
the HIV-infected cells. Subsequent experiments were carried out using doxycycline
concentrations that induced Tat-GFP mRNA at approximate levels seen in the HIV-
infected cells.

Results from the MTT cell viability assay previously demonstrated significant decrease in cell toxicity in the cell lines in the presence of increasing concentrations of SMX-HA while SMX is nontoxic when cells are incubated with the parent compound, SMX, under identical conditions. The constitutive expression of Tat increased SMX-HA-mediated cell toxicity, an effect similar to that seen in HIV-infected T cells. Differential expression of Tat also had a cooperative effect on SMX-HA and SMX toxicity, further significantly decreas viability as the expression of the TatGFP protein increased (Asif et al., 2005). These observations were the basis of the current project, to determine which region(s) of the Tat protein is (are) responsible for these results. For this several de protein were generated as stably transduced Jurkat T cell lines that were then evaluated in the MTT cell toxicity assay. The results show that the dif expression of Tat101 at physiologically relevant concentrations of doxycyc had an additive effect on cell toxicity in the presence of the highest SMX-HA concentrat As the doxycycline concentration increased, and the amount of Tat present inc degree of toxicity was correspondingly increased. Neither the parent compound (SMX) at a concentration of 200 µM nor the lower concentrations of SMX-HA (0-100 µM) metaboli caused significant cell toxicity, but there seems to be a growth inh the lower concentrations of SMX-HA which is in concordance with previo data (Hess et al., 1997). In the Tat101 cell line, significant toxicity began at 200 µM of SMX-HA when Tat101 mRNA is at levels equivalent to or higher tha HIV-infected cells. At 400 µM and 800 µM SMX-HA, the cell toxicities of the three doxycycline-induced Tat101 cell populations are significantly different fro

other, possibly due to the fact that this cell line expresses a moderate amount of Tat101 mRNA at 0ng/ml Dox (Figure 3.4), an amount only slightly less than 400ng/ml Dox. While doxycycline-induced Tat101 cell populations ar significantly different from one another, they are not significantly different from that of Jurkat E6.1 cells at 1200µM SMX.

The viability of the Tat101GFP cell line with 25µM began to decr populations of 0ng/ml and 100ng/ml doxycycline. As the SMX-HA increased, the viability of the 100ng/ml Dox cell populati was significantly different from the 0ng/ml Dox population. viability of the 500ng/ml Dox population remained largely unchanged at the lo SMX-HA (1000µM) and was not significantly different from the at any of the concentrations tested. The SMX-HA trajectory of cell viability in the Tat101GFP cell line where low expression of Tat101GFP r and high expression of the same protein enables no significa effect, a phenomenon that typically occurs in immunoassays antibody bind all the receptor sites, leaving nothing availa quite different from the results seen for the Tat101 cell could be due to the differences in the amount of Tat expressed in the two cell lines characterized by th mRNA levels. Besides the fact that the Tat101 cell line is leaky (0ng/ml Dox), the slope of Tat101 expression created by the increases in doxycycli as that found in the Tat101GFP cell line. The Tat101 mRNA at 400ng/ and 1000ng/ml Dox is only 1.4-fold and 3.5-fold greater, respectively than that se control cells (0ng/ml Dox). In comparison, the amount of Tat101GFP mRNA at

400ng/ml is a 10 fold greater while the amount at 200 ng/ml Dox is 160 fold greater than that at 0 ng/ml Dox. This means there is a much wider range of conce in the expression of Tat101GFP for the observance of the prozone effect.

Another possible explanation for the discrepancy between the toxicity results in the cells from the Tat101 cell line versus the Tat101GFP cell line is that the fusion of GF Tat101 has an effect on the results. Green fluorescent protein (GFP) is widely used in vitro as well as in vivo as a molecular marker. Although GFP is found in severa of marine invertebrates, GFP traditionally refers to the p jellyfish *Aequorea victoria*. GFP has also been identified in a variety of many corals where the protein is not bioluminescent. The purpose of GFP fluorescence in the jellyfish and in the coral is currently unknown (Bou-Abdallah et al., 2006). However, the fluorescent chromophore originates Ser-Tyr-Gly sequence that is post-translationally modified in the presenc oxygen to form a fluorescent ring structure that is maxima 475 nm and emits at 515 nm (Bou-Abdallah et al., 2006). While molecular oxyge is required for the post-translational formation of the protein, it is found in GFP corals where hyperoxia and reactive oxygen species (photosynthetic activity of algal symbionts (Bou-Abdallah et al., 2006). A recent study on the distribution and function of GFP in Caribbean corals, wild type GFP is s from the hydromedusa *Aequorea victoria*, may have the ability to quench superoxide radicals ($O_2^{\cdot-}$) and exhibit SOD-like activity by competing with cytoc for reaction with $O_2^{\cdot-}$. The authors found that this SOD-like activity of GFP does no the fluorescence properties of the protein and can provide supplementary a

protection to the cell. While this activity could potentially have a profound e outcome of the various experiments described herein, it is important to note two things. Firstly, the authors used wild type GFP in their experiments deletion mutants were fused to EGFP (enhanced green fluorescent protein) mutant that has been optimized for brighter fluorescence, higher expression in mammalian cells and to reduce clustering of the EGFP (Heim et al. 1995; Thastrup, 1997). Since the purpose of GFP in nature is still being deba EGFP mutation in relation to the original function is unclear. Secondly, the GFP generated by Bou-Abdallah and Chasteen (2006) suggests that wild type GFP has the ability to specifically quench O_2^- radicals, however a study by Gulow (2005) showed that there was no detectable O_2^- generation from the Jurkat T cell line incubation with Tat, but there was a significant increase in H_2O_2. Therefore, even i SOD-like activity of wild type GFP is maintained in the EGFP mutant, experiments by Gulow et al. (2005) suggest that fusion of EGFP to Tat and its deletion mu would have a minimal effect on the ROS generated by Tat that could lead to oxidative stress and cell death.

Therefore, in an effort to determine if the fusion of Tat to GFP has a significant effect on Jurkat cell viability, MTT experiments were carried out w line expressing GFP only. While there was the expected concentration-dependent decrease in cell viability in the presence of increasing SMX-HA, there was no significant effect on cytotoxicity upon induction of GFP expression, even at the concentration of Dox 100 ng/ml. The same pattern was seen in the cell Tat86GFP and Tat72GFP, where the effect of SMX-HA was clearly visible, but tha

the fusion proteins or a. The failure of Tat72GFP expression to add SMX-HA-mediated cell death is in contrast to previously published data (Aujla et al. 2009; Arp et al. 2005). This could be due to the fact that the sequence of the Tat protein used in the previously performed work was modified by amino acids compared to the Tat protein used in the current project. While both Tat variants may have basic Tat properties it is possible that amino acid differences in the Tat used previously confer a greater ability to mediate SMX-HA toxicity. An example is the substitution of Pro-58 in the Tat GFP fusion protein used previously (Aujla et al. 2009; Arp et al. 2005) with Ala-58 in the Tat constructs used in this chapter. The structure of the side chain in position 58 could affect protein tr nuclear localization as this residue is within the basic domain. Indeed, Peloponese et al. (1999) were among the first to show that differences in Tat could have an effect on activity. The authors chemically synthesized six d Tat proteins from HIV-1 isolates found in different parts of the world and compared some of their biological activities in different assays. They found that all six vari the ability to bind the TAR region as well as cross the membranes, to transactivate an HIV-LTR reporter gene in stably transfected He experiments showed that the high virulence of HIV-1 strains from Africa coul related to Tat activity. Since then a number of publications have dem acid variations between specific Tat proteins have different transactiv potentials in experiments using conventional reporter ge significant effect on the regulation of HIV-1 expression. Other studies have sho that the sequential differences in Tat primary monocytes,

leading to differential expression of genes including those TNF-α, CCR5, IL-4 and IL-10 (Desfosses et al., 2005; Gandhi et al., 2009; Samikkannu et al., 2009; Wong et al., 2010). To my knowledge there has not been a study looking differences in Tat regulation of genes that could affect SMX-HA toxicity such as those involved in redox regulation for instance glutathione s dismutase, two genes known to be modulated by Tat (Westendorp et al., 1995).

The Tat48GFP and ΔTatGFP cell lines are relatively more sensitive to the metabolite SMX-HA than both the parent cell line Jurkat E6.1 a expressing cell line. An important feature the Tat48GFP and ΔTatGFP cell lines have i common is that they are both missing the protein transduction. During HIV infection, cells infected with the virus produce Tat intra eventually able to leave the cell and enter uninfected cells. The ability of Tat to translocate across the mammalian cell membrane of the PTD which also contains the NLS. Without the PTD and NLS, thus both Tat48GFP and ΔTatGFP fusion proteins escape the nucleus and accumulate in the cytopl The best studied properties of the Tat protein is its transactivation activity on the viral genome and its ability to regulate the expression of various cellular genes. Among those genes are several of the cytoskeletal system which have been found to be down regulated by the Tat (Coiras et al., 2006). In addition, extracellular Tat has been found to interact with cytoskeletal proteins in the cytoplasm (Chen et al., 2002; de Mareuil et al., 2005). The link between microtubule polymerizati protein was first suggested a few years ago when it was observed that transiently transfected Tat interacts directly with tubulin dimers α? and polymerized microtubules, causing microtubul

the formation of abnormally stable microtubules and preventing microtubule depolymerization (Chen et al., 2002; Huo L, 2010). This ability of Tat require integrity of four amino acids in a conserved Tat core domain (amino acids 36-39). Experiments by de Mareuil et al. (2005) showed that the full-length Tat protein is not necessary to enhance tubulin polymerizatio central region had the same properties as the full-length counterpart. This demonstrated that the N-terminus and the C-terminus of Tat are not necessary fo interaction with tubulin. The functional consequences of Tat-induced changes of the dynamic property of the microtubules transduced by the Bim signal p apoptotic relay of Bcl-2 leading to mitochondrial apoptosis (Giacca, 2005; Matarre & Malorni, 2005). This is corroborated in preliminary experime incubation of Tat48GFP and GFP fusion Tat protein with SMX-HA leading to apoptotic cell death.

Both extra- and intracellular Tat display a different structur when synthesized inside the cell during HIV-1 infection than when it is rele infected cells and enters uninfected cells. One such difference is in the mo host cell cytoskeletal structure and function. The cytoskeleton is involved in ce proliferation and intracellular trafficking including the traf HIV-1 infection. The cytoskeleton is also involved in HIV-1-induced membrane fusio viral assembly and budding (Lopez-Huertas et al., 2010). In this context, intracellul has been found to induce down-regulation of several cytoskeletal proteins including protei and β-tubulin. While binding of extracellular Tat to cytoskeletal crucial in activating the mitochondria-dependent apoptotic pathway, the do

of these proteins by intracellular Tat can explain the absence of apoptosis despite the high expression of intracellular Tat, suggesting that intracellular Tat plays a role in the modification of the cytoskeleton str to HIV-1 infection (Coiras et al. 2006). Furthermore, a study looking at the effe Jurkat cells stably expressing either intracellular Tat101 or intracellular Tat72 cytoskeleton-related functions such as cell morphology, prolife actin polymerization, Lopez-Huertas et al. (2010) found that modifications of these functions were dependent on the second exon. One possible explanation fo differences in the behaviour of intracellular and extracellular Tat in HIV infected cells Tat localizes predominantly in the nucleus following its synthesis in the cytoplasm, but remains largely in the cytoplasm after being endocytosed where the protein c retained through the binding to cytoskeletal proteins such as tubulin and LIS1, leading to cellular apoptosis (Chen et al. 2002; Epie et al. 2005).

Neither case of intra- or extracellular Tat completely represent the "Tat48GFP" and "GFPTat" cell lines. While "Tat48GFP" and "GFPTat" are both synthesized intracellularly, and thus capable of down-regulating cytoskeletal genes, the absence of the NLS due to the loss of PTD in these constructs means that they the nucleus and the cytoplasm and are able to move freely between the t The higher concentration of these Tat mutants in the cytop bind to cytoskeletal proteins in a manner similar to that s But unlike in the case for extracellular Tat, the consequence of "GFP proteins being in the cytoplasm is higher sensitivity to SMX-HA toxicity rather than direct apoptosis. Also, the fact that "GFPTat" protein has an N-terminal mutants C

may still be able to alter tat-based cytoskeletal functions, making it more
cellular injury than the Tat48GFP cell line.

The ROS experiments mirrored the cell viability data
difference in the production of ROS was generated only by the Tat101 and Tat101
cell lines. The effect of ROS exposure on mitochondria can often lead to detrimental
consequences such as oxidative damage to mitochondria
mtDNA, which encodes proteins of the respiratory chain, would compromise
mitochondrial ATP production that ultimately triggers apop (Lenaz et al., 2002; Van
Houten et al., 2006). ROS are also known to be triggers of the intri
via interactions with components of the mitochondrial perme
an extra-large pore spanning the inner and outer mitochondrial membranes. Oxidative
modifications of the pore proteins, including cyclophilin-D, voltage-dependent anion chann
(VDAC) and adenine nucleotide translocase (ANT) will significantly impa
mitochondrial anion fluxes, leading to hyperpolarization of the mitochondrial inner mem
eventually the collapse of the mitochondrial membrane potenti (Brenner et al., 2007;
Kokoszka et al., 2004; Schinzel et al., 2005).

Another possible consequence of heightened cellular
intracellular signalling and regulation. Specifically, ROS-activated JNK
signalling pathway is associated with apoptosis. Upstream of JNK is the re
sensitive MAPK kinase, ASK1 whose activity is inhibited by
proteins such as thioredoxin-1 (Trx1) and heat shock proteins (Hsp90, Hsp (Saitoh et
al., 1998). Only reduced Trx1 binds ASK1 resulting in signalling, suggesting that AS
functions as a redox switch that senses cellular ROS and oxidizing

conditions (Fujino et al., 2007). ROS initiate Trx dissociation from the signalosome through oxidation of the Trx active-site, redox active cysteine. The ASK1 is thus activated and able to signal downstream JNK apoptosis, either via the mitochondria or AP-1-dependent transcription of pro-apoptotic signaling (Circu & Aw, 2010).

Given the ability of Tat to modulate cellular ROS balance by producing ROS, the role this plays in apoptotic signaling would be beneficial to explore the contribution of the HIV-1 protein to other post-translational mechanisms such as the formation of protein thiols as a mechanism of redox regulation. The oxidation state of peroxiredoxins that occurs in high concentrations (Chen et al., 2010) data in Chapter 4) would be one suitable endpoint in vitro for studies of this type. This could further the understanding of the development of mechanisms of AIDS during HIV-1 infection.

3.5 References

Adeyanju, K., Krizova, A., Dekaban, G. A. & Rieder, M. (20 HIV Tat potentiates cell toxicity in a T cell model for s induced adverse drug reactions. *Virus genes*, 38, 273-282.

Arp, J., Rieder, M. J., Urquhart, B., Freeman, D., Tucker, Lehmann, D. & Dekaban, G. A. (2005). Hypersensitivity of HIV-1-infected cells to reactive sulfonamide metabolites correlated to HIV-1 viral protein Tat. *The Journal of pharmacology and experim therapeutics*, 314, 1218-1225.

Baines, C. P., Kaiser, R. A., Sheiko, T., Craigen, W. J. & Molkent (2007). Voltage-dependent anion channels are dispensable fo mitochondrial-dependent cell death. *Nature cell biology*, 9, 550-555.

Bolte, S. & Cordelieres, F. P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *Journal of microscopy*, 224, 213-232.

Bou-Abdallah, F., Chasteen, N. D. & Lesser, M. P. (2006). Quenching of superoxide radicals by green fluorescent protein. *Biochimica et biophysica acta*, 1760, 1690-1695.

Carr, A., Tindall, B., Penny, R. & Cooper, D. A. (1993). In vitro cytotoxicity as marker of hypersensitivity to sulphamethoxazole in pat *Clinical and experimental immunology*, 94, 21-25.

Carroll, R., Martarano, L. & Derse, D. (1991). Identification of lentivirus tat functional domains through generation of equine infect virus/human immunodeficiency virus type 1 tat gene ch *Journal of virology*, 65, 3460-3467.

Chen, D., Wang, M., Zhou, S. & Zhou, Q. (2002). HIV-1 Tat targets microtubules to induce apoptosis, a process-promoted by t apoptotic Bcl-2 relative Bim. *The EMBO journal*, 21, 6801-6810.

Circu, M. L. & Aw, T. Y. (2010). Reactive oxygen species, cellular redo systems, and apoptosis. *Free radical biology & medicine*, 48, 749-762.

Coiras, M., Camafeita, E., Urena, T., Lopez, J. A., Caballe B., Lopez-Huertas, M. R., Perez-Olmeda, M. & Alcami, J. (2006). Modifications in the human T cell proteome induced by Tat protein expression. *Proteomics*, 6 Suppl 1, S63-73.

de Mareuil, J., Carre, M., Barbier, P., Campbell, G. R., La Esquieu, D., Watkins, J. D., Prevot, C., Braguer, D., P E. P. (2005). HIV-1 Tat protein enhances microtubule polymer *Retrovirology*, 2, 5.

Del Gaizo, V. & Payne, R. M. (2003). A novel TAT-mitochondrial signal sequence fusion protein is processed, stays in mitocho the placenta. *Mol. Ther.*, 7, 720-730.

Derse, D., Carvalho, M., Carroll, R. & Peterlin, B. M. (199 A minimal lentivirus Tat. *Journal of virology*, 65, 7012-7015.

Desfosses, Y., Solis, M., Sun, Q., Grandvaux, N., Van Lint Gatignol, A., Wainberg, M. A., Lin, R. & Hiscott, J. (20 Regulation of

human immunodeficiency virus type 1 clade-specific expression
Tat protein. Journal of virology 79, 9180-9191.

Epie, N., Ammosova, T., Sapir, T., Voloshin, Y., Lane, W.
Reiner, O. & Nekhai, S. (2005). HIV-1 Tat interacts with LIS1 protei
Retrovirology 2, 6.

Fujino, G., Noguchi, T., Matsuzawa, A., Yamauchi, S., Saitoh, M., Ta
& Ichijo, H. (2007). Thioredoxin and TRAF family proteins regu
reactive oxygen species-dependent activation of ASK1 through r
modulation of the N-terminal homophilic interaction of ASK1. Molecular
and cellular biology 27, 8152-8163.

Gandhi, N., Saiyed, Z., Thangavel, S., Rodriguez, J., Rao,
(2009). Differential effects of HIV type 1 clade B and clad
on expression of proinflammatory and antiinflammatory
primary monocytes. AIDS research and human retroviruses 25, 691-699.

Giacca, M. (2005). HIV-1 Tat, apoptosis and the mitochondria: a tub
Retrovirology 2, 7.

Gulow, K., Kaminski, M., Darvas, K., Suss, D., Li-Weber, M. & Krammer, P.
H. (2005). HIV-1 trans-activator of transcription substitutes for
signaling in activation-induced T cell death. J Immunol 174, 5249-5260.

Haga, N., Fujita, N. & Tsuruo, T. (2003). Mitochondrial aggregation preced
cytochrome c release from mitochondria during apoptosis. Oncogene 22,
5579-5585.

Heim, R., Cubitt, A. B. & Tsien, R. Y. (1995). Improved green fluorescence.
Nature 373, 663-664.

Hess, D. A., Bird, I. A., Almawi, W. Y. & Rieder, M. J. (199
The hydroxylamine of sulfamethoxazole synergizes with FK
cyclosporin A, inhibiting interleukin... The Journal of pharmacolog
and experimental therapeutics 281, 540-548.

Huo L, L. D., Sun L, Liu M, Shi X, Sun X, Li J, Dong B, Dor
(2010). Tat acetylation regulates its actions on microtubu
apoptosis in T lymphocytes. J Pathology

Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E.,
P., MacGregor, G. R. & Wallace, D. C. (2004). The ADP/ATP translocator
is not essential for the mitochondrial permeability tran
Nature 427, 461-465.

Lenaz, G., Bovina, C., D'Aurelio, M., Fato, R., Formiggini,
Giuliano, G., Merlo Pich, M., Paolucci, U., Parenti Cas
Ventura, B. (2002). Role of mitochondria in oxidative stress a
Annals of the New York Academy of Sciences 959, 199-213.

Lopez-Huertas, M. R., Callejas, S., Abia, D., Mateos, E., Dop
J. & Coiras, M. (2010). Modifications in host cell cytoskeleton structure
and function mediated by intracellular HIV-1 Tat protein are greatly
dependent on the second coding exon. Nucleic acids research 38, 3287-
3307.

Matarrese, P. & Malorni, W. (2005). Human immunodeficiency virus (HIV-1) proteins and cytoskeleton partners in viral life and host cell death. Cell death and differentiation, 12 Suppl 1, 939-41.

Peloponese, J. M., Jr., Collette, Y., Gregoire, C., Bailly, C. Meurs, E. F., Olive, D. & Loret, E. P. (1999). Full peptide synthesis, purification, and characterization of six Tat variants. Diff between HIV-1 isolates from Africa and other continents. The Journal of biological chemistry, 274, 11473-11478.

Rieder, M. J. (2009). Immune mediation of hypersensitivity adverse reactions: implications for therapy. Expert opinion on drug safety, 8, 331-343.

Rieder, M. J., Uetrecht, J., Shear, N. H., Cannon, M., Mille S. P. (1989). Diagnosis of sulfonamide hypersensitivity reactions by in vitro "rechallenge" with hydroxylamine metabolites. Annals of internal medicine, 110, 286-289.

Rieder, M. J., Uetrecht, J., Shear, N. H. & Spielberg, S. P. Synthesis and in vitro toxicity of hydroxylamine metabolites of su The Journal of pharmacology and experimental therapeutics, 244, 724-728.

Romani, B., Engelbrecht, S. & Glashoff, R. H. (2010). Functions of Tat: the versatile protein of human immunodeficiency virus type The Journal of general virology, 91, 1-12.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K Kawabata, M., Miyazono, K. & Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. The EMBO journal, 17, 2596-2606.

Samikkannu, T., Saiyed, Z. M., Rao, K. V., Babu, D. K., Rod Papuashvili, M. N. & Nair, M. P. (2009). Differential regulation of indoleamine-2,3-dioxygenase (IDO) by HIV type 1 clade B an protein. AIDS research and human retroviruses, 25, 329-35.

Schinzel, A. C., Takeuchi, O., Huang, Z., Fisher, J. K., Zh Hetz, C., Danial, N. N., Moskowitz, M. A. & Korsmeyer Cyclophilin D is a component of mitochondrial permeab mediates neuronal cell death after focal cerebral ischemia. Proceedings of the National Academy of Sciences of the United States, 102, 12005-12010.

Van Houten, B., Woshner, V. & Santos, J. H. (2006). Role of mitochondrial DNA in toxic responses to oxidative stress. DNA repair, 5, 145-52.

Westendorp, M. O., Shatrov, V. A., Schulze-Osthoff, K., Frank, R., Kraft, Los, M., Krammer, P. H., Droge, W. & Lehmann, V. (19 HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity b altering the cellular redox state. The EMBO journal, 14, 546-554.

Wong, J. K., Campbell, G. R. & Spector, S. A. (2010). Differential induction of interleukin-10 in monocytes by HIV-1 clade B and clade C Tat protei The Journal of biological chemistry, 285, 18319-18325.

Zinchuk, V., & Grossenbacher-Zinchuk, O. (2009). Recent advances in quantitative colocalization analysis: Focus on neurosci[...] Progress in histochemistry and cytochemistry, 44, 125-172.

CHAPTER 4: Detection of oxidant sensitive t
HIV-1 Tat-expressing cells by redox two-dimensional electrophoresis.

4.1 Introduction

During HIV-1 infection, the progression of the disease is ac increasing oxidative stress. A number of studies have shown that levels of intra the nucleophilic antioxidant, glutathione (GSH) in peripheral blood T cells ter markedly decreased at advanced stages of the disease (Roederer et al., 1992). Other studies have demonstrated decreased GSH levels in blood, brain and tissues in HIV-infected individuals (Castagna et al., 1995; Droge & Holm, 1997; Staal et al., 1992b; Staal et al., 1992a). Antioxidant depletion is an indicator of decreased immune fu as cells of the immune system generally require a higher other cells to retain redox balance, integrity and function (De la Fuente et al., 2002b; Stehbens, 2004). In HIV pathology, oxidative stress plays a responsiveness, apoptosis and depletion of CD4+ T cells. In addition, there is also association between decreased survival in HIV-infected individuals and low th in serum and CD4+ T cells (Bal et al., 2009).

This oxidative stress in large part is due to the HIV-1 transactivator of transcription (Tat). Besides being an important viral gene transactivator, Tat is secrete from HIV-1-infected cells and enters uninfected cells in a biologically ac regulates the expression of host cellular genes (Huigen et al., 2004; Rem et al., 2010). There are two ways for Tat to influence the cellular redox state, either by dec antioxidant concentrations or increasing the oxidant capacity. The ability of Tat t decrease antioxidant concentrations is linked to its ability to regulate cellular genes. For example, Tat suppresses the expression of manganese superoxide (Mn-SOD), a dis mitochondrial enzyme that is part of the cellular antioxid

stress (Flores et al., 1993; Westendorp et al., 1995). The HIV Tat protein is also able
directly decrease cellular GSH by down-regulating the enzyme required
synthesis, glutathione synthetase (Choi et al., 2000). Alternatively, Tat is able to induc
the production of reactive oxygen species (ROS) in a number of different cel
(Gulow et al., 2005; Price et al., 2006; Turchan-Cholewo et al., 2009). Furthermore, Tat
activation of astrocytes leads to the expression of inducib
leading to the overproduction of nitric oxide (Porcheray et al., 2005).

Reactive oxygen species can function as second
transduction, modulating a variety of biological responses
proliferation and apoptosis. The transduction of an oxidant signal in
response can be mediated by the oxidation of protein thiols. The thiol (SH) group
on the side chain of the amino acid cysteine is particular
and has been established as a redox sensor. Most protein thiols do not react w
under normal cellular conditions, however some thiols are
they are ionized at physiological pH due to their surrounding environment.
cysteine thiols can sometimes form transient reactive catalytic interme
of many enzymes. This allows oxidants to alter the act
modifying the redox state of functionally essential cysteine thiols
serves as a signal transduction mechanism that couples
functional activity. Many regulatory proteins contain reactive critical cysteine residues
that can interact with a variety of oxidants, to form many covalent c
modifications. These include intra- and intermolecular protein disulphides

mixed disulphides with GSH and are a major mechanism by which prot
be controlled and protected during oxidative stress (Eaton, 2006).

HIV-1 Tat is a protein made up of 101 amino acids, div
domains. One of those domains is the cysteine-rich domain which contains seven cysteine
residues and is highly conserved among the different HIV-1 strains.
redox state of this domain is known to be essential for its function and activity,
confirming the presence of at least one reactive cysteine thiol in this domain. We have
examined the protein disulphide formation by oxidation and identified reactive cysteine
thiols in the HIV-1 Tat protein as well as the effects of oxidants and the reactive
metabolite of SMX (sulphamethoxazole), SMX-HA (sulphamethoxazole-hydroxylamine)
by employing redox two-dimensional electrophoresis. Our objective was to esta
what effect the Tat protein, separately or in combination with SMX-HA, had
of highly expressed cellular thiol proteins.

4.2 Materials and Methods

4.2.1 Cell lines

The human T lymphocyte cell line Jurkat E6.1 (ATCC TIB-152), was obtained from American Type Culture Collection. The cell line was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, 2 L-glutamine, sodium pyruvate and 100units/ml of penicillin and streptom

Construction of plasmids and stable cell lines

The Tat gene was PCR-amplified from the pSV Tat plasmid. This plasmid encod full-length Tat gene and was a kind gift from K.T. Jean Molecular Virology Section, NIAID, NIH. The full-length Tat gene (Tat101) with two stop deletion mutants (Tat72 and Tat∆49-57) were cloned into the N1 plasmid (Clontech Inc.), resulting in Tat101GFP, Tat72GFP and Tat∆GFP fusion genes respectively. The fusion genes were excised from pEG NheI/NotI fragment and inserted into the NheI/NotI site of the plasmid pBIG2i, an ind doxycycline-responsive expression (Strathdee et al. 1999). The result was pBI Tat101, pBIG Tat72GFP and pBIG Tat∆GFP. As a control, the GFP gene fr N1 was also cloned into pBIG2i to create pBIG GFP.

pBIG Tat constructs or pBIG GFP were transfected into Jurkat E6.1 cells by nucleofection (Lonza, Amaxa Nucleofector) according to th to generate stable cell lines of Jurkat pBIG TatGFP and J, respectively. The expression of both TatGFP and GFP fusion proteins was induced by incubat pBIG2i-transfected cells with doxycycline (Sigma). Post-nucleofection, positive c

were selected using hygromycin B (Invitrogen) at a conce
clones were derived from single cells following limiting dilution in 96 ...es and
the resulting cell lines were maintained in complete RPM
supplemented with 1% L-glutamine, 10% fetal calf serum, 100units/m
streptomycin and 0.5mg/ml of hygromycin B.

4.2.2 Purification of polyclonal antibodies from serum of SMX-HA-KLH immunized rabbits

The polyclonal SMX-HA antibody was prepared by Dr. Jane
laboratory if Dr. Micheal Rieder at Robarts Reasearch Ins
Ontario. Post-immunization serum from New Zealand white rabb
SMX-HA conjugated to Keyhole Limpet Hemocyanin (KLH) wa
and stored at -20°C. The post-immunization serum samples (10 ml) w
overnight at 4°C, with continuous stirring, against 2L of PBS (pH 7
and 7 Dialysis membrane (1.8 cm/ml) (Spectrum, Texas). Dialyzed serum was puri
using a Protein G Sepharose 4 Fast Flow column (Amers
selectively capture all IgG antibody subtypes. The protein concentrations of ea
were determined using the Bio-Rad protein estimation assay
previously. Samples with protein concentrations greater than 0
overnight at 4°C, with continuous stirring, against 1L of PBS (pH 7
until needed.

4.2.3 Slot blots

Cells from the different cell lines were incubated with
treated with varying concentrations of SMX-HA for 2 h. The cells were collected

and lysed in RIPA buffer containing 50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Tr
100, 1% sodium deoxycholate, 0.1% SDS, 1mM EDTA (pH
protease inhibitor cocktail (Roche cat.# 1836170). The cell lysates were son
twice for 20 s each time then centrifuged at 13,000 rpm 4°C for 15
supernatant was collected and stored at -80°C. Samples of all the treatment con
of every cell line were generated on three separate occ
subsequently blotted thr

1 μg protein resuspended in cold PBS was applied to sl
microfiltration device (Bio-Dot SF Apparatus, Bio-Rad) according to the manufa
instructions. Briefly, after assembly of the Bio-Dot SF Apparatus with the p
nitrocellulose membrane, the appropriate samples were loaded into wells solution. T
flow valve was adjusted to allow the entire sample to filt
gentle vacuum then each well was washed with TBS buffer (50mM Tris
7.6, 150mM NaCl), again by pulling the wash liquid through
were completely drained, the membrane was removed.

The blots were first blocked with TBS-T (TBS with 1% Tween 20) conta
dry milk for 1h at room temperature with gentle shaking and then probed ove
4°C with anti-SMAH rabbit polyclonal antibody at a 1:1,000 dilution of TBS-T
The incubation with the primary antibody was followed by
for 10 min each time with TBS-T. The secondary antibody was HRP-conjugated goat
anti-rabbit antibody diluted to 1:35,000 in TBS-T. The blots were then treate
chemiluminescent agent (Thermo Scientific Pierce ECL We
visualized with a Fluorchem HD2 Imager (Alpha Innotech/Cell Biosciences). The b

then stripped and probed with anti-GAPDH monoclonal antibody ,000 (Sigma) followed by a donkey anti-mouse conjugated to HRP at 1:2000. Ban determined by the AlphaView software (Santa Clara, CA).

4.2.4 Sample preparation for redox 2D gel electrophoresis

Jurkat pBIG TatGFP cells (2.5 x 10^6) were induced with 0, 200 1000ng/ml of doxycycline for 40h at which point the cells were washe phosphate buffered saline (PBS) by centrifugation at 1500rpm for 5 min. The cells were resuspended in HEPES buffer and seeded at 10^6 cells/ml concentration. The cells were then treated with 200µM SMX-HA or the vehicle (DMSO) at concentration of 0.5% then incubated for 2h at 37°C. The cells were subs collected and lysed in RIPA buffer HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1mM EDTA (pH 8), 1% SDS, complete mini protease inhibitor cocktail (Roche cat. # 1 836 170) a Sigma). The cell lysates were sonicated on ice twice 20 s each time then centrif at 13,000rpm for 15 min at 4°C. The supernatant was collected and stored at -80°C for future analysis.

4.2.5 Protein assay

Protein concentrations were determined using the BioRad modification of the Lowry method. Bovine serum albumin (B

4.26 Non-reducing/reducing two-dimensional SDS-gel electrophoresis

The diagonal SDS-PAGE procedure was a modified version o
Sommer and Traut (1974). Briefly, protein samples were di
free of reducing agents and the proteins resolved on a 1.0mm thick, 10% p
gel by subjecting the gel to electrophoresis at a constant c for 1 h followed
by 1-25h at a constant current of 24mA. After electrophores
containing the separated proteins was excised from the gel and immerse
SDS sample buffer containing 100mM DTT (Dithiothreito
temperature with gentle rocking. The gel strip was then
running buffer then immersed in SDS sample buffer containing 100mM IA for 10 min at
room temperature with gentle rocking. After another wash
strip was applied horizontally to a 10% polyacrylamide g
23cm(L) x 20cm(W) in dimension. Electrophoresis was carried out in the sec
dimension at constant current 17mA approximately. Each treatment sample of every cell
line was subjected to the above redox 2D electrophoresis
times.

4.27 Silver staining and image analysis of 2D gels

Each set of 2D gels was silver-stained simultaneously using a stack o
freshly prepared solutions. After washing the gels in a solution of 50
methanol, the gels were sensitized with 0.02% sodium thio for 5 min and washed
with deionized water twice, 1 min. The sensitized gels were then sta
nitrate solution (0.25% silver nitrate and 0.05% formaldehyde) for min

washing with deionized water for an additional 20 [illegible]. The gels were conducted in a solution of 6% sodium carbonate, 0.012% s formaldehyde until the desired gel resolutions were obtain incubated in stop solution of 5% acetic acid for 5 min and later scanned by a scanner (Epson).

4.2.8 Statistics

Slot blot data were analyzed by GBStat. Means and standard errors (SE) were used to report continuous variables. Two-way ANOVA with Tukey's post procedure was used to compare mean differences between groups for concentration levels of each cell line.

4.3 Results

4.3.1 Quantification of SMX-HA-induced haptenation in Tat-expressing Jurkat T cells

Our current belief is that the mechanism of drug hyper... reactions (ADRs) is based on initial hapten formation, which may or may not lea[d to] hypersensitivity. In the case of sulphonamides (SMX), it is metabolically activated to chemically reactive SMX-HA, an electrophilic metabolite that can haptenate/becomes covalently conjugated to cellular proteins. After antigen processing and presentation, immune responses are directed against the hapten-protein conjugate. Previous data from the Pirmohamed laboratory have already shown that SMX-HA at concentrations of 25 µM and above reacts with cellular proteins (Manchanda et al., 2002). To determine if the expression of HIV protein Tat or its mutants in Jurkat T cells affect the SMX-HA-induced haptenation in Jurkat cells, western blot experiments were carried out.

The various cell lines were incubated for 40 h with different concentrations of doxycycline to facilitate expression of the Tat proteins, followed by 2 h treatment with various concentrations of the reactive metabolite, SMX-HA, and protein isolatio[n]. Proteins in cell lysates (15 µg) were subjected to SDS-PAGE and blotted with anti-SMX-HA antibody to visualize the haptenation pattern (Fig 4.1). The ... for each ... qualitative differences between the haptenation patterns o[f] ... decipher on the western blots, 2D gels in which identical amounts of the different protein samples were ... onto blots and the me[mbranes] probed with the anti-SMX-HA antibody preparation described above. The use of the

Figure 4.1 Representative film and slot-blot showing SMX-HA induced haptenation

(A) Image from a western blot of cells treated with 10-200 µM SMX-HA after a period of 2 h, protein extracted and resolved on 12% SDS-PAGE, transferred onto a P membrane which was then blotted with anti-SMX-HA antibody. Lanes 1-3: 10 µM; lanes 4-6: 100 µM and lanes 7-9: 200 µM SMX-HA. Lanes 1, 4, 7: Jurkat E6.1; 5, 8: Tat101GFP; lanes 3, 6, 9: Tat72GFP.

Alternatively, protein was obtained from the lysate of cells treated with the various concentrations of SMX-HA (lanes 0, 10, 50, 100 and 200 µM SMX-HA, respectively) and applied onto a nitrocellulose membrane via a slot blot apparatus. The membrane was then blotted for (B) SMX-HA, stripped then blotted for (C) GAPDH.

Figure 4.1

SMX-HA-specific polyclonal antibody preparation means that bands that could be detected on the membrane for the sa 0µM SMX-HA (solvent control) or 200µM SMX (negative control). The membranes were then stripped and probed with anti-GAPDH antibody and th intensity of the bands analyzed by densitometry to determine the degree of loading. The ratios of the band intensities were used to generate the graphs in F Figure 4.2

All three cell lines had baseline SMX-HA/GAPDH ratios of approximately 0. in the absence of SMX-HA (Figure 4.2). The ratios increased at least 2.5-3-fold when each of the cell lines were treated with 50µM SMX-HA, with further increases as the concentration of SMX-HA was increased. The parental cell line Jurkat E6.1 showed significant increase in SMX-HA/GAPDH ratio with the addition of the 50µM reactive SMX metabolite. While the ratios increased with the higher concentrations of SMX-HA, these increases were not significantly different from the effect with 50µM SMX-HA. Incubation of the cells with 1000ng/ml Dox prior to treatment with SMX-HA resulted in a lower SMX-HA/GAPDH ratio at 100 and 200µM, specifically 34% and 26% respectively (Figure 4.2).

Induction of Tat101 expression to levels approximating those seen in HIV-infected Jurkat cells by treatment with 100ng/ml Dox significantly increased the SMX-HA/GAPDH ratio at 50µM SMX-HA compared to that at 0ng/ml Dox; however, this increase was abrogated with 1000ng/ml Dox treatment. The ratios increased approximately 2-fold with a doubling of the concentration of SMX-HA to 100µM, but there was no significant effect between the various doxycycline concentrations other than at 1000ng/ml Dox. Similarly, the ratio at 0ng/ml Dox was increased at 40 and 400ng/ml

2-fold, respectively with the addition of 200µM SMX-HA. With SMX the ratio from th
1000ng/ml Dox treated sample at 200µM SMX-HA was double that observed at 50µM
SMX-HA, but this was still lower than 0ng/ml and 400ng/ml doxycycline samples a
200µM. Again, the higher concentration of doxycycline seeme
haptenation as the ratio at 200µM SMX-HA was not different than at 100µM

The Tat101GFP cell line showed a similar trend in that
dependent increase in the SMX-HA/GAPDH ratio with increasing concentra
SMX-HA (Figure 4.2C). There was, however, no significant diffe
treatment with the various doxycycline concentrations. As observed with the Jurka
and Tat 101 cell lines, the ratio from the 1000ng/ml Dox sample at 200µM SMX-HA was
unchanged from the 100µM SMX-HA data and lower than the ratios observed for the 0
and 200 ng/ml Dox samples seen at 200µM SMX-HA (Figure 4.2C).

For the Tat72GFP cell line there was a concentration-dependent increase of the
SMX-HA/GAPDH ratio at each concentration of SMX-HA for the 0ng/ml and
1000ng/ml Dox samples, though there were no significant differ
doxycycline samples (Figure 4.2D). There is also a ratio-dependent increase o
the SMX-HA/GAPDH ratio at 50 and 100µM SMX-HA for the 200ng/ml Dox sample
which approximates the amount of Tat mRNA in HIV infected cells, the ratio
decreases to the 50µM level at 200µM SMX-HA. The ratio at 100µM SMX-HA for the SMX
200ng/ml Dox sample was significantly higher than that seen for samples
also significantly higher than the 200ng/ml Dox sample at SMX-HA (Figure
4.2D). The protective effect of 1000ng/ml doxycycline was obs
SMX-HA concentration and was maintained at 200µM.

Figure 4.2 SMX-HA induced haptenation in Jurkat T cell lines.
doxycycline, cells from the (A) Jurkat, (B) Tat101GFP, (C) Tat101, (D)
(E) TatGFP or (F) GFP cell lines were then treated with SMX-HA for 2 h, protein
was isolated. The protein samples were applied onto memb
and then blotted for GAPDH and SMX-HA. The band intensities were measur
and the ratios plotted. Data are mean of three independent experimen p, P<0.05 vs.
400, 100 ng/ml Dox at 10 µM SMX-HA, respectively; P<0.05 vs. 400, 100 ng/ml Dox
at 50 µM SMX-HA, respectively; P<0.05 vs. 200 ng/ml Dox at 100 µM SMX-HA

Figure 4.2

Results from the GFP T cell line were somewhat different.
concentration-dependent increase of the ratio for each SMX-HA. At 50 and 100µM S
doxycycline samples, though not significantly different, there was
ratio for the 400ng/ml Dox sample compared to the other doxycycline samples at
100µM SMX-HA (Figure 4.2). At 200µM SMX-HA, the ratios from the 0ng/
1000ng/ml Dox samples were almost identical, while the 400ng/ml Dox sample, which
approximates the amount of Tat mRNA in HIV-infected cells, had a ratio that was hig
than that of the 0ng/ml sample (solvent control) (Figure 4.2). The ratios from the GF
expressing cell line did not show any differences between the
concentrations (Figure 4.2). Thus, taken together it does not appear t
Tat or its mutants causes significant differences in the SMX-HA mediated
haptenation, however it is possible that doxycycline at the concentration of 1000ng/ml did have
small to moderate protective effect against haptenation.

4.3.2 Detection of ROS-sensitive thiol proteins

While Tat did not affect the global level of SMX-HA mediated haptenation T
expression is known to alter the overall pattern of gene e
therefore may alter a set of genes to make the cellular environment more or less amenable
to protein haptenation. To explore this possibility, non-reducing/reducing SDS/2
electrophoresis was employed as it provides a direct method to identify
proteins that are redox regulated via oxidation of their cysteine groups protective pr
through formation of protein-protein mixed disulphides. Proteins that contain reduced
protein thiol (-SH) residues before being applied to the first dimension are resolved
on the diagonal after the gel is run in the second dimension, whereas proteins b

are not further reduced by DTT. In contrast, protein mixed disulfides are red
by treatment with DTT in the first dimension gel matrix, af
electrophoresis run, so that the thiols formed upon reduction of intermolecular disulphide
bonds are resolved below the diagonal line due to dissoc of protein dimers
disulphides and the attendant decrease in molecular mass afte
dimension. However, proteins with intramolecular disulphi
molecular mass and run above the diagonal line in the second dimension after reduction
with DTT.

In the present study, the primary objective was to identify protein mixed
disulphides formed in the presence of HIV-1 Tat protein and its deletion mutants and
more specifically to document oxidative changes (i.e. enh
protein mixed disulphides) as a result of treatment with th
Each treatment condition was analyzed on 2D gels at least three times
recorded only if it was present in at least two of the three gels.

A summary of the protein spots identified on the gels 4.1.
The first set of images Figure 4.3 shows 2D gels from protein samples of different
control cell lines, including untransfected Jurkat E6.1 cells incubated with
and without 1000 ng/ml doxycycline (Figure 4.3A and D, respectively), Jurkat T c
infected with HIV (Figure 4.3B) and the stably transfected Jurkat T ce
only EGFP (Figure 4.3C). Figure 4.4 again shows 2D gels of control, untransfected cells
Jurkat (Figure 4.4A) and HIV infected Jurkat (Figure 4.4B) as well as the gels from
vehicle control induced cells (0ng/ml Dox) of different cell (Figure 4.4C-F).
The non-reducing/ reducing 2D gel system revealed a number o

Table 4.1 - Summary of protein spots seen in the redox 2D gels
the presence of the corresponding protein spot in that cel
the presence of the corresponding protein spot in that c
200 µM SMHXA.

J-E6.1 + dox: Jurkat E6.1 incubated with 1000ng/ml doxyc
E6.1 cells infected with HIV. *Concentrations of doxycycli
TatGFP mRNA equivalent to that of HIV-infected Jurkat cells, for Tat101GFP
and Tat72GFP cell lines Dox was 200ng/ml, while Dox 400ng/ml was used for the
rest of the cell lines.

Table 4.1

Figure 4.3 Redox two-dimensional SDS-PAGE of thiol proteins from vario
cell lines; (A) Jurkat E6.1, (B) Jurkat-V, (C) Jurkat-GFP and (D) Jurkat E6.1 +
Dox. The sample from panel D was incubated with 1000ng
prior to drug treatment. Cells from each of the various line
DMSO for 2hrs, collected and the protein was isolated. 85µg of each prote
loaded onto the first dimension gel and run for 3hrs follow
second dimension gel. On the left side of each gel are molecular w
protein standards that are enumerated on both sides of the image. The gels from panels
and B are duplicated and used as reference in Figures 4.4-4.6

Figure 4.3

intermolecular disulphide bonds (below the diagonal), notably the spots most
just below 30 kDa circled (and labelled 1) which are putative peroxiredo
proteins. (High abundance proteins in this specific molecular
identified by matrix assisted laser desorption/ionization MS analysis of tryptic peptid
digests performed at the London Regional Proteomics Cent
cells (peroxiredoxin 1 and peroxiredoxin 2) and in HEK 293 cells (peroxire
peroxiredoxin 2, isoform A and peroxiredoxin 3) in the laboratory of Dr. Jack Bend at the
University of Western Ontario, London, Ontario [Al-Owaysheh, MSc Thesis, 20
shown in Figures 4.3-4.6, all the samples from the different cell lin
of induction displayed the same protein spots. An analysis of the patterns of the di
gels indicated that the average were similar, with most of the spots res
between 9-75 kDa but with differences in the number of individually resolved
spots. Of the control samples in Figure 4.3, the HIV-1 sample gel showing
the most protein spots (1-18 and 1-13, Figure 4.3B), indicative of
oxidative stress, with the majority having intermolecular
the diagonal line. The parental cell line Jurkat E6.1 (Figu
protein spots as HIV-1 Jurkat while Jurkat cells incubated w
doxycycline shows only five proteins (Figure 4.3D). Jurka
show only spots 1 and 7 (Figure 4.3C). The Tat101 and Tat72GFP cell lines
and 4.4, respectively) displayed the same number of disulphide proteins 0 µg/mL
doxycycline with five of the six proteins resolved below the line of the d
Both cell lines had proteins 1, 2, 4, 5 and 7 (Figure 4.4C and 4.4E). In the
Tat101GFP cell line (Figure 4.4D) had proteins 1, 2, 5, 7,

Figure 4.4 Redox-two-dimensional-PAGES of thiol proteins from (A) Ju

(B) J-Lat, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP and (F) 1µg/ml Tat

doxycycline. The redox 2D gels in panels A and B are replicas

4.3. Cells from each of the various lines were then treate

collected and the protein was isolated. 85µg of each prote

first dimension gel and run for 3hrs followed by an overnig

dimension gel. On the left side of each gel are molecular weight

standards that are enumerated on both sides of the image

Figure 4.4

Figure 4.5 Redox two-dimensional SDS-PAGE analysis of thiol proteins from (
E6.1, (B) HLV-Jurkat, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP, (F) with Tat
panels C-F induced for 40hrs with 400ng/ml doxycycline (C
doxycycline (D and E) prior to drug treatment. The redox 2D gels in panels A a
replicas of those seen in Figure 4.3. Cells from each of th
with 0.05% DMSO for 2hrs, collected and the protein was i
sample was loaded to the first dimension gel and run for 3hrs foll
run of the second dimension gel. On the left side of each gel are
molecular weight protein standards are numerated on both sides of t

Figure 4.5

Figure 4.6 Redox two-dimensional SDS-PAGE of thiol proteins from (A) Ju
(B) Jurkat-HIV, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP, and (F) Ta
panels C-F induced for 40hrs with 1000ng/ml doxycycline prior. The
redox 2D gels in panels A and B are replicas of those seen
of the various lines were then treated with 0.05% DMSO
protein was isolated. 85μg of each protein sample was loaded on
and run for 3hrs followed by an overnight run of the second. On the left
side of the diagonal on each gel are molecular weight
enumerated on both sides of the image.

Figure 4.6

protein only seen in the HIV infected cells, designated protein 13. The TatGFP sample had only two proteins (1 and 7, Figure 4.4).

I first examined the different cell lines treated with co that would induce the expression of Tat or TatGFP mRNA to approx of Tat mRNA that occur in HIV infected cells. Induction of Tat101 expression with 400ng/ml doxycycline (Figure 4.5C) led to a protein pattern on the gels similar to that of the vehicle control (0ng/ml Dox) s induced cells did not contain protein 3. The induction of Tat101GFP 200ng/ml doxycycline did not change the identity or number of proteins that resolve away from the diagonal compared to the 0ng/ml Dox sampl the Tat72GFP cell line with 200ng/ml Dox (Figure 4.5E) resulted in the disappeara protein no.4, leaving proteins no.1, 2, 5 and 7 as well as one new protein (designated protein no. 12). The protein patterns of samples from the TatGFP cell line induced with 400ng/ml Dox (Figure 4.5F) showed the appearance of protei below the diagonal of new protein no. 7. A (Figure 4.5F, nos 10 and 11 found above the diagonal line. These two proteins were doxycycline (Figure 4.3F), nor on any of the other gels. The gel showing dis samples from the GFP cell line consistently had few protein spots, one of doxycycline responsive cell lines, proteins 1, 2 and 7 were found in the treatment with at 400ng/ml Dox

Induction with 1000ng/ml doxycycline of the Tat101 cell line (Figure 4.6C) resulted in the loss of one of the proteins (no. 9) seen in gels of lowe concentrations of doxycycline. Similarly, the gel for the Tat72GFP 1000ng/ml sample

Dox (Figure 4.6E) shows the appearance of protein 4 in comparison to the ge
200ng/ml Dox (Figure 4.6B). The level of Tat-GFP expression induced at 10
doxycycline (Figure 4.6F) resulted in a protein pattern almost identi
400ng/ml doxycycline with the exception of the increased i 10 and
11. The induction of Tat101GFP at this time with 1000ng/ml doxycycline caused the
disappearance of protein 18 (Figure 4.6D). The Jurkat E6.1 cells showed proteins 1,
2, 4, 5 and 7, but a loss of 3 occurred upon incubation with 1000ng/ml
doxycycline (Figure 4.3D) compared to cells grown in the absence
(Figure 4.4A).

The control cell lines treated with 200uM SMX-HA are in Figure 4.7. One
of the obvious differences by the addition of SMX-HA is the dramatic reduc
in the intensity of the putative peroxiredoxin proteins 10 and 15 (Figure 4-7). In
cell lines treated with SMX-HA there is also loss of 2 and 3 and increase in the number
proteins resolved below the diagonal. Many of these proteins were also
absence of SMX-HA (Figure 4.4-6) and are numbered accordingly. The proteins that
are the result of the oxidative stress resulting from the addition of SMX-HA are
lettered as in the figures and in Table 4.1

Treatment of the parental cell line Jurkat E6.1 with SMX-HA had no effect on the S
resolution of any of the numbered proteins, but saw the addition of a, d, and
(Figure 4.7) to the profile. The only difference between the aforementioned cell li
(Figure 4.7) and SMX-HA-treated Jurkat E6.1 cells incubated with 1000ng/ml Dox
(Figure 4.7D) is the apparent reduction in the intensity of proteins 18 and 4 in the
latter indicating lower amounts of oxidized protein, (these) occur as prot

Figure 4.7 Redox two-dimensional SDS-PAGE of thiol proteins from vario
cell lines; (A) Jurkat-E6.1, (B) Jurkat-HIV1, (C) Jurkat-GFP and (D) Jurkat E6.1 +
dox. The sample from panel D was incubated for 40hrs with
to drug treatment. Cells from each of the various lines were t
SMX-HA for 2hrs, collected and the protein was isolated. 8
was loaded onto the first dimension gel and run for 3hrs fo
the second dimension gel. The gels from panels A and B are
reference in figures 4.8-4.10. On the left side of the diagonal on each
weight protein standards are enumerated on both sides of the ima

Figure 4.7

protein mixed disulphides) in the Jurkat E6.1cells treate
treatment of HIV-infected Jurkat cells with SMX-HA caused the loss of prot
and 4 and also dramatically increased the number of prote
a, f, g, h and i including proteins unique to this cell line,
Redox 2D gel electrophoresis of extracts from cell line ex
resolution of proteins 1, 2, 5, 7 and 10 (Figure 4.7C), but

With minimal Tat protein induction at 0ng/ml Dox, cel
fusion cell lines were treated with 200 µM SMX-HA and the results are shown
4.8. The "GFP Tat" cell line shows the most protein, a majority of (Figu
which are proteins with intermolecular disulphide bonds, r
of unresolved proteins on the gel. In the Tat101 cell line
vehicle control cells (0ng/ml Dox), resulted in a decreased intensity of prot
spot 4 and the resolution of proteins 3, 7, d, e, f and g (Figure 4.8C).

The Tat101GFP cell line saw the resolution of protein
but there were no changes to the proteins already seen in the absence of
SMX-HA treatment. There was a much more dramatic effect
with the addition of SMX-HA to the cells of the Tat72GFP cell line a
addition to the proteins seen in Figure 4.4E, proteins 3, 4, 6, 11 and SMX-
HA-specific proteins a, f and i (Figure 4.8E) were resolved.
the cells of the "GFP Tat" cell line at 0ng/ml Dox resulted in the res
3, 5, 8, 10, 11 and 12 (Figure 4.8F), proteins that were oxidized to disulphides in the
absence of SMX-HA at that concentration of doxycycline (Figur
specific proteins a, b, c, d, g and i were also resolved in t

The induction of mRNA expression in the different cell li
approximate levels found in HIV infected cells was followed by treatment with 200µM
SMX-HA and the results of redox 2D electrophoresis are documented in
Figure 4.9. In the Tat101 cell line the intensity of protein 4
reduced. In addition proteins 1, 2, 5, 7 and d were resolv
lysed proteins from the Tata101GFP cell line resolved pro
SMX-HA specific proteins a, i and m (Figure 4.9D). For th
combination of 200ng/ml Dox and 200µM SMX-HA resulted in the re
of proteins 1, 2, 5, 6, 7, 8, 10, 11 and 12 as well as pr
Expression of GFP at 400ng/ml Dox resulted in decreased inte
10 and 11 and the resolution of proteins 1, 2, 5, 6, 7, 12,
SMX-HA (Figure 4.9F). The control cell line expressing jus
saw the resolution of proteins 1, 2, 5, 7, 10 and 12 subsequent to redox
analysis.

Redox 2D electrophoresis of protein samples from ly
1000ng/ml Dox then treated with SMX-HA are shown in Figure 4.10. In the
line, proteins 1, 2, 3, 4, 5, 7 and 12, in addition to proteins
(Figure 4.10C) while the Tat72GFP cell line produced prot
f, g, i and s (Figure 4.10E). The Tat101GFP cell line at
SMX-HA only differed from the untreated sample in the decreased intensity of
18 and the addition of proteins a, f, i and r, the last of w
(Figure 4.10D). Once again the GFP cell line presented the most prot
severe oxidative stress. In addition to the resolution of proteins seen in the

reactive sulphonamide metabolite (Figure 4.6F) proteins 6 and q (Figure 4.10F) were also present. The last three of t this cell line.

In summary, the induction of Tat GFP expression with 400ng/ml or Dox produced proteins 10 and 11, two proteins otherwise from the Tat72GFP cell line were treated with SMX-HA. Tat GFP induction followed by treatment with SMX-HA resulted in protein no. 6 being reso also seen in the Tat72GFP cell line. Furthermore, the max GFP Tat72GFP in cells induced with 1000ng/ml Dox in combin SMX-HA produced the most oxidized proteins of any of the experiment a number of proteins unique to each cell line. This can als though there were not as many oxidized proteins produced lines. Differential induction of Tat101GFP did not appear to affe spots resolved away from the diagonal. There was however a fold increase in the number of spots seen when doxycycline induction is com SMX-HA in this Tat expressing cell line. There were also a numbe redox 2D gels of lysates of HIV-infected cells, in the absence and presence of SMX-HA that were unique to this cell line. Overall the control c as the parental Jurkat E6.1 cell line produced very few SMX-HA proteins in comparison to the other Tat-expressing cell lines and the HIV infected cell line.

Figure 4.8 Redox two-dimensional SDS-PAGE of thiol proteins from (A) Ju

(B) Jurkat-HIV, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP and (F) Tat 1ng/ml

doxycycline. The redox 2D gels in panels A and B are replicas

4.7. Cells from each of the various lines were then -HA for treat

2hrs, collected and the protein was isolated. 85µg of each

the first dimension gel and run for 3hrs followed by an overn

dimension gel. On the left side of the diagonal on each gel are m

standards that are enumerated on both sides of the image.

Figure 4.8

Figure 4.8 Redox two-dimensional SDS-PAGE of thiol proteins from (A) Ju

(B) Jurkat-HIV, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP and (F) Ta

panels C-F induced for 40hrs with 400ng/ml doxycycline (C

doxycycline (D and E) prior to drug treatment. The redox 2D gels in p

replicas of those seen in Figure 4.7. Cells from each of th

with 200µM H-SAMX for 2hrs, collected and the protein was iso

protein sample was loaded onto the first dimension gel and run for

overnight run of the second dimension gel. On the left side of the diagonal

are molecular weight markers that are annotated on both sides of

Figure 4.9

Figure 4.10 Redox two-dimensional SDS-PAGE of thiol proteins from (A) Ju

(B) Jurkat-HIV, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP, and the (F) last Tat

four panels showing samples induced for 40hrs with 1000ng

treatment. The redox 2D gels in panels A and B are replic

Cells from each of the various lines were then treated with 200µM SMX-HA for 2hrs,

collected and the protein was isolated. 85µg of each prote

first dimension gel and run for 3hrs followed by an ove

dimension gel. On the left side of the diagonal on each gel are molecular

standards that are enumerated on both sides of the image.

Figure 4.10

4.4 Discussion

The class of antimicrobials known as sulphonamides are broad spectrum antibacterials. In North America the combination Sulphamethoxazole-Trimethoprim (SMX-TMP, co-trimoxazole) is used to treat uncomplicated urinary and genital infections in HIV-negative individuals and is the first line treatment and prophylaxis for Pneumocystis pneumonia (PCP), a common opportunistic infection in HIV patients. Although very effective, SMX-TMP in HIV-infected patients has been implicated in the high incidence (up to 50% or more) of hypersensitivity ADRs, an incidence 10-fold higher than in the HIV negative population (25%). The mechanisms that lead to the development of ADRs are still unclear, but one of the explanations is the Hapten Hypothesis. This states that the initial step in the pathogenesis of hypersensitivity ADR involves the metabolic activation of the drug, in the case of SMX, oxidation of the parent drug to the hydroxylamine, SMX-HA, a reactive intermediate that can be further oxidized to nitroso derivative (SMX-NO). These reactive metabolites or haptens form covalent conjugates with proteins that can be recognized as ... Immune responses may be directed against the drug, the protein or conjugate of the ... Manchanda et al (2002) showed that concentrations of SMX-HA as low as 25 µM binds to cellular proteins. Given the high incidence of hypersensitivity in the HIV population, we decided to determine if the expression of a major HIV protein, Tat, wo[uld] increase the level of SMX-HA-induced haptenation in Jurkat T cells.

Our data confirm that protein haptenation occurs in Jurkat cells exposed to SMX-HA at concentrations as low as 50 µM and the level of haptenation is a...

the concentration of the reactive metabolite increased (Figure 4.1). Patients in the high dose Cotrimoxazole therapy have SMX plasma concentrations of 1.5 ... SMX-HA concentrations are 1-2% of these plasma concentrations (0.01-0.4 mM) and SMX-NO accounts for approximately 2% (Manchanda et al., 2002). The concentrations of SMX-HA used in this study were well within the therapeutic range attained in vivo.

Our data also showed that the induction of the metabolism to levels comparable to those seen in HIV-infected cells increased the SMX-HA level of haptenation of proteins (Figure 4.2). The reactions of drugs with proteins ... formation of bonds of different strengths, with hydrogen ... considered weak interactions and coordinate covalent bonds considered ... bonds. The interaction between a hapten and a protein res... covalent bonds, allowing the protein to function as ... of SMX ... reactive electrophiles containing aromatic ring susceptible to prote... haptenation via nucleophilic substitution. The main targets of small m... electrophiles such as SMX-HA are amino acid side chains with nucleophilic ... such as the cysteine sulfhydryl group, N-terminal amino group, or histidine imidazole (Dvorkovic et al., 2008). Proteomic studies have shown th... containing reactive cysteine thiol groups, whose nucleophilicity enhances ... ionization of the cysteine thiol, are preferred targets for haptenation rea... (Liebler, 2008; Wong & Liebler, 2008). Ornithine decarboxylase... protein has a cyste... rich domain (amino acids 2-237) that would make it a very attractive ... electrophilic metabolites of SMX. Further analysis by deletion and poin...

in the cysteine-rich domain has revealed that six of the seven Cy

for Tat transcriptional activity (Kalantari, 2009). However, the structural ba

this activity is still not well understood, though the trans

has been shown to be a monomer, and reducing agents dra

suggesting that Cys residues form two intramolecular disu

for transcriptional activity (Kalantari, 2009). Thus given that four of the seven

cysteine residues in the Cys domain form intramolecular disulphide bonds critical for

Tat activity, there remains free thiols that can act as nucleophil (Kalantari,

2008). Both control cells, the Jurkat E6.1 and the GFP-expressing cell line

showed significant haptenation as SMX-HA/GAPDH. This is their SMX

not unexpected as the SMX-HA can bind to several types of am

chains on cellular proteins that would by definition be in the control cell co

line and the doxycycline-responsive cell lines.

Whereas the expression of Tat in the different cell lin

SMX-HA/GAPDH ratios, there was no cell line that was consistently

different from the others or the control GFP. This probably reflects the fact

that all Tat constructs used in this study retain the cysteine-rich domain. One of th

differences seen is the discrepancy between the doxycycline-induced samples of the

Tat101 and Tat101GFP cell lines at 50 µM SMX-HA. This may be due at least in

the discrepancy in size of the Tat101 and Tat101GFP protein

latter adds 25kDa to the size of the protein. The ability of the SMX-HA to react

form of the target protein may be hindered or enhanced by the nucl

three-dimensional environment of the protein. For instance, a side chain may be

embedded amongst residues with non-polar hydrophobic side chains, making i accessible to hydrophilic chemicals. Similarly, the surrounding amin the pH of the local microenvironment making it dramatically different from the surrounding medium, thus greatly influencing the degree of reactivity c nucleophilic side chains (Divkovic et al., 2005). The degree of side chain ioniz also be altered depending on the site of haptenation as are more acidic than the cytoplasm (Divkovic et al., 2005).

Common to the Tat101 and Tat101GFP cell lines as we is the significant decline in HA/GAPDH SMX of the doxycycline 1000 ng/ml induced samples, particularly at 200 μM SMX. This can perhaps be attribu ability of doxycycline, a tetracycline, to bind non-microbial targets such as matrix metalloproteinases (MMP), facilitating its use in the treatment of oth (Golub et al., 1987; Gertifi et al., 2011). The oxygen-rich lower half of the doxyc molecule is critical for this binding and interference with this regio the effectiveness of the drug. This region is also import Binding of doxycycline to proteins may be greatly enhanc complexed with divalent metal ions Ca^{2+} or Mg^{2+} (Takahashi et al., 1986). The binding of doxycycline to MMPs is thought to be mediated by t and structural Zn^{2+} ions within the enzyme which in turn leads to MMP inhibi (Ryan et al., 2001). The relative affinity of tetracyclines for a given metal highly dependent on pH and the presence of other metal io (Berthon et al., 1983; Brion et al., 1985; Lambs et al., 1984). The relative superiority of doxycycline as a inhibitor is due to its increased affinity for Zn^{2+} as compared to other tetracycli

minocycline, another chemically modified tetracycline (Burns et al, 1989). Doxycycline can form coordinate bonds with nucleophilic centres in the prote comparable in strength to covalent bonds. In essence th chelated doxycycline molecule would bind to the nucleophile i the amount available for formation with the electrophilic SMX-HA. This in turn would mean less protein-SMX-HA complex formation. Hence less protein-SMX-HA complex would be detected by the antibody resulting in a lower SMX-GAPDH ratio.

Another consequence of the presence of SMX-HA in the cell is the ability o reactive metabolite to induce oxidative stress by generatin of oxidative stress, the most susceptible potential target is the thiol group in proteins with reactive cysteine thiol residues (PS⁻). We have shown previously increasing concentrations of SMX-HA increases ROS activity in the cell here (previous chapter). In the presence of increasing concentrati oxidation of ionized cysteine residues within a protein can result in a oxidative modifications, among them being the initial formation of sulphenic acid (PSOH) and subsequent oxidation to sulphinic (PSO_2H) or sulphonic acids (PSO_3H) (Dalle-Donne et al, 2009). The protein sulphenic acids are able to reac enzymatically with glutathione or with thiol-glutathionylated prote proteins (protein glutathione mixed disulphides) or protein-protein mixed disulphides where these can be either homodimeric or h These disulphide bridges are known as intermolecular whereas those between thi the same protein are termed intramolecular disulphides. The redox 2D electrophoresis system is an effective way to detect the quantity and quality

disulphide bonds formed in the HIV Tat protein and the effect of the reactive metabolite SMX-HA has on these interactions.

The presence of reactive cysteine thiol groups in Tat may act as a target for SMX-HA haptenation, but may also provide the chance to form intramolecular disulphides as well as intermolecular protein mixed disulphides. A glutathionylated proteins are not resolved by redox 2D gel because of the small change in molecular weight resulting from the addition o to the protein. However, formation of either Tat interm disulphide bonds is likely to result in a significant structural or functi that could be a contributing factor in the progression of HIV tow infected patients. The primary structure of Tat is 101 amino acids divided into functional domains. The cysteine-rich domain of Tat contains seven highly cons cysteine residues. Mutagenesis studies have shown that all but one cysteine residues are indispensable for the transcriptional activation function of HIV protein; indeed, the transcriptionally active form of Tat and reducing agents have been found to strongly inhibit its activity, suggesting that the residues i Tat form intramolecular disulphide bonds required for transcriptional activati (Koken et al., 1994). Also, increased transcription activity was seen in ba was subjected to protein refolding, which allows for the formation of disulphide bonds (Kalantari et al., 2008). These data suggest that the redox state of residues greatly affects the biological function of Tat. Furthermore, Kalantari et al. (2008) showed that three of the seven cysteine resi free thiol form and can form disulphide bonds independently

to maintain T cell activity. To achieve this, we carried out 2D gel electrophoresis experiments to determine the expression of Tax and its deletion mutant and the quantitative or qualitative nature of disulphide bonds, and what effect, if any, the a metabolite SMX-HA will have on the disulphide bonds in Jurkat T cells.

The most ubiquitous and prominent spot in all the gels is the putative peroxiredoxin. Peroxiredoxins (Prxs) are antioxidant enzymes found in all organisms with the single exception (a number of Borrelia species) (Hall et al., 2009). This family of antioxidant proteins are approximately 20-30 kDa in size and use specialized cysteine residues to decompose perox Prx towards peroxides in combination with their high expression mean proteins of the Prx family are a primary line of defence against p critical role as cellular antioxidants (Rhee et al., 2007). The physiological importan peroxiredoxins is illustrated by their relative abundance; Prx are the most abundant proteins in erythrocytes, after haemoglobin, with a wide cellular di different isoforms in the cytosol, nucleus, mitochondria, endopla plasma membrane. Mammalian cells contain at least six different isoforms of the Prx enzyme (Prx1 to Prx6) with all six found in Jurkat cells, Prx1 being the major cytoplasmic form (Chae et al., 1999). All Prx enzymes contai conserved active cysteine residue (designated the peroxidatic cys (C_P)) Prx1 to Prx4 also contain an additional conserved cysteine residue (designated the resolving cysteine, C_R). The Prx enzymes that contain only C_P are thus referred to as 1-Cys Prxs while Prx enzymes with two conserved cysteine residues are designated 2-Cys Prxs (Cho et al., 2010; Hall et al., 2009). The 2-Cys Prxs exist as non-covalently linked

homodimers, with the two monomers oriented head-to-tail (Seo et al., 2000). Peroxide detoxification by the 2-Cys Prxs proceeds in three main steps: peroxidation, re recycling (Figure 4.11). The first step is when the C_P-SH of one monomer is selectively oxidized by peroxides to the sulphenic acid intermediate C_P-SOH at the active site reactive cysteine. The resolution is when the C_R of the second monome reacts with C_P-SOH to form a disulfide-linked intermolecular Prx dimer and water. The catalytic cycle is completed when the disulphide is subsequently reduced, typically by thioredoxin, recycling the free thiol form of the C_P (Cox et al., 2010; Seo et al., 2000). Peroxiredoxins can also be over-oxidized to sulphinic or sulphonic acids when a fraction of the sulphenic acid catalytic cycle rather than forming the disulphide (Baty et al., 2005; Wagner et al., 2002). This leads to loss of peroxiredoxin activity and the formation of the dead end sulphinic acid competes with the resolution step o catalytic (Wood et al., 2003; Yang et al., 2002). Under mild oxidative conditio amount of inactivation caused by peroxiredoxin oxidation is possibly by de novo synthesis of the native, active enzyme. However, when exposed to stron oxidative stress, the normal form of Prxs disappears due inactivation by oxidation which effectively also indicates the (Rabilloud et al., 2002)

The presence of the Prx proteins sulphinic (protein 1a) in each of the 2D gels o Jurkat cell lysate samples demonstrates that the Jurkat E6.1 cells are being subjected to mild oxidative stress even before the treatment with SMX increase

Figure 4.1 The catalytic cycle of typical 2-Cys peroxiredoxins. 2-Cys Prxs are homodimers that reduce H_2O_2 in three main steps: peroxidation, resolut H_2O_2 is first reduced with a peroxidatic Cys to sulfenic acid (SOH) (1). The SOH then condenses with the resolving Cys of the other subunit to subsequently reduced by thioredoxin (TRX) recycling the homodime (3) (D'Autreaux & Toledano, 2007). In the event of excessive oxidation in enzyme can become overoxided (4), when a fraction of Cys-SOH or al SOH is further oxidized to sulphinic ($Cys-SO_2H$) or sulphonic acids ($Cys-SO_3H$) instead of forming disulphide. The formation of the sulphinic acid ($Cys-SO_2H$) is reversible by ATP-dependent reduction of $Cys-SO_2H$ by sulphiredoxin (SRX) (5) (D'Autreaux & Toledano, 2007). Additional oxidation of sulphinic acid ($Cys-SO_2H$) leads to the formation of the sulphonic acid ($Cys-SO_3H$) and the irreversible inactivatio enzyme (6). Image adapted from D'Autreaux and Toledano, 2007.

Figure 4.11

the Prx protein disulphide (protein 1) seen in the HIV-1-infected cell line suggests t
cell line is under enhanced oxidative stress compared to t
long established consequence of HIV infection (Buhl et al., 1989; Roederer, 1991).
The cell lines expressing Tat or HIV-1 deletion mutants showed also an apparent
decrease in the amount of protein 1 at every concentration of doxycycline
both the parental cell line and the HIV-1-infected there seems to
apparent decrease in the amount of the protein 1 between uninduced and ma
induced cells of the Tat-expressing cell lines. This suggests there was an inactivatio
the Prx enzyme, further compensation, oxidation of the peroxiredoxin and that
the expression of Tat or Tat deletion mutants resulted in additional ox
the Jurkat T cells.

The addition of SMX-HA to the different cell lines also lead to further
oxidation and inactivation of the peroxiredoxin enzyme in all the cell lines. A
comparison between the parent cell line, Jurkat E6.1, and
an apparent lower amount of the peroxiredoxin in the cells (Figure 4.7).
The treatment of the Tat-expressing cell lines with SMX-HA also resulted in th
inactivation of Prx and of an apparent decrease in the protein 1 compared
to the cells treated with the control (the parent). For the cell lines expres
Tat101, Tat72 and GFP-Tat, the decrease in the Prx spot after tr
HA looked to be more pronounced than that seen in the HIV-infected cells, though
perhaps roughly equivalent to that seen in the parent cel
(Figure 4.10). This was observed at every concentration of doxycycli
the HIV-infected cells are better able to compensate for the oxidative stres

SMX-HA than the cell lines expressing Tat1-72GFP. This is

probably due to two distinct factors; the first is the ability of HIV infection to u

the expression of the Prx1 protein in accordance with a ... Lane et al.

(2003). In that study the authors compared the gene expression in CD8+ T cells

of HIV seropositive bulk CD8+ T cells of seronegative individuals and found tha

Prx1 and Prx2 genes were expressed in greater quantity in bulk CD8+ T cells of HIV-

infected individuals. Additionally, they showed that these

amounts in the blood plasma of long-term non-progressors (LTNP) but not in persons

treated with ART or uninfected persons. They showed that these genes, no

regulated via oxidative stress, are up-regulated by stimulation with anti-CD3 in T

cells. As chronic immune activation of T cells is a consequence of untreat

infection, this could account for the elevated levels of P

HIV-infected individuals (Derdeyn & Silvestri, 2005). Furthermore, they showed

Jurkat T cells transfected with Prx1 and Prx2 DNA results in elevated levels of

Prx2 proteins that can inhibit HIV-1 replication. It has previously been shown that som

Prxs have antiviral activity against HIV-1 by decreasing free radical concen

their antioxidant enzyme function and down-regulating the NF-κB pathway, thereby

blocking transcription of HIV-1 (Matsuda et al., 1992; Okamoto et al., 1992)

A report by Mi et al. (2007) corroborated the finding about Prx2

showing significantly elevated serum levels of Prx2 from HIV-1 infected

individuals compared to their HIV-uninfected sexual partners and also comp

controls. Further RT-PCR analysis conducted on purified T cell subsets

from each cohort showed that Prx2 transcripts in CD8+ T cells and

to a lesser extent in the CD4+ T-cells. The reason for the apparent de
amount of the Prx disulphide after treatment of Tat-expressing cells with the SM
HA is that the HIV-1 Tat protein is able to suppress the expressio
as glutathione, a reduction in glutathione synthase or SOD, factors that wo
normally counteract the oxidative stress of SMX-HA (Flores et al., 1993; with
Westendorp et al., 1995b).

The HIV-infected cells showed the greatest number of protein spots wh
anticipated as this cell line expressed additional HIV proteins containing thiols such as
Vpr, CA, NC and PR that can potentially form disulphide bonds compared to the cell
lines expressing just Tat. The GFP cell line had the fewest protei
in the absence and presence of SMX-HA. This is probably due t
the fact that the variant of GFP used in this study is not k
thiol groups, although it could be potentially any antioxidant properties
overexpressed GFP possesses.

The "Tat-GFP" cell line consistently displayed more protein spots on the 2D
gels than the other Tat-expressing cell lines. This may be because of the
distribution of the Tat constructs that co-localised with SMX-HA, with the
exception of the aforementioned "Tat-GFP" construct, which is missing the basic d
(and thus the nuclear localisation signal, NLS). The other Tat proteins used in this
study have a predominantly nuclear distribution, whilst the Tat-GFP
construct expressed can be found in the nucleus, the loss of the NLS also means
readily detected throughout the cytoplasm. The intracellular distribution of
SMX-HA as determined by Manchanda et al. (2002) using fluorescent labelling and

confocal microscopy showed that the reactive metabolite was distributed cell cytoplasm. The anti-SMX antibody staining demonstrated that binding by p SMX-HA could be visualized inside the cell in vesicles near the cell membrane w five min of the cell incubation with SMX-HA. Sixty minutes after exposure to th metabolite, protein binding was visualized throughout the cytoplasm, concentrated discrete areas, but was observed in the nucleus. Thus, cytoplasmic localization of SMX-HA and "GFP" suggests that the GFP constructs might have a temporal advantage over the other constructs since the effects of exposed SMX-HA might accumulate more quickly due to its presence in the same compartment a addition, the reactive nature of SMX-HA suggests the majority of the introduced into the culture medium would be bound to cellular proteins in t cytoplasm before it is able to reach the nucleus.

The report by Manchanda et al. (2002) evaluated the cellular distribution of HA only at two time points, the longer of which was 1 h. Because treatment with S HA in this study was carried out for a period of 24 h, the cellular distribution showing SMX-HA binding to protein only in the cytoplasm may not be true at later time poin Indeed there have been studies showing that nuclear accu proteins can take up to several hours and, depending on the si h post-transfection (Konuma et al., 2009).

The nuclear envelope separates the nucleoplasm compartm thereby necessitating nucleocytoplasmic transport. All n imported from their site of synthesis in the cytoplasm whil the nucleus such as mRNA, need to be exported to the cy

exchange of small molecules and macromolecules occu
complexes (NPC), which are embedded in the nuclear enve
of material in two distinct modes: passive diffusion and fa
and Gorlich, 2001). Facilitated translocation can accommodate th
objects as large as several megadaltons and is often cou
energy that allows transport against a gradient of chemica
Gorlich, 2001). In contrast, passive diffusion does not require any specific
the diffusing species and components of the NPC, it is fa
macromolecules, but becomes increasingly inefficient as t
approaches a size limit of 20-40 kDa (Ribbeck and Gorlich, 2002). The delayed dynamics of pa
diffusion through the NPC could explain the quantitative and qualit
between the GFP-Tat expressing cells and the other cell lines. The fact that all the
Tat constructs have a presence in the nucleus would expla
the protein spots between the HA-Tat cell lines after SMX treatment.

The next step in this project would be to identify the
Tat-expressing cells after treatment with SMX and to characterize the re
sensitivities of the different protein targets. Future studies might also address
of oxidation on the function of the individual proteins. Baty et al. (2005) conducted a
similar study where they sought to determine whether cellular thiol oxidation of proteins
is selective or part of more global oxidative changes. They used a proteomic approach
identify thiol proteins that become oxidized with H_2O_2 in Jurkat T ce
that proteins with a wide variety of functions were oxidized. Of interest is the finding
the oxidation of GAPDH, a theme reinforced by previous s (Brennan et al., 2004;

Cummings et al 2004; Fratelli et al 2002). Given that GAPDH has a molecular weight of 36 kDA, there is no protein spot from the HA-SMX samples present in all the samples that matches this weight. This could be a reflection of the oxidizing agent used in the experiments as well as the concentration and duration of oxidant treatment. Other interesting proteins identified by Batey et al (2005) are moesin, heat shock protein 90², ubiquitin thiolesterase, all of which elo have estimated MW between 16 and 96 kDA according to their positions on the 2D gels. That is the same molecular weight range of proteins p, q, r and s that were unique to the "Tat-GFP"-expressing cell line after HA-SMX treatment. These proteins may be important as they are potential targets for Tat in Tat-GFP, the only Tat mutant with significant cytoplasmic distribution. Moesin is a protein involv cell structure, function as linkers between the plasma membran filaments. Heat shock protein 90² and ubiquitin thiolestera folding and degradation, respectively while elongation factor 2 is used in transl RNA processing. All four are proteins that primarily reside in the

Further proteomics research in the interactions betw cellular proteins can lead to increased understanding of the mechanisms behind the development of ADRs. One future direction of this study includes the use techniques such as mass spectrometry to isolate, purify ar the metabolite-HA-SMX in the context of Tat. It would also be benefic determine which proteins are involved in the formation of differences the expression of Tat. An example is the target expression

of Tat lead to mitochondrial proteins becoming a primary target for mixed disu bonds?

In summary, the results from these experiments suggest that expression of the HIV-1 Tat protein can have an effect on the amount of protein haptenation brought o by the reactive metabolite SMX-HA. However, the amount of SMX-HA induced haptenation may be less important than the specific cellular protein target distribution of the Tat protein may be a determining factor in specific protein targeting for haptenation which may contribute to cellular sensitivity to SMX-HA. The 2D experiments confirmed that SMX-HA is a potent oxidizing agent that is about the formation of mixed disulphides, bonds that could of proteins required for cellular homeostasis.

4.5 References

Akoumianaki, T., Kardassis, D., Polioudaki, H., Georgatos,
Theodoropoulos, P. A. (2009) Nucleocytoplasmic shuttling of sol
tubulin in mammalian cells. Journal of cell science 122, 1111-1118.

Baty, J. W., Hampton, M. B. & Winterbourn, C. C. (2005) Proteomic detection
of hydrogen peroxide-sensitive thiol proteins in Jurkat cells. The
Biochemical journal 389, 785-795.

Berthon, G., Brion, M. & Lambs, L. (1983) Metal ion-tetracycline interactions
biological fluids. 2. Potentiometric study of magnesium comple
tetracycline, oxytetracycline, doxycycline, and minocyc
of their possible influence on the bioavailability of the
plasma. Journal of inorganic biochemistry 19, 1-18.

Brennan, J. P., Wait, R., Begum, S., Bell, J. R., Dunn, M.
(2004) Detection and mapping of widespread intermolecu
disulfide formation during cardiac oxidative stress usi
diagonal electrophoresis. The Journal of biological chemistry 279, 41352-
41360.

Brion, M., Lambs, L. & Berthon, G. (1985) Metal ion-tetracycline interactions
biological fluids. Part 5. Formation of zinc complexes
some of its derivatives and assessment of their biological significance.
Agents and actions 17, 229-242.

Buhl, R., Jaffe, H. A., Holroyd, K. J., Wells, F. B., Mastran
Cantin, A. M. & Crystal, R. G. (1989) Systemic glutathione deficienc
symptom-free HIV-seropositive individuals. Lancet 2, 1294-1298.

Burns, F. R., Stack, M. S., Gray, R. D. & Paterson, C. A. (
purified collagenase from alkali-burned rabbit corneas. Investigative
ophthalmology & visual science 30, 1569-1575.

Campbell, G. R. & Loret, E. P. (2009) What does the structure-function
relationship of the HIV-1 Tat protein teach us about developing
vaccine? Retrovirology 6, 50.

Carr, A. & Cooper, D. A. (2000) Adverse effects of antiretroviral thera
356, 1423-1430.

Castagna, A., Le Grazie, C., Accordini, A., Giulidori, P., Cavalli, G
Bottiglieri, T. & Lazzarin, A. (1995) Cerebrospinal fluid S
adenosylmethionine (SAMe) and glutathione concentrat
infection: effect of parenteral treatment with SAMe. Neurology 45, 1678-
1683.

Chae, H. Z., Kim, H. J., Kang, S. W. & Rhee, S. G. (1999) Characterization of
three isoforms of mammalian peroxiredoxin that reduce
presence of thioredoxin. Diabetes research and clinical practice 45, 101-
112.

Cho, C. S., Lee, S., Lee, G. T., Woo, H. A., Choi, E. J. & Rhee, S. G. (
Irreversible inactivation of glutathione peroxidase 1 an
inactivation of peroxiredoxin II by H_2O_2 in red blood c
redox signaling 12, 1235-1246.

Choi, J., Liu, R. M., Kundu, R. K., Sangiorgi, F., Wu, W., Maxson, R. & Forman, H. J. (2000). Molecular mechanism of decreased gluta content in human immunodeficiency virus type 1 Tat-transgenic mice. The Journal of biological chemistry, 275, 3693-3698.

Cumming, R. C., Andon, N. L., Haynes, P. A., Park, M., Fischer, W. H Schubert, D. (2004). Protein disulfide bond formation in the cy during oxidative stress. The Journal of biological chemistry, 279, 21749-21758.

Dalle-Donne, I., Rossi, R., Colombo, G., Giustarini, D. & Milz A. (2009). Protein S-glutathionylation: a regulatory device from bacte Trends in biochemical sciences, 34, 85-96.

D'Autreaux, B. & Toledano, M. B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeosta Nature reviews, 8, 813-824.

De La Fuente, M., Miquel, J., Catalan, M. P., Victor, V. M. (2002). The amount of thiolic antioxidant ingestion neede several immune functions is higher in aged than in adu Free radical research, 36, 119-126.

Derdeyn, C. A. & Silvestri, G. (2005). Viral and host factors in the patho of HIV infection. Current opinion in immunology, 17, 366-373.

Divkovic, M., Pease, C. K., Gerberick, G. F. & Basketter, D Hapten-protein binding: from theory to practical application in of skin sensitization. Contact dermatitis, 53, 189-200.

Droge, W. & Holm, E. (1997). Role of cysteine and glutathione in HIV and other diseases associated with muscle wasting and immunologic dysfunction. Faseb J, 11, 1077-1089.

Eaton, P. (2006). Protein thiol oxidation in health and disease: te measuring disulfides and related modifications in comp mixtures. Free radical biology & medicine, 40, 1889-1899.

Flores, S. C., Marecki, J. C., Harper, K. P., Bose, S. K., N McCord, J. M. (1993). Tat protein of human immunodeficiency v 1 represses expression of manganese superoxide dismu cells. Proceedings of the National Academy of Sciences of the U States of America, 90, 7632-7636.

Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberin Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E., Ba Vandekerckhove, J., Gianazza, E. & Ghezzi, P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidat human T lymphocytes. Proceedings of the National Academy of of the United States of America, 99, 3505-3510.

Geiben-Lynn, R., Kursar, M., Brown, N. V., Addo, M. M., Shau, H., Lieberman, J., Luster, A. D. & Walker, B. D. (2003). HIV-1 antiviral activity of recombinant natural killer cell enhancing fa NKEF-A and NKEF-B, members of the peroxiredoxin family. The Journal of biological chemistry, 278, 1569-1574.

Golub, L. M., McNamara, T. F., D'Angelo, G., Greenwald, R
Ramamurthy, N. S. (1987). A non-antibacterial chemically-modified tetracycline inhibits mammalian collagenase activity. *Journal of dental research*, 66, 1310-1314.

Griffin, M. O., Fricovsky, E., Ceballos, G. & Villarreal, F. (2010). Tetracyclines: a pleitropic family of compounds with promising therap
Review of the literature. *American journal of physiology. Cell physiology*, 299, C539-548.

Gulow, K., Kaminski, M., Darvas, K., Suss, D., Li-Weber, M. & Krammer, P. H. (2005). HIV-1 trans-activator of transcription substitutes for
signaling in activation-induced T cell death. *J Immunol*, 174, 5249-5260.

Hall, A., Karplus, P. A. & Poole, L. B. (2009). Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. *The FEBS journal*, 276, 2469-2477.

Hanson, G. T., Aggeler, R., Oglesbee, D., Cannon, M., Cap
R. Y. & Remington, S. J. (2004). Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *The Journal of biological chemistry*, 279, 13044-13053.

Huigen, M. C., Kamp, W. & Nottet, H. S. (2004). Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection. *European journal of clinical investigation*, 34, 57-66.

Johri, M. K., Mishra, R., Chhatbar, C., Unni, S. K. & Singh
(2011). Tits and bits of HIV Tat protein. *Expert opinion on biological therapy*, 11, 269-283.

Kalantari, P., Narayan, V., Henderson, A. J. & Prabhu, K. S. (2009). 15-Deoxy-Delta12,14-prostaglandin J2 inhibits HIV-1 transactivating protein, Tat, through covalent modification. *Faseb J*, 23, 2366-2373.

Kalantari, P., Narayan, V., Natarajan, S. K., Muralidhar, K
Vunta, H., Henderson, A. J. & Prabhu, K. S. (2008). Thioredoxin reductase-1 negatively regulates HIV-1 transactivating protein Tat-dependent transcription in human macrophages. *The Journal of biological chemistry*, 283, 33183-33190.

Koken, S. E., Greijer, A. E., Verhoef, K., van Wamel, J., B
Berkhout, B. (1994). Intracellular analysis of in vitro modified
protein. *The Journal of biological chemistry*, 269, 8366-8375.

Lambs, L., Brion, M. & Berthon, G. (1984). Metal ion-tetracycline interactions in biological fluids. Part 3. Formation of mixed-metal ternary complexes of tetracycline, oxytetracycline, doxycycline and minocyc
and magnesium, and their involvement in the bioavaila
antibiotics in blood plasma. *Agents and actions*, 14, 743-750.

Lazarou, J., Pomeranz, B. H. & Corey, P. N. (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studi
Jama, 279, 1200-1205.

Liebler, D. C. (2008). Protein damage by reactive electrophiles: tar
consequences. *Chemical research in toxicology*, 21, 117-128.

Manchanda, T., Hess, D., Dale, L., Ferguson, S. G. & Ried
Haptenation of sulfonamide reactive metabolites to cel
Molecular pharmacology, 62, 1011-1026.

Masutani, H., Naito, M., Takahashi, K., Hattori, T., Koito, A., Takatsuki, K
Go, T., Nakamura, H., Fujii, S., Yoshida, Y. & et al. (1
Dysregulation of adult T-cell leukemia-derived factor (ADF)/thioredox
HIV infection: loss of ADF high-producer cells in lymphoid tissues
patients. *AIDS research and human retroviruses* 8, 1707-1715.

Mehta, U., Durrheim, D. N., Blockman, M., Kredo, T., Goun
K. I. (2008). Adverse drug reactions in adult medical inpatie
African hospital serving a community with a high HIV/AIDS prevalence
prospective observational study. *British journal of clinical pharmac* 65, 396-406.

Misse, D., Yssel, H., Trabattoni, D., Oblet, C., Lo Caputo,
Pene, J., Gonzalez, J. P., Clerici, M. & Veas, F. (2007). IL-22
participates in an innate anti-HIV-1 host-resistance network through ac
phase protein induction. *J Immunol* 178, 407-415.

Okamoto, T., Ogiwara, H., Hayashi, T., Mitsui, A., Kawabe,
(1992). Human thioredoxin/adult T cell leukemia-derived factor activates
the enhancer binding protein of human immunodeficien
thiol redox control mechanism. *International immunology* 4, 811-819.

Peskin, A. V., Low, F. M., Paton, L. N., Maghzal, G. J., Hampton, M. B. &
Winterbourn, C. C. (2007). The high reactivity of peroxiredoxin 2
H(2)O(2) is not reflected in its reaction with other oxi
reagents. *The Journal of biological chemistry* 282, 11885-11892.

Pirmohamed, M. (2007). *Drug Hypersensitivity*. Basel: Karger.

Pirmohamed, M. & Park, B. K. (2001). HIV and drug allergy. *Current opinion in allergy and clinical immunology* 1, 311-316.

Pocernich, C. B., Sultana, R., Mohmmad-Abdul, H., Nath, A. & Butterfiel
A. (2005). HIV-dementia, Tat-induced oxidative stress, and antiox
therapeutic considerations. *Brain research* 50, 14-26.

Price, T. O., Uras, F., Banks, W. A. & Ercal, N. (2006). A novel antioxidant N
acetylcysteine amide prevents gp120- and Tat-induced oxidative stress in
brain endothelial cells. *Experimental neurology* 201, 193-202.

Rabilloud, T., Heller, M., Gasnier, F., Luche, S., Rey, C.,
Benahmed, M., Louisot, P. & Lunardi, J. (2002). Proteomics analysis of
cellular response to oxidative stress. Evidence for in vivo overoxidati
peroxiredoxins at their active site. *The Journal of biological chemist* 277, 19396-19401.

Ribbeck, K. & Gorlich, D. (2001). Kinetic analysis of translocation thro
nuclear pore complexes. *The EMBO journal* 20, 1320-1330.

Rieder, M. J. (2009). Immune mediation of hypersensitivity adverse
reactions: implications for therapy. *Expert opinion on drug safety* 8, 331-343.

Roederer, M., Ela, S. W., Staal, F. J., Herzenberg, L. A. &
(1992). N-acetylcysteine: a new approach to anti-HIV therapy. *AIDS research and human retroviruses* 8, 209-217.

Roederer, M., Staal, F. J., Osada, H., Herzenberg, L. A. &
(1991). CD4 and CD8 T cells with high intracellular glutat

selectively lost as the HIV infection progresses. International immunology 3, 933-937.

Romani, B., Engelbrecht, S. & Glashoff, R. H. (2010) Functions of Tat: the versatile protein of human immunodeficiency virus type... The Journal of general virology 91, 1-12.

Ryan, M. E., Usman, A., Ramamurthy, N. S., Golub, L. M. & G... (2001) Excessive matrix metalloproteinase activity in dia... by tetracycline analogues with zinc reactivity. Current medicinal chemist... 8, 305-316.

Seo, M. S., Kang, S. W., Kim, K., Baines, I. C., Lee, T. H. & Rhee, ... Identification of a new type of mammalian peroxiredoxi... intramolecular disulfide as a reaction intermediate. The Journal of biological chemistry 275, 20346-20354.

Sommer, A. & Traut, R. R. (1974) Diagonal polyacrylamide-dodecyl sulfate gel electrophoresis for the identification of ribosomal prot... methyl-4-mercaptobutyrimidate. Proceedings of the National Acade... Sciences of the United States of America 71, 3946-3950.

Staal, F. J., Ela, S. W., Roederer, M., Anderson, M. T., He... Herzenberg, L. A. (1992) Glutathione deficiency and human immunodeficiency virus infection. Lancet 339, 909-912.

Staal, F. J., Roederer, M., Israelski, D. M., Bubp, J., Mole, L. A., McShane, D., ... Deresinski, S. C., Ross, W., Sussman, H., Raju, P. A. ... Intracellular glutathione levels in T cell subsets decre... HIV-infected individuals. AIDS research and human retroviruses 8, 305-311.

Stehbens, W. E. (2004) Oxidative stress in viral hepatitis and AIDS... Experimental and molecular pathology 77, 121-132.

Strathdee, C. A., McLeod, M. R. & Hall, J. R. (1999) Efficient control of tetracycline-responsive gene expression from an autoregula... bi-directional expression vector. Gene 229, 21-29.

Takahashi, M., Altschmied, L. & Hillen, W. (1986) Kinetic and equilibrium characterization of the Tet repressor-tetracycline complex by fluoresc... measurements. Evidence for divalent metal ion require... energy transfer. Journal of molecular biology 187, 341-348.

Turchan-Cholewo, J., Dimayuga, F. O., Gupta, S., Keller, J. N... Hauser, K. F. & Bruce-Keller, A. J. (2009) Morphine and HIV-Tat increase microglial-free radical production and oxidative stres... role in cytokine regulation. Journal of neurochemistry 108, 202-215.

van der Ven, A. J., Koopmans, P. P., Vree, T. B. & van der ... Adverse reactions to co-trimoxazole in HIV infection. Lancet 338, 431-433.

Wagner, E., Luche, S., Penna, L., Chevallet, M., Van Dorsselaer, A... Wagner, E. & Rabilloud, T. (2002) A method for detection of overoxidation of cysteines: peroxiredoxins are oxidized... active-site cysteine during oxidative stress. The Biochemical journal 366, 777-785.

Westendorp, M. O., Shatrov, V. A., Schulze-Osthoff, K., Frank, R., Kraft, ... Los, M., Krammer, P. H., Droge, W. & Lehmann, V. (19...

potentiates TNF-induced NF-kappa B activation and cytotoxicity b
altering the cellular redox state. The EMBO journal, 14, 546-554.

Woo, H. A., Kang, S. W., Kim, H. K., Yang, K. S., Chae, H.
(2003). Reversible oxidation of the active site cysteine of
cysteine sulfinic acid. Immunoblot detection with antib
hyperoxidized cysteine-containing sequence. The Journal of biological
chemistry, 278, 47361-47364.

Yang, K. S., Kang, S. W., Woo, H. A., Hwang, S. C., Chae,
Rhee, S. G. (2002). Inactivation of human peroxiredoxin I duri
as the result of the oxidation of the catalytic site cyst
sulfinic acid. The Journal of biological chemistry, 277, 38029-38036.

CHAPTER 5: Discussion and Conclusion

5.1 Discussion and Conclusions

Prior to 1998, 7 antiretroviral drugs were available for the treatment of HIV infection and therapy consisted of treating opportunistic diseases ar arising from immunodeficiency (Fischl et al., 1987). In that environment, the antimicrobials used for treatment or prophylaxis against o the most attention as the most common drugs responsible for hypersens AIDS patients (Carr & Cooper, 1995). Cotrimoxazole, used to treat Pneumocystis jirovecii pneumonia was the most commonly cited drug, but a significan incidence of hypersensitivity was observed with other sul antimicrobials (Carr & Cooper, 1995; Coopman et al., 1993). For reasons which we unclear, the rate of ADRs among people with HIV infection, notably when they h progressed to clinical AIDS was elevated over that of the general population

The advent of antiretroviral therapy (ART) almost a d drastically changed the course of HIV infection in North America from a rapidly fatal o to one which is now a chronic disease (Pirmohamed, 2007). The use of ART has a resulted in a change in the epidemiology of ADRs as immune reconstitution mea need for prophylactic therapy of opportunistic diseases is reduced and subsequently, th problems associated with it have decreased (Pirmohamed, 2007). As well, among patients with controlled viral loads, the rate of A appears to be much closer to that of the general population. However, ADRs to antimicrobial dr have been replaced by ADRs to antiretroviral agents inclu protease inhibitors as well as anticonvulsants and other drugs used to treat the symptom of HIV-associated dementia (Carr & Cooper, 2000; Pirmohamed & Park,

In the general population ADRs are responsible for 3% of all hospit and occur in 10-20% of hospital inpatients (Lazarou et al, 1998). They are a major pub health problem due to their frequency, potential for major morbi also the impact they have on the development (Rieder et al., 2009) of the developing world, disease prevalence, access to medications, d drug management are markedly different from those of developed countries, impacting on the incidence and nature of ADRs in these countries. One of the first studies to determine incidence and nature of ADRs in Sub-Saharan Africa during t HIV/AIDS pandemic, Mehta et al (2008) found the incidence of ADRs in their study population to be 14%. South Africa has one of the highest HIV prevalence rates in t world with approximately 12% of the total population-infected 60% of those individuals on ART. In their study, Mehta et al (2008) observed that ADRs to antiretroviral drugs were more frequently reported than in de and severely-ill HIV-infected individuals, who were receiving antiretrovirals, frequently developed ADRs to drugs used to treat opportunistic infections.

Cotrimoxazole is associated with hypersensitivity ADRs in 1-3% of the HIV negative population (van der Ven et al, 1991). In HIV-positive patients, ADRs oc 30% of patients at prophylactic doses and 50% of patients at (Carr & Cooper, 1995). Cotrimoxazole is a combination agent composed of sulphonamide sulphamethoxazole (SMX). SMX is believed to be the aetiologic for the hypersensitivity ADRs to cotrimoxazole and thus represents an ideal model for investigating the mechanisms of

hypersensitivity DRs in HIV-positive patients given our considerable knowled
its disposition and metabolism (Pirmohamed, 2007).

The research embodied in this thesis was carried out to assess the contributio
HIV-1 Tat to Jurkat T cell toxicity induced by the reactive sulphonamide metabolite,
SMX-HA. To study these issues, we used a vector system to differentially induce t
expression of Tat and then to analyze the effects of treatment with SMX-HA. The data
in Chapter 2 demonstrate that the expression of the first exon of Tat in transfected
Jurkat T cells produced a cooperative effect on SMX-HA toxicity, an effect dependent o
the level of Tat expression. We also demonstrated that apoptosis was the primary
mechanism of cell death, that the expression of Tat had no effect on cellular
GSH/GSSG concentrations. We concluded that the first exon of Tat wa
affect the redox state of Jurkat T cells. We concluded that oxidative str
primary mechanism driving cell death.

After obtaining full-length Tat protein from different viral isolates, we
constructed deletion mutants in an effort to determine the
was responsible for the toxicity enhancement that was observed in Chapter 2. Full-length Tat
and the deletion mutants were stably transfected into Jurkat T cells and these cell lines
were used in cell viability and ROS assays, as described in Chapter 3. The cell viability
assays showed that differential Tat expression did not have a significant effect on SMX-
HA-induced cell toxicity. However, the ROS assay showed that
expression of full-length Tat protein increased the ROS generated by t
Jurkat T cells with SMX-HA, consistent with a pro-oxidant role. Of significance,
the deletion mutants had a similar effect to that of full-length Tat

protein is required for increased ROS in Jurkat cells. Previous reports have shown that the Tat mutants (Tat72 and Tat86) augment ROS production in cultured cells, however this was observed only with the addition of recombinant extracellular Tat protein to the culture (Aksenov et al., 2006; Gu et al., 2005; Liu et al., 2005).

Evaluation of the consequences of the oxidative stress induced by SMX-HA was studied in Chapter 4 by analyzing changes to the redox proteome. Redox 2-dimensional (2D) electrophoresis of lysates of Tat-expressing cell lines produced numerous spots that arose from proteins with intermolecular disulphide bonds. One of the proteins that was highly expressed was the peroxiredoxin enzyme, the apparent intensity of which illustrated that HIV-infected cells were under less oxidative stress than the Tat-expressing cell lines without exposure to SMX-HA. Exposure of the cell lines to SMX-HA dramatically increased the oxidation of cysteine thiols in proteins in the redox 2D gels.

A major obstacle to the eradication of HIV-1 is the persistence of the virus in reservoirs. In patients that have not been treated with ARVs, 90% of plasma virus is mainly generated by repeated cycles of de novo infection of activated CD4+ T cells (Coiras et al., 2009). The residual virus due to viral replication from cellular reservoirs produces HIV-1 at lower rates, e.g. resting T cells, infected dendritic cells and macrophages, which are more resistant to virus-induced cytopathic effects, and anatomical sites with low pharmacological activity for ARVs, due to the inability of the ARVs to penetrate tissues such as the central nervous system (Alexaki et al., 2008; Chun et al., 2008; Coiras et al., 2009; Gupta et al., 2006; Ruiz et al., 1999).

Ongoing viral replication in CD4+ T cells cannot be fully excluded in HIV-1 infection, even in patients undergoing antiretroviral therapy. Consequently, HIV-1 Tat protein is continuously produced and its basic domain allows the protei infected cells and enter uninfected bystander cells. When Frankel and Pabo (198 demonstrated this unique property of Tat, their results su was followed by translocation into the nucleus in transcriptionally active form determined that it took 6 h for approximately 80% of the in in the nuclear compartment. This means there is a substantial portio cytoplasm for up to 6 h. Furthermore, the basic domain allows Tat to including the heart, liver, lung, spleen, kidney, brain and s (Schwarze et al., 1999).

Therapy with SMX produces a system wide distribution of the drug and while the liver is quantitatively the primary site of bioactivation fo also been shown to occur in other organs, including lung and cells of the ski immune system (Cribb et al., 1990; Vyas et al., 2006). The cytoplasmic distributi the Tat-GFP cell line was likely responsible, in part, for the increased sensit of the Jurkat CD4+ T cells to SMX-HA treatment (Chapter 3). The 2D electroph of Jurkat CD4+ T cells expressing Tat-GFP and treated with SMX-HA unique protein spots that were probably due to the cytoplasmic distribution of Tat-GFP. The Tat fusion protein invokes a scenario where full-length (wild type) Tat protein is released from HIV infected cells, enters uninfected cells and localizes to the cytop where, in the presence of SMX-HA, it lowers the threshold to apoptosis leading t increased cell death of both the HIV-infected CD4+ T cells and of uninfect

various types (Figure 5.1). The loss of the CD4+ T cell compartment h immune deficiencies for, but that HIV could potentially also lead ADRs due to the loss of the regulatory CD4+ CD25+ T cel mirror the situation described by Kish et al (2007) for contact hypersensitiv Contact hypersensitivity also known as allergic contact dermatitis (ACD) hypersensitivity ADR. It is a common inflammatory skin dis to repeated skin exposure to non-protein chemicals, or haptens 2,4-dinitrofluorobenzene (DNFB) and oxazolone (Oxa) (Vocanson et al, 2009). CHS responses are mediated by IFN-γ and IL-17 producing CD8+ T cells primed b dendritic cells - mostly hapten-presenting Langerhans cells (LC) migrating skin to the draining lymph nodes (Gorbachev & Fairchild). Although CD4+ T cells not required to mediate the CHS response, either as ef regulatory CD4+CD25+ T cells have been found to restrict specific CD8+ T cell expansion for CHS responses (Kish et al, 2007). One of the major differe between CHS and hypersensitivity ADRs induced by SMX nature of the effects caused by SMX involving multiple organs in contrast to the area of skin that is sensitized by the allergen in CHS. Thi the severity of the SMX induced reactions. The wide spread distribution of increases the probability of Tat being in proximity to SMX-HA, raising the cellular toxicity and increasing the incidence of ADRs in th Preliminary data from experiments looking at the externalization of apoptosis show that the cell death observed in the GFP and Tat48GFP cell lin the presence of SMX-HA is mediated via apoptosis. This is in keepi

Figure 5.1 The model for increased incidence of ADRs in HIV pati
the SMX dose is metabolized to SMX-HA and then SMX-NO which are both able to
haptenate cellular proteins. The haptenated protein is then processed by an
antigen presenting cell (APC), leading to recognition by a T cell receptor and immune
response. During HIV infection, Tat is released by infected
of uninfected cells where it changes the disulfide proteom
for cell death. In the presence of SMX-HA, this could lead to increased antigen
presentation to CD8+ T cells and the development of an ADR to SMX.

Figure 5.1

found in Ade et al. (2009).

Another consequence of increased apoptosis in the proposed model (Figure 5.1) is that these apoptotic cells can be a source of immunologic may influence subsequent immune responses. Specifically, it has been shown t derived from phagocytosed dying or dead cells may be processed by professional antigen presenting cells (APCs) to generate MHC I-restricted peptides that are then activation of CD8+ T cells (Shen et al., 2006). Classically, MHC I molecules antigens synthesized within the cell to CD8+ T cells, whil via endocytic uptake are loaded onto MHC II molecules for The process by which APCs acquire and process exogenous antigens into the pathway for presentation to CD8+ T-cells is known as cross-presentation, and th resulting stimulation of CD8+ T cell responses in vivo is termed cross-priming (Rock & Shen, 2005)

It is not clear exactly how antigens from apoptotic cells are released and acqui by the APCs, though there is now evidence that the im presentation to monitor tissues for foreign antigens or tumors from viruses. There is also evidence that in individuals with chronic HIV infecti T cells seem to activate a wide repertoire of CD8+ T cells epitopes in vivo (Rawson et al., 2007). Indeed the magnitude of the CD8+ T directed against apoptotic-derived peptides correlated with the decline CD4+ T cells. Rawson et al. (2007) suggest that the presence of these CD8+ HIV-infected individuals with depleted CD4+ T cells and the decreased CD4+ T cell help may be due to the capacity of dendritic cells to be independently stimula

variety of signals including apoptotic T cells expressing the CD40 ligand (C

(Propato et al., 2001). Dead or dying cells used as a source of antigens for cross-

presentation is not limited to CD4+ T cells, antigen derived from intern

apoptotic cells has been shown for models of class I-restricted, antigen

cells that support HIV infection and are permissive for Tat expression (Alberts, 2004;

Fleeton et al., 2004; Marten et al., 2003). In the model of ADRs in HIV p

(Figure 4.5), the metabolism of SMX leads to the formation of SMX-HA and S

both shown to haptenate to cellular proteins in T cells (Manchanda et al., (C

2002) and most likely in other cell types. The cellularity of these haptenated cel

to the presentation of SMX-related antigens and an immune response man

clinically as an ADR to SMX.

5.2 Future studies

One of the major objectives of this thesis was to build upon the work pub

Adeyanju et al. (2009), specifically to ascertain the region of the HIV-1 Tat protein of t

needed to amplify the cell toxicity caused by HA-SMX. The HIV-1 Tat used in that

report and the experiments in Chapters 3 and 4 were from two different sources

result had amino acid sequence differences. It would help clarify

the cell viability data in Adeyanju et al. (2009), Chapter 2 and the results describ

Chapter 4 if the two Tat variants were compared in HIV-1 LTR-Luc (HIV-1 long terminal

repeat luciferase) reporter assays. The transcriptional activity assay would determin

of the different Tat proteins. Regardless of any potential differences in

two Tat variants, the form of cell death appears to remain the same.

We have determined that the stable transfection of the HIV-1 Tat protein only
mutant that retains the protein transduction domain (PTD
nuclear localization sequence (NLS) perhaps exclusively, to distribu
protein in the nucleus. Indeed the results from the fluorescence micro
experiments suggest that these proteins (Tat101GFP, Tat8
ventured out of the nucleus to impact other organelles s. To
biochemically verify these results, we could carry out mitoch
subfractionation of cell extracts by differential centrifugation followed by PAGE
and immunoblotting with anti-Tat and/or GFP antibody.

The data in Chapters 2 and 3 showed that the stably transfected Jurkat T cell
lines showed a decrease in cell viability when treated with
HA. However, in the Jurkat cell line expressing Tat101GFP, the cells had a
small additive effect on cell viability when treated with SMX-HA, but no significant
effect with the expression of any of the other Tat mutants, Tat86GFP, Tat72GFP,
Tat48GFP" or GFP (Chapter 3). It would be interesting to determine if
differences in cell viability between the full-length Tat protein and the deletio
they were applied to the T cell culture, both in the presence of SMX-HA. In
other words, determine what effect extracellular Tat has on a T c
without SMX-HA. The extracellular application of Tat and its de
also mean that the number of Tat molecules in a particula
this amount could then be multiplied many fold to determine wha
expression of Tat might have. The use of the deletion m
particular region of Tat had a synergistic effect on T cell viabil

Another potential area for research is to evaluate the cytokine profile produced by the incubation of peripheral blood mononuclear ce
protein first followed by SMX-HA. A similar study by Ho et al. (2001) showed that SMX-HA reduced mitogen-stimulated PBMC production of th
inflammatory cytokines IL-1 and TNF-α. The authors also found no change in IFN-γ production, but a significant decrease in IL-4 production, leading them to conclude
PBMC exposed to SMX-HA could lead to a heightened T_H1 immune response. A T_H1 immune response is thought to account for some of the characteris
hypersensitivity ADRs such as delayed onset and fever. Given that Tat
monocytes to produce pro-inflammatory T_H1 cytokines, including IL-1, IL-6 and TNF-α (Chen et al., 1997; Nath et al., 1999), such an experiment would establish if the add
of the HIV-1 protein could lead to a more robust T_H1 response. This might account, at least in part, for the higher incidence of hypersensitivity ADRs in HIV-infected patients

5.3 Conclusions

Drug hypersensitivity ADRs contribute substantially to patient m
mortality, especially in HIV-infected patients. They are a major public health pro
developed countries and increase the burden and cost of
overstretched healthcare system. The mechanisms underlying the development of hypersensitivity ADRs are complex, but ill-defined. Studies have shown a contribution of the Tat protein in the HIV populat
this thesis lay the groundwork for understanding the role played by the cytoplasmic distribution of Tat in the sensitivity of CD4+ T cells to the reactive sulphonamide metabolite, SMX-HA.

5.4 References

Adeyanju, K., Krizova, A., Gilbert, P. A., Dekaban, G. A. &
HIV Tat potentiates cell toxicity in a T cell model for s
induced adverse drug reactions. *Virus genes* 37, 273-282.

Aksenov, M. Y., Aksenova, M. V., Nath, A., Ray, P. D., Mac
Booze, R. M. (2006) Cocaine-mediated enhancement of Tat toxicity in rat
hippocampal cell cultures: the role of oxidative stress
receptor. *Neurotoxicology* 27, 217-228.

Albert, M. L. (2004) Death-defying immunity: do apoptotic cells influ
antigen processing and presentation? *Nat Rev Immunol* 4, 223-231.

Alexaki, A., Liu, Y. & Wigdahl, B. (2008) Cellular reservoirs of HIV-1 and their
role in viral persistence. *Current HIV research* 6, 388-400.

Bailey, J. R., Sedaghat, A. R., Kieffer, T., Brennan, T., Le
Rotolo, M., Haggerty, C. M., Kamireddi, A. R., Liu, Y., Lee, J.
D., Gallant, J. E., Cofrancesco, J., Jr., Quinn, T. C., W
S. C., Siliciano, J. D., Nettles, R. E. & Siliciano, R. F
human immunodeficiency virus type 1 viremia in some
antiretroviral therapy is dominated by a small number
rarely found in circulating CD4+ T cells. *Journal of virology* 80, 6441-6457.

Carr, A. & Cooper, D. A. (1995) Pathogenesis and management of HIV-
associated drug hypersensitivity. *AIDS clinical review*, 65-97.

Carr, A. & Cooper, D. A. (2000) Adverse effects of antiretroviral thera
Lancet 356, 1423-1430.

Chen, P., Mayne, M., Power, C. & Nath, A. (1997) The Tat protein of HIV-1
induces tumor necrosis factor-alpha production. Implications for HIV-1-
associated neurological diseases. *The Journal of biological chemistry* 272,
22385-22388.

Chun, T. W., Nickle, D. C., Justement, J. S., Meyers, J. H.
Hallahan, C. W., Kottilil, S., Moir, S., Mican, J. M., Mu
D. J., Kovacs, J. A., Mannon, P. J. & Fauci, A. S. (200
HIV in gut-associated lymphoid tissue despite long-term antiretroviral
therapy. *The Journal of infectious diseases* 197, 714-720.

Coiras, M., Lopez-Huertas, M. R., Perez-Olmeda, M. & Alcami, J. (2009).
Understanding HIV-1 latency provides clues for the eradicatio
term reservoirs. *Nature reviews* 7, 798-812.

Coopman, S. A., Johnson, R. A., Platt, R. & Stern, R. S. (1
disease and drug reactions in HIV infection. *The New England journal of
medicine* 328, 1670-1674.

Cribb, A. E., Miller, M., Tesoro, A. & Spielberg, S. P. (199
dependent oxidation of sulfonamides by monocytes and
humans and mice. *Molecular pharmacology* 38, 744-751.

Eckstein, D. A., Penn, M. L., Korin, Y. D., Scripture-Adams, D. D., Zack, J. A.
Kreisberg, J. F., Roederer, M., Sherman, M. P., Chin,
Goldsmith, M. A. (2001) HIV-1 actively replicates in naive CD4(+
residing within human lymphoid tissues. *Immunity* 15, 671-682.

Fischl, M. A., Richman, D. D., Grieco, M. H., Gottlieb, M. S
A., Laskin, O. L., Leedom, J. M., Groopman, J. E., Mil
Schooley, R. T. & et al. (1987). The efficacy of azidothymidine (AZ
the treatment of patients with AIDS and AIDS-related complex. A double-
blind, placebo-controlled trial. The New England journal of medicin 317,
185-191.

Fleeton, M. N., Contractor, N., Leon, F., Wetzel, J. D., De
Kelsall, B. L. (2004). Peyer's patch dendritic cells process viral antigen
from apoptotic epithelial cells in the intestine of reovirus-infected mice. The
Journal of experimental medicine 200, 235-245.

Frankel, A. D. & Pabo, C. O. (1988). Cellular uptake of the tat protein fr
human immunodeficiency virus. Cell 55, 1189-1193.

Gorbachev, A. V. & Fairchild, R. L. CD4+CD25+ regulatory T cells utiliz
as a mechanism to restrict DC priming functions in cut
responses. European journal of immunology 40, 2006-2015.

Guadalupe, M., Sankaran, S., George, M. D., Reay, E., Verhoeven, D
Shacklett, B. L., Flamm, J., Wegelin, J., Prindiville, T
(2006). Viral suppression and immune restoration in the g
mucosa of human immunodeficiency virus type 1-infected patients
initiating therapy during primary or chronic infection. Journal of virology 80,
8236-8247.

Gulow, K., Kaminski, M., Darvas, K., Suess, D., Li-Weber, M. & Krammer, P.
H. (2005). HIV-1 trans-activator of transcription substitutes for
signaling in activation-induced T cell death. J Immunol 174, 5249-5260.

Hess, D. A., O'Leary, E. F., Lee, J. T., Almawi, W. Y., Mad
M. J. (2001). Inhibition of cytokine production and interfere
receptor-mediated Jak-Stat signaling by the hydroxylamine metabolite o
sulfamethoxazole. Faseb J 15, 1855-1857.

Kish, D. D., Gorbachev, A. V. & Fairchild, R. L. (2005). CD8+ T cells produce
IL-2, which is required for CD(4+)CD25+ T cell regulatio
T cell development for contact hypersensitivity responses. Journal of
leukocyte biology 78, 725-735.

Kish, D. D., Gorbachev, A. V. & Fairchild, R. L. (2007). Regulatory function of
CD4+CD25+ T cells from Class II MHC-deficient mice in contact
hypersensitivity responses. Journal of leukocyte biology 82, 85-92.

Lazarou, J., Pomeranz, B. H. & Corey, P. N. (1998). Incidence of adverse drug
reactions in hospitalized patients: a meta-analysis of prospective studi
Jama 279, 1200-1205.

Liu, K., Chi, D. S., Li, C., Hall, H. K., Milhorn, D. M. & K
(2005). HIV-1 Tat protein-induced VCAM-1 expression in human
pulmonary artery endothelial cells and its signaling. American journal of
physiology 289, L252-260.

Mandron, M., Martin, H., Bonjean, B., Lule, J., Tartour, E.
(2008). Dendritic cell-induced apoptosis of human cytomegalovi
infected fibroblasts promotes cross-presentation of pp65 to CD8+ T
The Journal of general virology 89, 78-86.

Mehta, U., Durrheim, D. N., Blockman, M., Kredo, T., Goun
K. I. (2008). Adverse drug reactions in adult medical inpatie
African hospital serving a community with a high HIV/A
prospective observational study. British journal of clinical pharmac
65, 396-406.

Nath, A., Conant, K., Chen, P., Scott, C. & Major, E. O. (1999). Transient
exposure to HIV-1 Tat protein results in cytokine production
macrophages and astrocytes. A hit and run phenomenon. The Journal of
biological chemistry, 274, 17098-17102.

Pirmohamed, M. (2007). Drug Hypersensitivity. Basel: Karger.

Pirmohamed, M. & Park, B. K. (2001). HIV and drug allergy. Current opinion in
allergy and clinical immunology, 1, 311-316.

Propato, A., Cutrona, G., Francavilla, V., Ulivi, M., Schiaff
Dunbar, R., Cerundolo, V., Ferrarini, M. & Barnaba, V. (2001).
Apoptotic cells overexpress vinculin and induce vinculin-specific cytotoxic
T-cell cross-priming. Nature medicine, 7, 807-813.

Rawson, P. M., Molette, C., Videtta, M., Altieri, L., France
T., Finocchi, L., Propato, A., Paroli, M., Meloni, F., Mas
d'Ettorre, G., Sidney, J., Sette, A. & Barnaba, V. (200
presentation of caspase-cleaved apoptotic self antigens in HIV
Nature medicine, 11, 1431-1439.

Rieder, M. J. (2009). Immune mediation of hypersensitivity adverse
reactions: implications for therapy. Expert opinion on drug safety, 8, 331-
343.

Rock, K. L. & Shen, L. (2005). Cross-presentation: underlying mechanism
role in immune surveillance. Immunological reviews, 207, 166-183.

Ruiz, L., van Lunzen, J., Arno, A., Stellbrink, H. J., Schne
Castella, E., Ojanguren, I., Richman, D. D., Clotet, B., Tenner-Racz, K.
& Racz, P. (1999). Protease inhibitor-containing regimens compared
nucleoside analogues alone in the suppression of persistent HIV-1
replication in lymphoid tissue. AIDS (London, England), 13, F1-8.

Schwarze, S. R., Ho, A., Vocero-Akbani, A. & Dowdy, S. F. (1999). In vivo
protein transduction: delivery of a biologically active p
mouse. Science (New York, N.Y.), 285, 1569-1572.

Uhl, M., Kepp, O., Jusforgues-Saklani, H., Vicencio, J. M., Kroemer,
Albert, M. L. (2009). Autophagy within the antigen donor cell f
efficient antigen cross-priming of virus-specific CD8+ T cells. Cell death
and differentiation, 16, 991-1005.

van der Ven, A. J., Koopmans, P. P., Vree, T. B. & van der
Adverse reactions to co-trimoxazole in HIV infection. Lancet, 338, 431-433.

Vocanson, M., Hennino, A., Rozieres, A., Poyet, G. & Nicolas, J. F. (2009
Effector and regulatory mechanisms in allergic contact
64, 1699-1714.

Vyas, P. M., Roychowdhury, S., Koukouritaki, S. B., Hines,
K., Williams, D. E., Nauseef, W. M. & Svensson, C. K. (2006). Enzyme-
mediated protein haptenation of dapsone and sulfameth

keratinocytes: II. Expression and role of flavin-containing monooxygenases and peroxidases. The Journal of pharmacology and experimental therapeutics 319, 495-505.

Zhang, Z., Schuler, T., Zupancic, M., Wietgrefe, S., Staskus, K. A., Reinhart, T. A., Rogan, M., Cavert, W., Miller, C S., Notermans, D., Little, S., Danner, S. A., Richman, Wong, J., Jordan, H. L., Schacker, T., Racz, P., Tenner-Racz, K., Letvin, N. L., Wolinsky, S. & Haase, A. T. (1999). Sexual transmission and propagation of SIV and HIV in resting and activate Science (New York, N.Y.) 286, 1353-1357.

APPENDIX

Curriculum Vitae

Name: Kaothara Oluwakemi Adeyanju

Post-secondary Education and Degrees:
Honours Bachelor of Science
Department of Biochemistry
University of Ottawa
Ottawa, Ontario, Canada
1996-2000

PhD, Microbiology and Immunology
University of Western Ontario
London, Ontario, Canada
2004-2011

Honours and Awards:
CIHR HIV/AIDS Research Initiative Doctoral Award- Biomedical/Clinical Stream

Schulich Graduate Scholarship, 2006-2010.

Western Graduate Scholarship, 2004-2010.

Related Work Experience
Research assistant
The University of Ottawa Heart Institute
2002-2004

Research skills include

- Flow cytometry and analysis
- ELISA; Enzyme-Linked Immunosorbent Assays
- Virus culture, viral plaque assays and titration
- Mammalian cell culture; transfections and colony i
- Western, Northern and Southern blots
- Recombinant DNA techniques; subcloning
- Polymerase Chain Reaction and Real-Time PCR
- Isolation of genomic and plasmid DNA
- Gel electrophoresis
- Restriction enzyme analysis
- Redox-two-dimensional SDS-PAGE

Publications:

Paper

Adeyanju K., Krizova A., Gilbert P.A., Dekaban G.A. and
potentiates cell toxicity in a T cell model for sulphamethoxazole-induced adverse dru
reactions. Virus Genes 38(3):382-92.

Adeyanju K., Dekaban G.A. and Rieder M. (2011) Cytoplasmic distribution of HIV-1 Tat
sensitizes Jurkat T cells to sulphamethoxazole hydroxylamine-induced tox
(Manuscript in preparation).

Adeyanju K., Dekaban G.A. and Rieder M. (2011). Detection of oxidant sensitiv
proteins in HIV-1 Tat-expressing cells by two-dimensional electrophore
(Manuscript in preparation).

Abstracts

Wang, H.W., Antillon V., Hartman K., Adeyanju K., and Va
of Brain Na,K-ATPase by Adenovirus. Can J. Cardiol. 18(Suppl. B) 175B, 2002.

Hou X., Theriault S., Adeyanju K., Lingrel J. and Van Huys
Pressor Response to Ouabain in α2 Na,K-ATPase Deficient Mice. Can J.
(2003)

Adeyanju K., Krizova A., Rieder M. and Dekaban G.A. Margaret P. Moffat Gra
Research Day. London, Ontario. May 2005. The contributio
adverse drug reactions induced by Sulphamethoxazole-hydroxylamine.

Adeyanju K., Krizova A., Rieder M. and Dekaban G.A. The [...] of the HIV
Tat protein to adverse drug reactions induced by sulphona
Conference 2006.

Adeyanju K., Krizova A., Rieder M. and Dekaban G. A. S
cellular toxicity in HIV-1 Tat-expressing cells is dependent on the full length
protein. 4th Canadian Therapeutics Congress, May 2007.

Adeyanju K., Rieder M. and Dekaban G. A. Differential HIV-1 Tat Protein Exp
by T cells Confers Differential Sensitivity to Sulphamethoxazole-Hydroxylamine. The
IXth World Conference on Clinical Pharmacology and Therape

Oral Presentations:

Adeyanju, K. The Contribution of the HIV Tat protein to
induced by Sulphamethoxazole-hydroxylamine. MICROIMM 540y, Depa
Micorbiology and Immunology, The University of Western O
April 2005.

Adeyanju, K. The Contribution of the HIV-1 Tat protein to Adverse Drug R
induced by Sulphonamides. MICROIMM 540y, Departmen
Immunology, The University of Western Ontario. London, Ontari

Adeyanju, K. The Contribution of the HIV-1 Tat protein to Adverse Drug R
induced by Sulphonamides. Guest Speaker for the BioTh
Robarts Research Institute. London, Ontario. January, 2006.

Adeyanju, K. The HIV Tat protein and Adverse Drug Re
utilizing sulphamethoxazole-hydroxylamine. MICROIMM 9250y, Depa
Microbiology and Immunology, The University of Western Ontario. Lond
November 20, 2006.

Adeyanju K. Understanding the mechanism behind adver
AIDS Patients. MICROIMM 9250y, Department of Microbi
The University of Western Ontario. London, Ontario. October, 2008.

Adeyanju K. Differential Expression of HIV-1 Tat Protein by T cells Co
Differential Sensitivity to Sulphamethoxazole-Hydroxylamine. Guest speaker i
Pharmacology Grand Rounds, Department of Medicine, The University of Weste
Ontario. London, Ontario. April, 2009.

Adeyanju K. Understanding the mechanism behind adver
AIDS Patients. MICROIMM 9250y, Department of Microbi
The University of Western **Ontario.** London, November, 2009.

Activities and Contributions:

I was a member of the governing council of the Society
representative of the department of Microbiology and Imm
Western Ontario. 2005-2008

I also sat on the Academic Committee of the Society of G
charged with organizing the Western Research Forum. Esta
Research Forum is an annual, multidisciplinary conferen
Academic Committee and held at UWO. The conference features both pos
presentations from graduate students in the arts, bioscienc
sciences. 2005-**2008**

I was on the editorial board of the Western Graduate R
publication that profiles the outstanding research conduct
variety of disciplines at UWO. The Western Graduate Revi
resource for current graduate students, as well as those **interested in pursuing** a
graduate degree. It can be viewed at: http://www.uwo.ca/sogs/WGR/index.h 2005 -
2008.