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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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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology
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THE HIV-1 Tat PROTEIN AND ADVERSE DRUG REACTION
A MODEL SYSTEM UTILIZING JURKAT T CELLS AND SULPHAMETHOXAZOLE-HYDROXYLAMINE

(Spine title: The Tat and ADVERSE DRUG REACTIONS)

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by

Kaothar 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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entitled:

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

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Date ______________________________

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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
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
Previous work from our lab has shown that the HIV 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 Tat 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 cells were then induced
expression and then used in assays for cellular toxicity and
and presence of SMX. We found that cellular toxicity was depen
of Tat used. In the presence of the first exon of the Tat protein
augment T cell death caused by SMX, and addition of SMX death c
via apoptosis. This cell death took place without alteration

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

Keywords: HIV-1 Tat, sulphamethoxazole, sulphamethoxazole hydroxylamine, hypersensitivity adverse drug reactions, Jurkat T cell lines, two-dimensional 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 the 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 the 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 received here wi serve me well in my future endeavours and the friendship enriching experience.

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2D	Two second dimension
ADR	Adverse Drug Reaction
ANT	adenine nucleotide translocase
AIDS	Acquired Immunodeficiency Syndrome
APC	antigen presenting cell
ART	antiretroviral therapy
ARV	antiretrovirals
BSA	Bovine serum albumin
CBP	CRE-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 inhibitors
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,-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	sulphyl groups
SMX	Sulphamethoxazole
SMX-HA	Sulphameth-oxazole-hydroxylamine
SMX-NO	sulphameth-oxazole-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 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, a group of RNA viruses that infect a range of diverse 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 period (Levy, 1998). They are transmitted as single-stranded, enveloped, 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 illnesses such as Pneumocystis pneumonia and tuberculosis (Mercer et al., 2004; Steel et al., 2005).

There are two strains of HIV, HIV-1 and HIV-2, that share approximately amino acid sequence homology in the 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 the massive elimination of cells the failure of the immune system and susceptibility of opportunistic illnesses and cancers. HIV-2 infection has been associated with lower transmissibility and pathogenic potential probably due to lower levels of circulat virus at all stages of the disease (Berry et al., 1998; Popper et al., 1999). Though HIV-2 is associated with a clinical spectrum similar to HIV-1, there is a marked

reduced rate in the loss of CD4+ T cells and disease progression. The reasons for this resistance to HIV is not clear at present and further will be 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 bilayer membrane that surrounds a cone-shaped nucleocapsid core (Sierra et al., 2005). The nucleocapsid contains the genomic RNA, the viral proteins protease (PR), reverse transcriptase (RT), integrase (IN), Nef, and some cellular factors (Sierra et al., 2005). The HIV-1 genome consists of two identical single-stranded RNAs, 9.2 kb in length with open reading frames encoding nineteen proteins.

HIV infection begins with the attachment of the virion to the cell by an interaction between the extracellular domain of gp120 and cellular receptors. The major receptor for HIV-1 is the CD4 molecule, and the co-receptors are the seven-transmembrane chemokine receptors, CCR5 and CXCR4 (Clapham & McKnight, 2002). Binding to the receptors is followed by the fusion of the membranes which leads to the injection of the viral nucleocapsid. Viral uncoating is carried out by cellular factors as well as the matrix (MA), Nef and Vif. The viral RNA genome is reverse transcribed into full-length double-stranded DNA (dsDNA) by the viral RT (Harrich & Hooker, 2002). The viral dsDNA associates with viral and cellular proteins to form a preintegration complex (PIC) in the cytosol of the infected cell (Arhel, 2010). Amongst the viral proteins in the PIC, the proteins Vpr, IN and MA play a significant role in crossing the nuclear membrane by taking advantage of the cellular import machinery,

is required for integration of the viral DNA into host chromosome (Accola, 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 transactivator 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, et al., 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 that 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 Gag-Pol, Env proteins and Gag migrate to the p

membrane where the Env proteins insert into the membrane and vira

take place (Derdowski, et al., 2004; and Sfur, 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 conformational maturation to form a 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 regulatory (Karn, 1999; Sodroski et
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; full length HIV-1 Tat
exists predominantly in two different lengths: 86 or 101 residues. The 101
amino acid form of Tat is the predominant form 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 encode 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 passaging (Neuveut & 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 the full-length 101 amino acid protein. The second form is evident after the onset of Rev expression, which exports singly spli viral transcripts to the cytoplasm to be translated. The action of Rev means that the shorter form of Tat encoded by the first exon, 72 amino acids in length (Jones & Peterlin, 1994; Malim et al., 1988). In vitro, both variants are able to transactivate the LTR efficiently but affect HIV-1 infection differently.

Transcription of the integrated HIV-1 provirus is characterized by an independent and a later, Tat-dependent phase. HIV-1 specific transcription in the presence of Tat is increased several hundred-fold and is essential for viral repl lentiviral LTR is composed of U3, R and U5 regions with divided into the modulatory, enhancer and promoter regions (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 enhancer region 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 co-regulation of the HIV 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 (Williams & Greene, 2007). The κB and Sp1 binding sites are required for HIV while other sites enhance it, they are not (Williams & Greene, 2007). Members of the NF-κB/Rel family can have both positive and on transcription depending on which NF-κB is in the LTR (Brigati et al. 2003; Williams & Greene, 2007). During HIV-1 infection, p50 homodimers bind the LTR and promote recruitment of tr histone deacetylases (HDACs) (Ito et al. 1996; Williams & Greene, 20 response to cellular stimulation, such as binding of TNF or cytokin IL-1β and NF-κB, NF-κB proteins bind the LTR cooperatively transcription synergistically (Perkins et al. 1993; Pei et al. 1994; Popik & Pitha, 1 Prince et al. 1999; Sun & Baltimore, 1996). NF-κB heterodimers (RelA/p50 displace the p50 homodimers and also recruit the cellular CREB-Binding protein (CBP) complex which has histone acetylat addition, RelA directs recruitment of cellular RNA polym including P-TEFb (positive transcriptional elongation factor transcriptional (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 structure that binds to RNA polymer RNA stem loop structure is located at the 5′ end of all na Tat, however, this transcript 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 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 as the regulatory subunit. 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 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 bond formation (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 in HIV-1, and the RK HIV-2 and SIV Tat, though its function is still unknown (Chen et al., 2002). Perhaps the best studied region of Tat is the fourth basic domain (amino acids 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 functional domains have been identified in Tat. The domains are the (1) N-terminal, (2) cysteine-rich, (3) core (4) basic (5) glutamine-rich and (6) C-terminal domains.

Figure 1.2

protein is the sequence that allows extracellular Tat to be released by an infected cell and taken up by other cells (Ensoli et al., 1989; Ensoli et al., 1993; Frankel & Pabo, 1988; Hauber et al., 1989). 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 which has the RGD (arg, gly, asp) se and the highly conserved ESKKKVE motif. The RGD-binding site 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, HIV 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 (Briggs et al., 2003). Tat is able to enter uninfec through interactions of its basic domain with heparin 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 tumor 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 which in turn the result in the establishment of persistent infection (Hoxie et al., 1993; Vernhes et al., 1998; Weissman, 1998).

1.4 HIV Infection and Pathogenesis

The Joint United Nations Program on HIV/AIDS (UNAIDS ... 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 ... (UNAIDS, 2010). The course of HIV-1 infection can be divided into three phases. The primary acute ... begins with symptomatic illness (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 cell counts 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 ... several years ... eventually results in 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 the 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 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 rever 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, pharmacokinetic effects of the drugs

1.5 HIV and Adverse Drug Reactions

The use of antiretroviral therapy has had a significan 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 on with

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 the 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; Stanley et al., 2006). 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 rechallenge recurs usually within re 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 (Roychowdhury & Svensson, 200 The 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 starting ART with a very low C count (<200 cells/µl) and a concomitant opportunistic infection. At the beginning of antiretroviral treatment, patients frequently present with a variety of adve drug effects such as rashes, hyperpigmentation, hypers reactions such as erythema multiforme, Stevens-Johnson syndrome (SJS) or epidermal necrolysis (TEN) (Borrás-Blasco et al., 2008). Overall HIV-infected patients have a higher risk of developing cutaneous reactions to dr (Hernandez-Salazar et al., 2006; Lanza 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 cutaneous reactions is greatly and patients with AIDS have up to 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 illnesses encountered by HIV infected persons is *Pneumocystis* pneumonia (PCP) which is caused by *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) (Morris et al., 2004). During the early years of the epidemic, PCP was the initial 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 declined, PCP remains the most common serious opportunistic illness in t One study showed that of those who died from an HIV associated illness, the mortalit *Pneumocystis* pneumonia was 38% for those not receiving ART, 3% those receiving ART (Jain et al., 2003; Kaplan et al., 2000). The situation in th developing world is even worse with prevalence of up to infected 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 in 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 the 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 manifestation of hypersensitivity to TMP-SMX are 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 T-cell-mediated component of th the immune system in the pathogenesis of drug-induced cu (Rieder, 2009). There are currently two major hypotheses that attempt to e

Figure 1-3. Folate synthesis and SMX-TMP interaction. Sulphamethoxazole (SMX) is a structural analogue of para-aminobenzoic acid and inhibits the formation of the intermediary dihydrofolic acid from its precursors. Trimethoprim (TMP) is a structural analogue of the pteridine section of dihydrofolic acid and inhibits dihydrofolate reductase and thus the production of tetrahydrofolic acid from dihydrofolic acid (adapted from Masters et al., 2003).

Figure 1.3

first is the Hapten Hypothesis whose premise is that chemically reactive drugs or drug metabolites act as haptens when they covalently bind to proteins or peptides processed by an antigen presenting cell (APC) and presented in conjunction with MHC as modified peptides or neoantigens (Figure 1.4). These small molecular weight co by themselves are believed to be unable to elicit an immune response. However, v drug compounds are themselves chemically reactive drugs. SMX must be metaboliz a reactive species prior to haptenation (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 is N-acetylation in the liver (Figure 1.5), a detoxification. A small fraction of a SMX dose undergoes cytochrome P450 bioactivation by enzyme 2C9 to 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 reactive metabolite (Gerber & Pichler, 2006; White 2006). 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 hydroxylamine 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 4.1. Proposed mechanism of the hapten hypothesis in -induced drug reactions. Metabolism of the parent compound 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
sulphameth-oxazole hydroxylamine that is subsequently bio
sulphameth-oxazole. 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 (Vyas & Pichler, 2007). In extra-hepatic tissues, many of the defenses against intracellular 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, 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, although this did not reach 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 and anti-SMX antibodies in patients (Bircher et al., 2000; Gruchalla et al., 1998; Naisbitt et al.

2003). Reactive SMX metabolites can stimulate T cell prolife or cytotoxicity in a MHC-restricted manner (Schnyder et al. 1998; 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 cutaneous 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 the TCC were obtained from already sensitized patients with manifestations of the drug, they may not be relevant in reflecting the pathogenesis underlying primary sensitization.

Despite this evidence, a few questions are about the validity of t hypothesis. For example, for several drugs, the T cell activation kinetics appears to be too fast for any involvement of antigen 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 step 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 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 incubatin cells, there
was no stimulation of the T cells by the drugs lidocaine, carbamazepine,
SMX (Pichler et al, 2006; Zanni et al, 1998). However, the reactive-metabol
NO was able to stimulate these T cells as it is 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 TCR instead of specific antigen or covalently binding the
MHC-peptide complex (Figure 16), leading to an immune response dire
drug alone (Pichler et al, 2006). Such a drug-TCR interaction would be metabolism and
processing-independent, but the MHC-peptide complex would still be nece
cell activation and would in fact mimic drug interactions with immunological
receptors. This model was elucidated for TCRs, but the authors suggest it is possible 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 the conserved His81 in the HLA-DR α chain 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 finding that immediate, drug-induced skin reactions can occur within a few hours after administrat exposure to the drug. This is the case with radiocontrast media (RCM), a medical ra contrast agent used to improve the visibility of internal bo in X-ray-based imaging techniques such as CT (computed tomography), or (Christiansen, 2002; Christiansen et al., 2000). The kinetics of the RCM-induced reaction is much fast to be explained by the induction of a classical primar Moreover, 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 peptide-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 an 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 from Pichler et al. 2006.

Figure 1.6

would explain the higher incidence of ADRs during infections
The p-i concept implies that ADRs are actually due to cross-reactive peptide-spe
memory T cells to certain drugs. In line with this notion, HIV, is
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 methods, notably with
our ability to detect very reactive and thus very unstable metabolites (Lavergne et al.
2008). As well, although the majority of quantitative 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 the drug-metabolizing enzymes than hepatocytes

counterbalanced by the larger weight and surface area of t explain why the skin is a primary target in hypersen ADRs (Lavergne et al., 2008). In addition, while most important hepatic CYPs are exp several CYPs that are much more abundant in the skin and the liver (Sanders 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 hepatic T cell activati is more likely to result in tolerance rather than a pathogenic immune response (Sanders 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-HA and SMX-NO). They also showed that there were 3 patterns of T cell specific stimulation; first, clones that were metabolite specific; second, clones that were stimulated by the parent drug alone; and third, clones stimulated by SMX and metabolites. The authors also showed data that concluded responsive T cells were stimulated through a hapten mech APC.

The strongest evidence against the pi-concept is the finding of asso

between certain drug hypersensitivity reactions and HLA-class I alleles. The major

patients with ADRs to abacavir, approximately 94%, carry HLA-B*5701 allele,

compared to only 1.7% of controls. The association of carbam

treatment with the appearance of Stevens-Johnson 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 alt production

of reactive oxygen/nitrogen species (ROS/RNS respectiv

cellular anti-oxidant defences, leading to a disruption of redox c

or molecular damage (Jones, 2006). These reactive species target a la

biological molecules such as nucleic acid, unsaturated lip

with the latter being the most common target making up

oxidized molecules in the cell (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 of the protein (Dalle-Donne et al. 2007)

Reactive oxygen/nitrogen species can target lipid me peroxidation and the formation of multiple aldehydes, including 4-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, which means that HAD is now the leading caus dementia in adults younger than 60 years 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 and the related reactive oxygen nitrogen species, including enzymes like superoxide dis reductase, peroxiredoxin and catalase (Haslett 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 antioxidants that lead to added oxidative stress in HIV-infected patients. The concentratio decreased in serum, the liver and CNS of HIV-infected patients while adminis the GSH precursor, N-acetyl-cysteine (NAC) to patients in a pre-ART study was

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-1 activity and HIV replication while supplementation with vitamins C red infection and produced a downward trend in viral load in HIV 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 ROS is thought to be 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 the extent of 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 [illegible] (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 Tat (Hayman et al., 1993).

induced neurotoxicity is believed to be mediated through two distinct excito mechanisms involving (Mattson et al., 2005). Tat is capable of depolari hippocampal neurons and human cortical neurons, increasing intracellular Ca^{2+} followed by mitochondrial calcium uptake, generation of RO apoptosis (King et al., 2006). In neurons, Tat also activates phosphatid-kinase increases levels of IP_3, release of calcium from IP_3-sensitive endoplasmic reticulu internal stores, and 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 ($ONOO^-$) (Kaul et al., 2001; Liu et al., 2002). Tat also induces TNF-α, which in turn induces iNOS, leading to the incre 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 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 activation and TNF-mediated cell death. As this was inhibited suggested to the authors that the activity of Tat was linked. The further observed that Tat, either produced intracellularly significantly reduced RNA and protein levels of manganese-dependent superoxid

dismutase (Mn-SOD), 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 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 can undergo various reversible and alterations in response to ROS and/or RNS (Dalle-Donne et al., 2007). Thiols are a class of organic sulphur 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 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 cysteine proteins 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 form of two-dimensional 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

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 layered onto the second gel and elect
90^0 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 non-
(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 death, defining diseases such as AIDS. 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 10-15 years. However, hypersensitivity adverse drug reactions (ADRs) to many drugs, used to treat HIV, 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 Objectives</u>:

1.a) Establish T cell lines in which the HIV-1 Tat protein (Tat101) and mutants (Tat86, Tat72, Tat(Δ45-74) and Tat) can be differentially induced characterize these 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 the reactive metabolite, SMX-HA, SMX.

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

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

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

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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 along with the appearance of the
manifestations of AIDS (Douek, 2003). The link between the disease an
suggests there is a contribution of 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 (Brigati 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 single-spliced viral transcript, the produci
one-exon form of the protein with 72 amino acids (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
cells phenotype by modulating the expression of cellular (Fanales et al., 2002a-b; Weiber et al., 2000). The protein has been found to stimulate
immune-regulatory cytokines, including the production of IL-6, dysregulation
which is implicated in the pathogenesis of several AIDS associated

psoriasis, B cell lymphoma and Kaposi's sarcoma (Amoroso et al., 1997). In another study, Tat was able to increase tumor necrosis factor-beta (TNF-β) expression by activating the TNF-β 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 HIV-1 conditio infection (Choi et al., 2000; Flores et al., 1993). Additionally, this situati exacerbated by a systemic decrease in glutathione in infected cont 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 HIV-1 loss o 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 one of the most common of these diseases is Pneumocystis jirovecii fungus, responsible for the lung infection known as pneumocystis pneumonia (PCP) (Morris et al., 2004). Without treatment, over 75% of people with AIDS de 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) in patients with AIDS usually present as a high fever cutaneous eruption (Rotunda & Stern, 1994). The risk for patients with A approximately 50%, significantly more prevalent than seronegative population at 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 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 et al., 2006).

Previous data indicates that hypersensitivity ADRs to mediated by the reactive metabolites of SMX, particularly 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. (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+ T cell line infected with HIV is more susce toxicity by SMX-HA than the uninfected parent cell line (Rieder et al., 1995). The metabolite produced concentration-dependent toxicity in cultured CEM HIV

human T lymphoblasts, a result significantly greater than -infected 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 (Asante et al., 2005). The 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 dynamic significant increase in the viral load of a patient at the la indicative of a similarly considerable overall increase in t extracellularly. This in turn might have a profound effect on the hypersensit and its metabolites (Carr 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 Method

2.2.1 Cell lines

The human T lymphocyte cell line Jurkat, clone 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 Roswell Park Memorial Inst 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 10% fetal calf s 1mM sodium pyruvate and 100 units/ml of penicillin and stre

Construction of plasmids and cell lines

The Tat gene was amplified from the plasmid pSV2tat72. encodes the first exon (72 aa) of the HIV-1 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), resulting in a Tat-GFP fusion gene. was excised from pEGFP-N1 as a Nhe I/Not I fragment and inserted i digested plasmid pBIG2i, an inducible, high expression ve (Strathdee et al 1997). The result was pBIG TatGFP. GFP was cut from pEGFP-N1 was also cloned into pBIG2i to create pBIG GFP.

pBIG TatGFP or pBIG GFP were transfected into Jurkat cells by Ju 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 dilutions in 96 well plates and the resulting cell lines we complete RPMI 1640 medium (Invitrogen) supplemented with 10% glutamine, 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 incubat 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, Inc

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 manufactur time-PCR reactions were performed in the absence of cDNA t used as a housekeeping gene. Real-time PCR analysis was performed using 7900HT Sequence Detection System (Applied Biosystems Relative expression levels were determined by the compar (Wong & Medrano, 2005).

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 resolved by SDS-PAGE gel containing 6M urea (Arp et al., 2005). After transferring to a PVDF (pol difluoride) membrane, the proteins were probed with mouse anti-Tat and β-actin monoclonal antibodies (Advanced Biotechnologies Inc. a Another membrane was probed with rabbit polyclonal anti-GFP antibody (Chemicon Bound antibody was detected using goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and enhanced (Amersham). β-actin was used as a control for protein loading.

Apoptosis and SMX-HA 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 SMX-HA 0, 50 or 200 μM 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, 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) o anti-caspase 3 rabbit monoclonal antibody (Cell Signaling Technology). The 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 goat anti-rabbit HRP-conjugated secondar antibody respectively (Jackson ImmunoResearch Laboto chemiluminescence (Amersham). The consistency of protei β-actin monoclonal antibody.

2.25 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-wt-TH-GFP c plates and incubated in culture with 100 µM SMX (parent drug) (Sigma-Aldrich) or 25-800 µM of SMX-HA (active drug metabolite) for 2 hours. Af removed, the cells were resuspended in media and allowed at 37°C for 18 hrs. Next, 1mg/ml MTT was added 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-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 gated events were measure each sample. Flow cytometry data was analyzed using Flo appropriate, camptothecin (6μM)-induced apoptosis 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 with 0, 100 and 200μM for 8hrs. The cells were then washed in PBS, formaldehyde, permeabilized with cold 70% ethanol and th 4^{0}, in the dark. After removing the ethanol, the cells were with phycoerythrin-conjugated (PE) rabbit anti-active caspase-3 monoclonal antibod (BD Biosciences) for 1hr. PE-Caspase-3 and GFP 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 -way ANOVA with
Tukey's post hoc procedure was used to compare mean for groups 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 -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 an respectively.
This was followed by a 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-induced ADR. To (SM
end, the exon 1 of the Tat coding region was amplified by
fragment was inserted into an EGFP plasmid to create the fusi
product was then inserted into a doxycycline-inducible vector (pBIG2i
1999). The control vector consisted of the GFP fragment of
into the pBIG2i vector. After transfection and selection with G418 of 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
resulted in the respective induction of GFP and TatGFP
microscopy images showed GFP fluorescence throughout
pBIG GFP and pBIG TatGFP (Figure 2.1A and Figure 2.1B, respectively).
Immunoblotting of 38 h of induction with 1000ng/mL of doxycy
(Figure 2.1C) and anti-Tat (Figure 2.1D) antibodies showed that Jurkat pB
cells not induced with doxycycline did not produce the Ta
Figure 2.1C and 2.1D).

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

Figure 2-1. Expression of GFP and the TatGFP fusion protein. Confocal images of 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 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 used in the 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 SIX HA activity compared with the uninfected cell lines (Arp et al., 2005; Reiter 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. Increases in the concentration of doxycycline resu
expression.

(A) Time course of TatGFP induction upon exposure of Ju
doxycycline. Jurkat pBIG Tat-GFP cells (5×10^5) 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 Tat protein. Cell lysates obtained fro
TatGFP cells treated with 0 or 1000ng/ml of doxycycline were probed wit
or anti-actin antibodies. Data are representative of three ind

(C) Total RNA was isolated and reverse 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 2.2C).

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

Earlier studies demonstrated that SMX-HA toxicity was amplified in the presence of Tat (Asensio et al., 2005). To determine if there was a relatio increasing levels of Tat-protein and SMX-HA toxicity, we 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 non-toxic effect of the parent drug (SM 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 2.3). 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 Dox) (Figure 2.3). 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. Tat-GFP expression decreases cell viability upon exposure to SMX. The MTT cell viability assay was used to show profile of cell population exposed to increasing concentrations of SMX. Data are representative of three independent experiments performed in triplicate. *P<0.01, Jurkat E6.1 vs. Jurkat E6.1 vs. TatGFP: 0, 31, 62, 125 and 500ng/ml dox groups, &P<0.01, Jurkat E6.1 vs. TatGFP: 31, 62, 125 and 500ng/ml dox groups, #P<0.01, TatGFP vs. TatGFP: 31, 62, 125 and 500ng/ml dox groups, ‡P<0.01, TatGFP: 0ng/ml dox vs. all other groups, §P<0.01, TatGFP: 31ng/ml dox vs. all other groups, ¶P<0.01, TatGFP: 31ng/ml dox vs. TatGFP: 125 and 500ng/ml 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 using 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 vial 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 stair treatment of cells with SMX-HA in a dose dependent fashion, followi TatGFP expression (Arrington et al., 2005). Cells not treated with drugs (0 μM SMX-HA) were highly viable and the initial stages of apoptosis were not SMX 100 μM, again confirming the lack of toxic effect of the parent compound (Arrington et al., 2005). These previous experiments demonstrated dose-related toxicity of SMX-HA at concentrations of 50 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 in cytochrome c and caspase-3 western blot assay. Cytochrome c is a protein normally confined to the intermem

Table 1-2. Expression of target genes. Values were obtained after a MTT data using the GrapdPad Prism 4 software. Data are of three independent experiments. *P < 0.01 vs. doxycycline. #P < 0.01 vs. pBIG, GFP: 500 ng/ml dox. &P < 0.01 vs. pBIG TatGFP: 500 ng/ml dox, pBIG TatGFP: 31 ng/ml dox.

Table12

Cell Populatio	EC_{50}(uM)	95% Confic Intervals
Jurkat E6.1	175.3	16.00 19.20
pBIG GFP: 500ng/	146.0*	13.62 15.64
pBIG TatGFP: 0n	143.0*	13.55 15.01
pBIG TatGFP: 31	68.02	63.20 73.22
pBIG TatGFP: 62	58.02	53.74 62.64
pBIG TatGFP: 12	51.51	47.16 56.27
pBIG TatGFP: 50	52.50	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 end result
of this cascade is the activation of the executioner caspase-3 and apoptosis. After cas
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 exp 0 µM SMX-HA
contained 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
cells as the concentration of doxycycline increases from 3
exposure to 50 µM SMX-HA as assessed by densitometry (data not shown). These 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 cleaved-activated 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 - 31 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 31 ng/ml to -fold 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 and SMX-HA apoptosis. After inducing TatGFP ex the Jurkat pBIG TatGFP cells were treated with SMX-HA for 8 h, fixed, permeabi and stained for active 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 (Arp et al., 2005) the western blo experiments described above, the results showed a dose-dependent increase in toxicity in all cell populations which was further enhanced with inc expression as demonstrated by the increased active caspase-3 fluorescence coinciding with higher levels of TatGFP-associated 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
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. Uninduc
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 an increase in caspase-3 expression
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 an 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 (Bhul et al. 1992; Davis & Shearer, 2008; Lin et al., 2006). 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 also has an effect on the incid
of ADRs, there is evidence that maintaining control of vir
appears to lower the risk for drug hypersensitivity ADRs (Carr et al., 2001). In this
study, we tested whether a differential level of Tat expre
cellular sensitivity to SMX-HA in a Tat-dependent manner in a T-cell mode
HA induced ADR. To do this, we used a system to differentially induce th
Tat and then assess cell death upon exposure to SMX-HA.

The inducible pBIG vector developed by Strathdee (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 equivalent to that seen among HIV-infected cells (Appel et al, 2005). Cell viability experiments the MTT assay demonstrated no toxicity to the parental d significant decrease in cell viability with increasing concentrations of 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, a significant decrease 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 toxicity with SMX-HA that 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 (10-25 μM) (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 sulfonamide-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). Thus, this 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 et al. (1995) and Park et al. (2001), could contribute to the increased incidence of ADRs in HIV in potentially an example of apoptosis deregulation, which h other conditions such as neurodegeneration and autoimmunity (Jana et al., 2004; Liu et al., 2004; Rego & Oliveira, 2003).

Tat protein expression has been linked to an increase and mitochondrial damage (Choi et al., 2000; Reidel et al., 2002). Specifically, in th association between Tat and SMX-HA-induced toxicity was established by Arp et al (Arp et al., 2005); 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 increasing Tat-expressing cells

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, spliced) 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 further addressed (Muthumani et al., 2003; Yedavalli et al., 2005). Our data suggests that Tat, in addition to conferring increa… mediator of apoptosis (Huigsen et al., 2004; Yang et al., 2003a), also confers an increa… sensitivity to the intrinsic activation of apoptosis involving caspase 3 in… 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 HIV infection to AIDS should predis the infected individual to hypersensitivity ADRs related t important in the care of patients living with HIV infection.

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

3.1 Introduction

One consequence of HIV-1 infection is the gradual decline of the immune system which allows for vulnerability to opportunistic infections. The fungus Pneumocystis jiroveci takes advantage of this immunodeficiency to cause Pneumocystis pneumonia, a lung infection that is commonly treated with sulphamethoxazole-trimethoprim (SMX-TMP). This combination antimicrobial is also used to treat urinary tract infections in HIV-negative populations. The SMX moiety of the drug is associated with an incidence of 50% or more adverse drug reactions (ADRs) in HIV-1 infected patients, but only 2-5% in patients not infected with HIV-1. Adverse drug reactions can be defined as a response to a drug which is noxious and unintended, occurring at doses normally used for prophylaxis or therapy (Rieder, 2009). The most serious of these is the hypersensitivity ADR which usually presents as a delayed reaction characterized by the onset of fever and proceeded by the development of a skin rash such as Stevens-Johnson syndrome or toxic epidermal necrolysis. The liver and kidney can also become involved, presenting clinically as 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 suggests that 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 nitroso-SMX. Both metabolites are known to be toxic to cells in vitro, however this cytotoxicity is minimized by co-incubation with glutathione

(GSH), an antioxidant, and its precursor N-acetylcysteine (Rieder et al., 1988). Indeed, the in vitro toxicity was the basis of the initial 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 non-hypersensitive patients with this reaction (Rieder et al., 1989). A similar finding was noted 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 synthesis of full-length HIV transcripts (Romani et al., 2010).

The coding sequences of the Tat gene are 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 encoded 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 nonsynonymous single nucleo polymorphism creating a stop codon in the second exon a (Campbell & Loret, 2009). Though a few laboratory strains that exp

amino acid form of Tat is, though this represents 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

domains. They are the N-terminal domain (aa 1-21), cysteine-rich domain (aa 22-37),

core domain (aa 38-47), basic domain (aa 48-60), the glutamine-rich domain (aa 61-72)

and the C-terminal domain (aa 73-101) (Campbell & Loret, 2009; Johri et al 2011). The first

three domains (aa 1-48) together circumscribe the minimum activation domain of the

protein which mediates its interactions with cellular machine (Cardelli et al 1991;

Derse et al 1991; Roman 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 (aa 1-72) with the first five domains

represents the combination of the activation domain and binding regions that i

required for functional HIV-1 transactivating activity.

In this study we sought to determine which region of th

cellular toxicity (HAND and SMX). Furthermore, to determine if the sam

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[ned in] Complete RPMI 1640 medium (Invitrogen) supplemented w[ith] 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[00 units/ml] penicillin and streptomycin, was obtained from ATCC.

Construction of plasmids and cell lines

The Tat gene was PCR-amplified from the plasmid pSVTat. T[his] encodes the full-length Tat gene and was a kind gift from Dr. KT J[...] of Molecular Microbiology, the Molecular Virology Sectio[n] [...] length Tat gene (Tat101), with or without stop codons, an[d] Tat72, Tat48 and Tat86(Δ49-57) were cloned into the plasmid pEGFP-N1 (Clontech Inc.), resulting in Tat101, Tat101GFP, Tat86GFP, Tat86ΔGFP, Tat72GFP, Tat[...] gene, respectively. The fusion genes were excised from pE[GFP-N1] as Nhe I/Not I fragments and inserted into the Nhe I/Not I digested pla[smid] doxycycline-responsive expression vector (Strathdee et al., 1999). The results were th[e] following plasmid constructs; pBIG Tat101, pBIG Tat101GFP, pBIG Tat86GFP, [...] Tat72GFP, pBIG Tat48GFP and pBIG Tat86ΔGFP. As a control, the GFP ge[ne ...] pEGFP-N1 was also cloned into pBIG2i to create pBIG GF[P].

constructs or pBIG GFP were transfected into Jurkat E6.1 T cells by nucleofec

(Lonza, Amaxa Nucleofector) according to the manufactur

stable cell lines of Jurkat pBIG TatGFP and Jurkat pBIG G, respectively. Cos7 cells

were also transfected with the pBIG TatGFP constructs for studies.

The expression of TatGFP fusion protein or GFP in either cell type was induced

by incubating the pBIG2i-transfected cells with doxycycline (Sigma).

Selective media

Post-nucleofection, 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 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

Cos7 pBIG TatGFP cells were incubated with 500ng/ml o

incubated with 6µM MitoTracker Far Red (Invitrogen) for 30 min at 37°C and

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 doxycycline for 40hr
the cells were analyzed on a FACSCalibur (Becton Dickinson) flow cytometer
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 FACSCalibur. Both the dose respons
course experiments were carried out at least twice. 10,000 events were measured for each
cytometry data was 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 pBG TatGFP
treated 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 Jurkat HTLV-I cells. Total
(10¼g) was reverse transcribed using the High Capacity cDNA Archive
Biosystems) according to the manufacturer's instructions. PCR
reactions were performed in the absence of cDNA template
control housekeeping gene, GAPDH. Real time PCR analysis was performed using
PRISM 7900HT Sequence Detection System (Applied Biosys

Relative expression levels were determined by the compar
(Wong & Medrano, 2005).

3.2.5 Immunoblot analysis

To characterize the expression of the Tat-GFP fusion
lines, cells from each cell line were differentially induced
which the cells were washed twice in cold PBS 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 second bursts at 30 percent duty cycle
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 protei

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

3.2.6 MTT cell viability assay

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

for 40h, washed twice with PBS then $7.5x10^4$ cells/well were seeded in 96-well plates and incubated in hepes buffer with 0-200µM SMX (Sigma-Aldrich) or 0-800µM of SMX-HA for 2 h. After the drug was removed, medium was added and the cells were 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 solution (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 doxycycline induction for 40h, 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 96well 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 half an hour after which the plate was centrifuged at 1500r for 5min and the supernatant decanted. The wells were refilled with 100µl HBS background fluorescence read at 485nm excitation and 527nm emission. SMX-HA or SMX added to the wells in quadruplicates and the plate incubated at 37°C with fluorescence readings at 30min intervals for 2h. This assay was performed on three sep for each cell line.

3.2.8 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 betw concentration levels. The IC_{50} values were calculated using GraphPad P, where the X values (concentration of HA) were transformed using log X and the Y values viability) were normalized to define 0% and 100% as the respectively. This was followed by sigmoidal 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 cell type to of imaging analysis than the small Jurkat 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 cells were treated with 500ng/ml doxycycline for 48h, incubated with 200nM of mito specific MitoTracker Deep Red 633 for 3 min and Hoechst 33258 which stains for D

The confocal images showed that the fluorescence of Tat101GFP, Tat86GFP Tat72GFP constructs co-localized with the Hoechst stain in the nuc (Figure 3.1A-C). The mito-tracker marked mitochondria were found to be evenly distributed throughout th the cytoplasm. In contrast, both TatGFP and Tat48GFP constructs sho 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 aggregating around th nucleus in the cells expressing the TatGFP construct (Figure 3.1E). The non-punctate/diffuse nature of the GFP fluorescence in the TatGFP and Tat48GFP-expressing cells suggests no co-localization between the GFP and relatively structured mitochondria in these cells (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 it could simply the presenc 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 in the cell. 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 TatGFP 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 and mounted. Cells were analyzed using a LSM 510 META confocal microscope.
scale bar represents 20µm.

Figure 3.1

3.3.3 Characterization of the TatGFP fusion constructs

The next set of experiments involved the characterization Jurkat Tat cell lines. Time course experiments were carried out to det induction of protein expression by different doxycycline co as well as the time point at which the cell lines expressed the maximal levels of their v TatGFP fusion proteins. 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, prior to the Tat101GFP cells was substantially free of detectable GFP expression (Figure 3.2A). At the 12h time point analyzed, the inc concentration of doxycycline 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 cells expressing GFP at 500ng/ml and 1000ng/ml doxycycline reached a maximum of approximat 90% of the cells analyzed. This value then held steady until 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 v kinetics of GFP induction similar 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) Tat61GFP lines were treated with various concentration
incubated for 72 h. Aliquots of cells were analyzed by flo
expression at six different time points. Data are means of three independent

Figure 3.2

incubation (Figure 3.2B). The exact percentage 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 55.7% the Tat72GFP and T
cell lines respectively. Furthermore, the maximal percentage of
the Tat101GFP and Tat86GFP cell lines is maximal at 500
Dox for the lower three cell lines. In addition, GFP expression generally b
36 h time point and is maintained for at least the 72 h per
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 T
GFP at the maximal level of induction by 1000ng/ml doxy
Tat86GFP 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 in Tat-GFP fusion pro
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 antibodies showed the presence of t
each of the cell lines. The western blots showed that the
even in the absence of doxycycline in the Tat86GFP cell line
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. GFP in the Tat101GFP cell line (Figure 3.3E), respe
exception to the general trend is the Tat48GFP cell line
Tat48GFP expression even in the absence of doxycycline
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 PCR of HIV Tat and TatGFP specific mRNA

In an effort to determine if the expression of the engineered
is comparable to infection of HeLa cells, quantitative PCR was performed
This was necessary as the Tat protein in the T-REx Jurkat cells was
undetectable via western blots. Using primers specific
quantitative PCR and RNA from all the cell lines, Tat and GFP expressing
proteins as well as HIV infected Jurkat cells.

Figure 3. Expression of the proteins from the various Tat co
induction. Cells from the (A) Tat101GFP, (B) Tat86GFP, (C
and (E') GTat1 lines were treated with different concentrati
1000ng/ml) 40 h and then the cells were lysed and protein
were analyzed by SDS-PAGE and membranes blotted with anti-Tat and anti-GAPDH
was used as a loading control. Data are representative of two independe

Figure 3.3

The Tat101 cell line showed Tat mRNA in the absence of Dox (0ng/ml Dox, Figure 3-4) that is already more than half of the amount in HIV cells. There was an increase in the Tat mRNA as the concentration of doxycycline was increased to a maximum at exposure concentration of approximately 400ng/ml Dox that the Tat101 cell Tat mRNA reached the level of Jurkat HIV cells. These Tat101 GFP cells showed a 4-fold lower amount of mRNA at 0ng/ml Dox compared to the HIV Jurkat cells showed a much steeper increase in Tat mRNA in response to increasing doxycycline. Furthermore, Tat101 GFP cells had a much lower Tat mRNA overall compared to the Tat86 GFP cells. 1000ng/ml Dox was sufficient to reach Tat mRNA seen in HIV Jurkat cells.

In the Tat86 GFP cell line, a small, but detectable Tat mRNA amount at 0ng/ml Dox was approximately 100 fold lower than amounts seen in HIV Jurkat cells and concentration of 200ng/ml Dox was sufficient to reach Tat mRNA amounts similar to those in HIV Jurkat cells (Figure 3-4). The Tat86 GFP cell line showed a clear increase in Tat mRNA with the increase in doxycycline with the maximal Tat mRNA occurring with 800ng/ml Dox was a subsequent decrease of the mRNA levels at 1000ng/ml Dox. The cell line showed gradual increase in Tat mRNA as the doxycycline increased with a maximal amount at the highest concentration (Figure 3-4). Like the previous cell line, the Tat72 GFP cell line reached approximately 200ng/ml Dox which is comparable to that seen in HIV Jurkat cells.

Figure 34. 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 specific primers. Data are
mean of three independent experiments.

Figure 3.4

The Tat48GFP cell line-expressed amount of Tat mRNA at 0ng/ml Dox (Figure 3.4E) in uninduced cells. However, there increase in Tat mRNA levels as doxycycline concentration is inc cell line also had considerably less Tat mRNA than the descr lines, needing 1000ng/ml Dox to reach the levels of Tat mRNA Tat GFP cell line follows the general trend as Tat mRNA tracking with increases in doxycycline concentration (Figur mRNA levels coincide with the cells between 200 and 40 Dox. PCR analysis was also carried out using RNA from un well as Jurkat T cells expressing only GFP and not the other 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 that toxicity up incubation 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 protein and deletion mutants used above were evaluated. These cell lines were employed in MTT assays activity to test the effects of SMX and its reactive metabolites SMX. 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 the highest mRNA in the cell line.

Previous studies have shown that SMX has no significant effect on the viability of Jurkat T cells (Figure 3.5). In each of the Tat constructs assayed by the MTT assay, SMX was then used to assay the viability of differentially induced Tat101 Jurkat E6.1 T cells in the presence of various concentrations of five metabolites of SMX-HA. Indeed, cell viability remained largely unchanged for the cell populations until the addition of SMX-HA. At 200µM of SMX stably transfected Jurkat cells induced with 1000ng/ml Dox showed a trend towards lower cell viability when compared to Dox and the untransfected Jurkat E6.1 T cells. The difference between the viability of the experimental cell line E6.1 and Tat101 was significant at 400µM of SMX. At this concentration there was no significant difference in the differentially induced Tat101 cells.

For the Tat101GFP cell line doxycycline concentrations of 100ng/ml and 500ng/ml were tested. The vehicle DMSO (0µM SMX-HA) had no discernable effect on the viability of the mentioned cells. However, there was a dose dependent decrease in cell viability with the increase of SMX-HA (Figure 3.6). This concentration in the Jurkat E6.1 cell line and the doxycycline induced cell populations. The toxicity of the Jurkat E6.1 cells was significantly different with 100ng/ml Dox and 200-400µM SMX-HA. At 500µM of SMX-HA, the decrease in cell viability in cells with 100ng/ml doxycycline began to be compared to both the Jurkat E6.1 cells and the induced for Tat101

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 0ng/ml Dox and 100ng/ml doxycycline cell

populations and this was also for 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 SMX-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), in 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. Tat in the p
expression was induced for 40 h from (A) Tat101, (B) Tat
Tat72GFP, (E) Tat48"GGFPPolin(F) by treatment with various c
of doxycycline (1000ng/ml) then incubated with 200µM SM
concentrations of (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 cells are the average of 25 different
carried out in conjunction with the different Tat constructs over a cours
months. *P ≤ 0.001 versus 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 when compared to Jurkat E6.1 ce 200 and 400 µM SMX-HA. Furthermore, a significant decrease in viability doxycycline-induced cell populations becomes evident at 50 µM SMX-HA, a lower exposure dose than seen with any of the cell lines described above. At 100 µM SMX-HA, the viability of the 0ng/ml population had dropped to approximately 40%. At this concentration, the cells induced with 200ng/ml Dox showe 30%. When the cells were induced with 100ng/ml Dox, cell viability dropped but was not significantly different from control cells (0ng/ml Dox). Cell viability continu to decrease at the higher concentration of SMX-HA, but there was no signi difference in the differentially induced cells. Similarly, there was no sign difference between the differentially induced populations of the GFP Tat expressing cell line (Figure 3.5). The main difference between this cell line a above is the general sensitivity of these cells to SMX-HA. These cells show approx 70% and 30% cell viability with 100 µM and 50 µM SMX-HA, respectively, concentrations at which the cell lines expressing Tat101, Tat101GFP, T Tat72GFP displayed cell viabilities of 90% and above.

Comparing the LC_{50} (lethal concentration, 50%) of each of the 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, Tat10 and Tat72GFP cell lines were in the 300 µM SMX-HA concentration range, while that of Tat48GFP and "GFP Tat" was 2-fold to six-fold lower at 106.67 µM and 59.52 µM

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

the cells used in the experiments were established 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. The LC_{50} values were calculated using GraphPad Prism v.5

the methods.

Table 13.

Cell Line	0ng/ml Ddx	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
Tat31GFP	59	52	57
Pooled Tat48	161	162	137
Pooled Tat31GFP	149	146	138

SMX-HA respectively. In an effort to determine if this phenomenon-specific,

the pBIG Tat48GFP" and pBIG-GFP DNA was used to transfect Jurkat

the pool of stably selected transfectants rather than sing

MTT assay. The LC$_{50}$ was found as 1760µM and 1493µM respectively. Wh

in both cases this is higher than the LC$_{50}$ found in the assay using specific cl

much lower than the other cell lines demonstrating selective toxicity of SMX-HA in these 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-HA or SMX 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 1.8- and 4-fold increase in ROS produced by -HA concentrations of 50 and 200µM, respectively. Furthermore whereby increase in the 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 H-A ... various Tat constructs. After induction with doxycycline, c... (B) Tat86GFP, (C) Tat72GFP, (D) Tat48GFP or (E) GFP cells were seeded ... concentration of 1×10^6 cells/ml and incubated with 10 µM DCFH at 37^0C for 1 h. There ... followed an incubation with different concentrations of H-A for 2 h which ... µM ... the fluorescence was determined on a plate reader. *P<0.01 vs. 0 (control cells), #P<0.05 vs 200 mg/l D.O. 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 significant, 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 (Figure 3.6B). The initial increase in ROS occurs with cells induced with 10ng/ml Dox produced significantly more ROS than was seen from treated with the 50µM SMX-HA. This is followed by a decrease in ROS producti 200ng/ml Dox, a decrease that is significant for 50µM 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 Dox and 50 and 200µM SMX-HA, respectively.

Production of ROS from the rest of the cells confirms that SMX-HA furth produced significantly more ROS than the parent drug, SM 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 ROS-generating effect of SMX-HA was still evident in

Tat48GFP (Figure 3.6) and Tat GFP (Figure 3.6) cells, in the expression of t
construct had no significant effect on ROS production.

3.4 Discussion

HIV-1 currently infects 33 million people world. 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; the primary function anti-retrovirals (ARV) is the suppression of HIV replication. This is carried out mo effectively when combination ARV are used. However, these drugs 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 drug, individual, and interaction with othe drugs. One of the most common drugs producing adverse effects in HIV-infected individuals is the antimicrobial SMX. ADRs 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 may be one such factor as the expression of the protein correlates w metabolite of SMX, SMX-HA (Asensi et al, 2005). We have also demonstrated that differentia expression of this protein in cultured cells alters 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 were conducted 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 (nuclear

localization signal (NLS)). There was minimal GFP fluorescence detected in

cytoplasm with the Tat101GFP cells. The deletion mutants Tat86GFP and Tat72GFP and

showed the same cellular distribution. In contrast, the Tat48GFP and

Tat GFP fusion proteins were distributed throughout the cell, the 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 pattern of the Tat-GFP proteins an

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 transduction domain that allows the

proteins to pass through the nuclear 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 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 proteins and cytoc

c release that lead to caspase activation. Studies have also shown that mitochondrial

dynamics have a significant role in regulating apoptosis. I (2003)

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

The characterization of the cell lines in 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. As 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. 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 Tat protein leaves Thus, the full-length Tat101 (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 allows Tat101 to cross all cell membranes. In addition the use of Q-PCR allowed the comparison of the Tat mRNA produced at different doxycycline concentrations in the Tat-expressing cell lines to the Tat mRNA in the HIV-infected cells. Subsequent experiments carried out using doxycycline concentrations that induced Tat-GFP mRNA to approximate the level in the HIV-infected cells.

Results from the MTT cell viability assay have previously demonstrated significant toxicity using the cell line in the presence of increasing concentrations of SMX-HA while SMX demonstrates no toxicity when cells are incubated with the parent compound, SMX, under identical conditions. The constitutive expression of Tat increases SMX-HA-mediated toxicity, an effect similar to that seen in HIV-infected T cells. The 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 was increased (Apparao, 2005). These observations were the basis of the current project, which region(s) of the Tat to dete protein is (are) responsible for these results. For this several de protein were generated and stably transfected into 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- metaboli 100 µM) caused significant toxicity, indeed 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 begins at 200 µM of SMX-HA when Tat101 mRNA is at levels equivalent to or higher than 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 the cell line expresses an amount of Tat101 mRNA at 0ng/ml Dox (Figure 3.4) an amount only slightly less than 400ng/ml Dox. While the doxycycline induced Tat101 cell populations ar significantly different from one another, the cell viability is significantly different from that of Jurkat E6.1 cells, starting at 200µM SMX-HA.

The viability of the Tat101GFP cell line begins at 25µM 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. The viability of the 500ng/ml Dox population remained largely unchanged at the lo SMX-HA (100µM) and was not significantly different from the at any of the concentrations of SMX-HA tested. The trajectory of cell viability in the Tat101GFP cell line where low expression of Tat101GFP r and high expression of the same protein resembles a prozone effect, a phenomenon that typically occurs in immunoassays antibody bind all the receptor sites, leaving nothing availa quite different from the results seen and could be due to the differences in the amounts of Tat expressed in the two cell lines characterized by th mRNA levels. Besides the fact that the Tat101 cell line is leaky 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 of Dox at 2000 ng/ml is 160 fold greater than that at 10 ng/ml Dox. This means there is a much wider range of conce 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 many a variety corals where the protein is not bioluminescent. The purpose of GFP fluorescence in the jellyfish and in the coral currently remains unclear (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 505 nm (Bou-Abdallah et al. 2006). While molecular oxyge is required for the post-translational formation of the protein, 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 s wild type GFP 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 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 here, 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 i
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 of GFP is unclear. Secondly, the
generated by Al-Abdullah and Chao (2006) suggests that wild type GFP has the
ability to specifically quench $O_2^{\cdot-}$ radicals, however a study by Gulow (2005)
showed that there was no detectable generation of $O_2^{\cdot-}$ 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,
by Gulow et al. (2005) suggests 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 EGFP to Tat proteins has a
significant effect on Jurkat cell viability, MTT experiments were carried out w
line expressing only EGFP. While there was the expected concentration-dependent
decrease in cell viability in the presence of SMX-HA, there was no significant
effect on cytotoxicity due to induction of EGFP expression, even at the
concentration of 1000 ng/ml Dox. 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. The failure of Tat72GFP expression to add SMX-HA-mediated cell death is in contrast to previously published da[ta] ([illegible] et al. 2009; Arp, 2005). This could be due to the fact that the sequence of the protein used in the above publications differed by seven 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 the amino acid differences in the Tat used previously confer a greater ability to induce SMX-HA toxicity. As an example the substitution of Pro-58 in the Tat-GFP fusion protein used previously ([illegible] et al. 2009; Arp, 2005) with Ala-58 in the Tat constructs used in this chapter. The cyclic structure of the proline side chain in position 58 could affect protein tr nuclear localization as the residue is within the basic domain. Peloponese et al. (1999) were among the first to show that differences in Tat could have an effect on Tat 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. The authors found that all six vari the ability to bind the TAR region as well as cross the membranes, to transactivate an HIV-1 LTR-reporter gene in stably transfected HeLa. The 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 clade-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 in primary monocytes,

leading to differential expression of genes including those for 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 t metabolite SMX-HA than both the parent cell line Jurkat E6.1 a expressing cell lines. An important feature the Tat48GFP and ΔTatGFP cell lines have i common is that they are both missing the protein transduction During an HIV infection, cells infected with the virus produce Tat intra eventually able to leave the cell as extracellular Tat and enter uninfected cells of Tat to translocate across the mammalian cell membrane of the PTD which also contains the NLS. Without the PTD and NLS, both the Tat48GFP and ΔTatGFP fusion proteins escape the nucleus and accumulate in the cytopl The best studied properties of the Tat protein include its transactivation of the viral genome and its ability to regulate the expression of various cellular genes. Amongst those genes are several of the cytoskeleton which have been found to be down-regulated by Tat (Carpenter 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 in a transiently transfected ... Tat interacts directly with α/β-tubulin dimers and polymerized microtubul

the formation of abnormally stable microtubule and prevention of microtubule depolymerization (Chen et al., 2002; Huo L, 2010). The ability of Tat require integrity of four amino acids in the 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 Tat. This demonstrated that the N-terminus and C-terminus of Tat are not necessary fo interaction with tubulin. The functional consequences of the Tat-induced loss of the dynamic property of the microtubules is sensed by the Bim signal p apoptotic relative of Bcl-2, to mitochondria (Giacca, 2005; Matarre & Malorni, 2005). This is corroborated in preliminary experime incubation of Tat48GFP and Tat GFP fusion protein with SMX-HA leading to apoptotic cell death.

Both extra- and intracellular Tat display the 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. 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 protei and β-tubulin. While binding of extracellular Tat to cytoskeletal crucial in activating the mitochondrial-dependent apoptotic pathway, the do

of these proteins by intracellular Tat appears to stabilize cell and explains the absence of apoptosis despite the high expression, suggesting that intracellular T 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 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 rather than being endocytosed. Where the protein c retained through the binding to cytoskeletal proteins such as tubulin, leading to cellular apoptosis (Chen et al., 2002; Epie et al., 2005).

Neither case of intra- nor extracellular Tat completely represent the Tat48GFP and Tat64GFP cell lines. While Tat48GFP and Tat64GFP are both synthesized intracellularly, and thus capable of regulating cytoskeletal genes, the absence of the NLS due to the loss of the 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 a higher sensitivity to SMX-HA toxicity rather than direct apoptosis. Also, the fact that the GFP-Tat protein has a C-terminus mutants

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 to mitochondria can often lead to detrimental consequences such as oxidative damage to mitochondria mtDNA, which encodes proteins of the respiratory chain, which can 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 membrane. 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 in mitochondrial mem eventually the collapse of the mitochondrial membrane potenti (Baines et al., 2007; Kokoszka et al., 2004; Schinzel et al., 2005).

Another possible consequence of heightened cellular intracellular signalling and regulation. Specifically, ROS activate JNK, signalling pathway is associated with cell 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 signalosome that AS functions as a redox switch that senses cellular ROS and oxidizing

conditions (Fujino et al., 2007). ROS initiate Trx dissociation from th[e] signalosome through oxidation of Trx-1 active site redox-active cysteines. Th[e] AKS1 is thus activated and able to signal downstream JNK apoptosis, either via the mitochondria or AP-1-dependent transcription of pro-apopt[otic] signaling (Circu & Aw., 2010)

Given the ability of Tat to modulate cellular redox balance by producing ROS, the role this plays in apoptotic signaling would be beneficial to exp[lore] contribution of the other HIV-1 proteins to post-translational mechanisms such as the formation of protein thiols as a mechanism of redox state regulation. The oxidation of peroxiredoxins that occur at high concentrations in Jurkat cells (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 AIDS during HIV-1 infection.

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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 tissues in HIV-

infected individuals (Castagna et al., 1995; Droge & Holm, 1997; Staal et al., 1992; Staal et al., 1992). 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 and integrity (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 of HIV-infected individuals and low th

in serum and CD4+ T cells (Ball et al., 2009).

This oxidative stress in large part is due to the HIV-1 transactivator of

transcription (Tat). Besides being a potent viral gene transactivator, Tat is secrete

from HIV-1-infected cells and enters uninfected cells in a biologically ac

regulates the expression of cellular genes (Huigen et al., 2004; Rom et al., 2010).

There are two ways for Tat to influence the cellular redox state, either by decreasing the

antioxidant concentrations or increasing the oxidant capacity. The ability of Tat t

decrease antioxidant concentrations is linked to its ability to regulate genes. For

example, Tat suppresses the expression of manganese superoxide dismutase (MnSOD), a

mitochondrial enzyme that is part of the cellular antioxid

stress (Flores et al., 1993; Westendorp et al., 1995). The HIV 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 (Pocernich 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 catalytic intermediates of many enzymes. This allows oxidants to alter the act modifying the redox state of functionally and catalytically essential thiols serves as a signal transduction mechanism that couples p functional activity. Many regulatory proteins contain critical reactive cysteine residues that can interact with a variety of oxidants, to form many covalent c modifications. These include inter- and intra-molecular disulphide protein

mixed disulphides with GSH 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. The
redox state of this domain is known to be essential to protein function and activity,
confirming the presence of 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 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, SMX-HA separately or in combination, had on oxidation
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 the American Type Culture Collection. The cell line was maintained in RPMI 1640 medium (Invitrogen) supplemented with 2 mM L-glutamine, 10% fetal calf seru sodium pyruvate and 100units/ml of penicillin and streptom

Construction of plasmids and stable cell lines

The Tat gene was amplified by PCR from the pSV Tat plasmid. This plasmid encod full-length Tat gene and was a kind gift from Molecular Virology KT Jean Section, NIAID, NIH. The full-length Tat gene (Tat101) with two stop deletion mutants (Tat72 and TatΔ(49-57)) were cloned into the N1 plasmid (Clontech Inc.), resulting in Tat101GFP, Tat72GFP and TatΔGFP fusion genes respectively. The fusion genes were excised from NheI/NotI pEG fragment and inserted into the NheI/NotI sites of plasmid pBIG2i, an ind doxycycline-responsive expression (Strathdee et al., 1999). The result was pBIG Tat101, pBIG Tat72GFP and pBIG TatΔGFP. 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 fusion and GFP 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 dilutions and
the resulting cell lines were maintained in complete RPM
supplemented with 1% glutamine, 10% fetal calf serum, 100 units/m
streptomycin and 0.5 mg/ml of hygromycin B.

4.2 Purification of polyclonal antibodies from serum of SMAXHA immunized rabbits

The polyclonal SMAXHA antibody was prepared by Dr. Jane
laboratory if Dr. Micheal Rieder at Robarts Reasearch Ins
Ontario. Pre-immunization serum from New Zealand white rabb
SMXHA conjugated to Keyhole Limpet Hemocyanin (KLH) wa
and stored at 20°C. The pre-immunization serum samples (10 ml) w
overnight at 4°C with continuous stirring, against 2 L of PBS (pH 7
and Dialysis membrane (1.8 cm/ml) (Spectra/Por). The immune serum was puri
using a Protein G Sepharose 4 Fast Flow column (Amersh
selectively capture all IgG antibody isotypes. 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 1 L of PBS (pH 7
until needed.

4.3 Slot blot

Cells from the different cell lines were incubated with
treated with varying concentrations of SMXHA for 2 hours. The cells were

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). The cell lysates were son
twice for 20 s each time and then centrifuged at 13,000 rpm 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 (Dot-Blot Apparatus, Bio-Rad), according to the manufa
instructions. Briefly, after assembling the Dot-Blot apparatus with the p
nitrocellulose membrane, the apparatus was loaded with the samples and vacuum
flow valve was adjusted to allow the entire sample to filt
gentle vacuum then each well was washed with 150 µl of TBS (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 1 hour at room temperature with shaking and then probed ove
4°C with anti-SMAD4 rabbit polyclonal antibody, 1:1000 dilution of
The incubation with the primary antibody was followed by w
for 10 min each time with TBS-T. The anti-SMAD4 antibody was visualized by
anti-rabbit antibody diluted to 1:15,000 dilution with TBS-T then treate
chemiluminescent agent (Thermo Scientific Pierce ECL We
visualized with a Fluorchem imaging system (Alpha Innotech).

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

4.24 Sample preparation for redox 2D gel electrophoresis

Jurkat pBIG TatGFP-3 cells (2.5 x 10^6 cells) were induced with 0, 200 1000 ng/ml of doxycycline for 40 h at which point the cells were washe phosphate buffered saline (PBS) by centrifugation at 1500 rpm for 5 min. The cells were resuspended in HEPES buffer and seeded at 1 x 10^6 cells/ml concentrat. The cells were then treated with 200 µM SMXHA or the vehicle (DMSO) at concentration of 0.5% then incubated for 2 h at 37 ^oC. The cells were subs collected and lysed in RIPA buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA (pH 8.0), 1% SDS, and complete mini protease inhibitor cocktail (Roche cat. # 1 836 170) a Sigma). The cell lysates were sonicated on ice twice for 20 s then centrif at 13,000 rpm for 15 min at 4 ^oC. The supernatant was collected and stored at -80 ^oC for future analysis.

4.25 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-PAGE

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 were resolved on a 1.0mm thick, 10% p
gel by subjecting the gel to electrophoresis at a constant current of 24mA for about
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 fresh SDS sample buffer containing 100mM DTT
room temperature with gentle rocking. After another wash w
strip was applied horizontally to a 10% polyacrylamide g
23cm(L) x 20cm(W) x 1.0mm(T). Electrophoresis was carried out in the sec
dimension at constant current of 24mA for about 1.5h. Each treatment of every cell
line was subjected to the above redox 2D electrophoresis
times.

4.27 Silver staining and analysis of 2D gels

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

washing with deionized water for an additional 20 min. The development of the gels were conducted in a solution of 6% sodium carbonate, 0.012% s formaldehyde until the desired gel resolutions were obtain incubated in a 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 GBS. Means and standard errors (SE) were used to report continuous variables. A two-way ANOVA with Tukey's post procedure was used to compare differences between groups for concentration levels and 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 reaction (ADR) is based on initial hapten formation which may or may not lea hypersensitivity. In the case of sulphonamide SMX, it is metabolically activated to chemically reactive SMX-HA, an electrophilic metabolite that acts as a hapten and becomes covalently conjugated to cellular proteins. After antigen processing presentation, immune responses are directed against the hapten-protein conjugate. Previous data from the Rieder 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 protein, followed by 24 h treatment with various concentrations of the reactive metabolite SMX-HA, and protein isolation. Proteins in cell lysates (15 µg) were subjected to SDS-PAGE and blotted with an anti-SMX-HA antibody to visualize the haptenation pattern for each (Fig. 4.1). The qualitative differences between the haptenation patterns o decipher on the western blots. To quantify the gels, identical 2D amounts of the different protein samples were loaded on slot blots and the mem 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 induction

(A) Image from a western blot of cells treated with 10-200 µM SMX-HA for a period of 2 h, protein extracted and resolved on a 12% SDS-PAGE, transferred onto a P membrane which was then blotted with the 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 the cells treated with 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 (C) for GAPDH.

Figure 4.1

SMX-HA-specific polyclonal antibody preparation means that there were no bands that could be detected on the membrane for 0µM SMX-HA (solvent control) or 200µM SMX (negative control). The membranes were then stripped and probed with anti-GAPDH antibody and the 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 Figure 4.2.

All the cell lines had baseline SMX-HA/GAPDH ratios of approximately 0. in the absence of SMX-HA treatment (Figure 4.2). The ratios increased 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 Jurkat cell line and E6.1 showed a 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% lower respectively (Figure 4.2).

Induction of Tat101 expression to levels approximating those seen in HIV-infected Jurkat cells with 400ng/ml Dox treatment 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 400ng/ml

2-fold, respectively and with the addition of 200μM SMX the ratio from th 1000ng/ml Dox treated sample at 200μM SMX-HA was double that observed at 50μM SMX-HA, 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 Jurk and Tat101 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 concentration-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 the HIV infected cells, the ratio decreases to the 50μM level at 200μM SMX-HA. The ratio at 100μM SMX-HA for the 200ng/ml Dox sample was significantly higher than that seen for untreated samples and also significantly higher than the 200ng/ml Dox sample at 200μM 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 6.1, (B) Tat101 GFP, (C) Tat101, (D) (E) Tat GFP or (F) GFP cell lines were treated with SMX-HA for 2h and then protein was isolated. The protein samples were applied onto memb and then blotted for GAPDH and SMX. The band intensities were measur and the ratios plotted. Data are mean of three independent experimen p, $p < 0.05$ vs. 0, 400, 1000 ng/ml Dox at 0 µM SMX-HA, respectively. $P < 0.05$ vs. 0, 400, 1000 ng/ml Dox at 5 µM SMX-HA, respectively. $P < 0.05$ vs. 200 ng/ml Dox at 0 µM SMX-HA

Figure 4.2

Results from "GFP Tat" cell line were somewhat different. concentration-dependent increase of the ratio for each. At 50 and 100µM SMX-HA 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 in vivo, that was hig than that of the 0ng/ml sample (solvent control) (Figure 4.2). The ratios from the GFP-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 HA-mediated haptenation. It also appeared that doxycycline at the concentration 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 haptenation T expression is known to alter the overall pattern of gene therefore may alter a set of genes to make the cellular environment more vulnerable to protein haptenation. To explore this possibility, non-reducing/reducing SDS/2 electrophoresis was employed as it provides a direct system to both identify proteins that are redox regulated via oxidation of cysteine thiol groups protective pr through formation of protein-protein mixed disulphides. Proteins that contain reduce 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; 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 protein dimerisation disulphides and the attendant decrease in molecular mass afte dimension. However, proteins with intramolecular disulphides show an increase in 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 or its deletion mutants and more specifically to document oxidative changes (i.e. enh protein mixed disulphides) as a result of treatment with th -HA. 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 of protein samples from different control cell lines, including untransfected Jurkat cells, Jurkat E6.1 incubated with and without 1000ng/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 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

200µM 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 seen in 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-HIV, (C) JFP 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. The gels from the panels and B are duplicated and used as reference in Figures 4.4-4.6

Figure 4.3

intermolecular disulphide bonds (below the diagonal), most notably the spots located just below 30 kDa circled (and labelled 1) which are the 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 isoform 2, and peroxiredoxin 3) from work in the laboratory of Dr. J. Beck at the University of Western Ontario [London, Ontario; Alwaysheh, MSc Thesis, 20 shown in Figures 4.3-4.6, all the samples from the different cell lin of induction displayed the same protein spot patterns. An analysis of the di gels indicated that overall the gels 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 gel showing the most protein spots (around 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 4E, respectively) displayed the same number of disulphide proteins at 0 µg/ml doxycycline with five of the six proteins resolved below the line on the d Both cell lines had proteins 1, 2, 4, 5 and 7 in common (Figure 4.4C and 4.4E). The Tat101GFP cell line (Figure 4.4D) had proteins 1, 2, 5, 7,

Figure 4.4. Redox-dimensional PAGE of thiol proteins from (A) Ju
(B) Ju-HKa/t, (C) at101, (D) at101GFP, (E) at72CGFP and (F) Tat
doxycycline. 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 overni
dimension gel. On the left side of the diagram 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) Jurkat, (C) HIV-Tat101, (D) Tat101GFP, (E) Tat72GFP, with (F) Tat
panels C-F induced for 40hrs with 400ng/ml doxycycline (C
doxycycline (D and E) prior to 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 on the first dimension gel and run for 3hrs foll
run of the second dimension gel. On the left side of the diagonal are
molecular weight proteins that 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 with (F) Ta
panels C-F induced for 40hrs with 1000ng/ml doxycycline prio. 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 onto
and run for 3hrs followed by an overnight run. On the left of the seco
side of the diagonal on each gel are molecular weight that are
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 4.4H).

I first examined the different cell lines treated with co[ncentrations] that would induce the expression of Tat or TatGFP mRNA to approx[imate the levels] 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 13. The induction of Tat101GFP... 200ng/ml doxycycline did not change the number of proteins that resolve... away from the diagonal compared to the 0ng/ml Dox sample of... the Tat72GFP cell line (induced with 200ng/ml Dox) (Figure 4.5E) ... the disappeara[nce of] protein no.4, leaving proteins no. 1, 2, 5 and 7 as well as a new protein (designated protein 12). The protein pattern of samples from the TatGFP cell line induced with 400ng/ml Dox (Figure 4.5F) showed the appearance of protei[n] below the diagonal of protein 7. A new protein (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 gels showing dis... samples from the GFP cell line consistently had few protein spots of ... doxycycline responsive cell lines, proteins 1, 2 and 7 were found in the... treatment with at 400ng/ml Dox

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

Dox (Figure 4.6E) shows the appearance of a protein m in comparison to the ge 200ng/ml Dox (Figure 4.6E). The level of GFP at expression induced at 10 doxycycline (Figure 4.6F) resulted in a protein pattern almost identi 400ng/ml doxycycline with the exception of the increased 10 and i 11. The induction of Tat101GFP in the cells with 1000ng/ml doxycycline caused the disappearance of protein m (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 4.3A).

The control cell lines treated with 200µM SMX-HA for 2 hours. One of the obvious differences caused by the addition of SMX-HA is the dramatic reduc in the intensity of the putative peroxiredoxin proteins 10 and 11 (Figure 4.7) in the (cell lines treated with SMX-HA. There is also a 2 to 3 fold increase in the number proteins resolved below the diagonal line. Many of these proteins were also absence of SMX-HA (Figure 4.4 - 4.6) and are numbered accordingly. The proteins that are the result of oxidative stress resulting from the addition of SMX-HA are lettered in the figures and in Table 4.1.

Treatment of the parental cell line Jurkat E6.1 with SMX-HA affected 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 protein spots 1 and 4 in the latter indicating lower amounts of oxidized proteins, as occur as prot

Figure 47. Redox-dimensional PAGE of thiol proteins from vario
cell lines; (A) Jurkat E6V1, (B) GJRuP 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 f
the second dimension gel. The gels from panels A and B are
reference in figure 48. The left side of the diagonal on each
weight proteins that are enumerated on both sides of the ima

Figure 4.7

protein mixed disulphides) in the Jurkat E6.1cells treate treatment of the HIV-infected Jurkat cell line 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 the lettered proteins

With minimal Tat protein induction at 0ng/ml Dox, cel fusion cell lines were treated with 200µM SMX-HA and the results are shown 4.8. The "GFP Tat" cell line shows the most proteins, the majority of (Figu which are proteins with intermolecular disulphide bonds, ru of unresolved proteins on the gel. In the Tat101 cell line vehicle control cells (0ng/ml Dox), resulted in the appearance 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 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 4, 8, 10, 11, 13 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 disulphides in absence of SMX-HA at that concentration of doxycycline (Figur SMX-HA-specific proteins a, b, c, d, g and i were also resolved in t

The induction of RNA expression in the different cell li
approximately found in HIV infected cells followed by treatment with 200 μM
SMX-HA and the results of redox 2D electrophoresis of cell
Figure 4.9. In the Tat101 cell line the intensity of proteins 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). The
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
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 (Figure
analysis.

Redox 2D electrophoresis of protein samples from ly
1000ng/ml Dox then treated with SMX-HA as shown in Figure 4.10. In the
line, proteins 1, 2, 3, 4, 5, 7 and 12, in addition
(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 cells in the intensity of
18 and the addition of proteins a, f, i and r, the last of w
(Figure 4.10D). again the GFP cell line presented the most prot
severe oxidative stress, the addition 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 Tat72GFP expression with 400ng/ml or Dox produced proteins 10 and 11, two proteins otherwise from the Tat72GFP cell line were treated with SMX-HA. Tat72GFP induction followed by treatment with SMX-HA resulted in protein no. 6 being reso also seen in the Tat72GFP cell line. Furthermore, the max 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 over-expressing cell line. There were also a numbe redox 2D gels of lysate of HIV-infected cells, both in the absence and presence HA that were unique to this cell line. Overall the control c as the parental Jurkat E6.1 cell line produced very few proteins, -HA, in comparison to the other Tat-expressing cell lines and the HIV-infected cell line.

Figure 4.8 Redox-dimensional PAGE of thiol proteins from (A) Ju
(B) Jurkat V, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP and (F) Tat
doxycycline. redox 2D gels in panels A and B are replicas
4.7. Cells from each of the various lines were heat 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 r
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) Ju-HIV-Cat, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP and (F) Tat72'GFP, with 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-HSAM 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 standards that are numerated on both sides of

Figure 4.9

Figure 4.10 Redox-two-dimensional-PAGE-SDS of thiol proteins from (A) Ju
(B) Jurkat-HIV, (C) Tat101, (D) Tat101GFP, (E) Tat72GFP, and (F) Tat72GFP the last
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 used as 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 first line treatment and prophylaxis of Pneumocystis pneumonia (PCP), a common opportunistic infection in HIV patients. Although ve 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 (2-5%). The mechanisms that lead to the developm are still unclear, but one of the explanations is the Hapten Hypothesis. This states that the initial step in the pathogenesis of hypersensitivity ADR is the metabolic activation of the drug, in the case of SMX, oxidation of the parent dr the hydroxylamine (SMX-HA), a reactive intermediate that can be further oxidized nitroso derivative (SMX-NO). These reactive metabolites or haptens are able to form covalent conjugates with proteins that can be recognized as responses may be directed against the protein, the drug conjugate or both of the Manchanda et al. (2002) showed that concentrations of 25 μM and above SMX-HA 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 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 on high dose Co-trimoxazole therapy have peak SMX plasma concentrations of 1.5 SMX-HA concentrations can be 1-20% of these plasma concentrations 0.015-4 mM and SMX-NO accounts for approximately 2% (Manchanda et al., 2002). The concentrations of SMX-HA used in this study therefore fall within the therapeu range attained in vivo.

Our data also showed that the induction of Nef to levels comparable to those seen in HIV-infected cells increased the HA level haptenation of protein (Figure 4.2). The reactions of drugs with proteins formation of bonds of different strengths, such as ionic and hydrogen considered weak interactions, and coordinate covalent bonds considered bonds. The interaction between a hapten and a protein res covalent bonds, allowing the production of an antigen. The metabolites of SMX reactive electrophiles containing a benzene ring and are 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 group (Divkovic et al., 2005). Proteomic studies have shown th containing reactive cysteine thiol groups, which have enhanced nucleophilicity du ionization of the cysteine thiol, are therefore preferred sites for hapten rea (Liebler, 2008; Wong & Liebler, 2008). The Nef protein has a cyste- rich domain (amino acids 2-37) that would make it a very attractive electrophilic metabolites of SMX-HA. Functional analsysis 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-rich domain form intramolecular disulphide bonds for criti
Tat activity, there remains free thiols that can act as nucleophil (Kalantari et al.,
2008). Both control cells, the Jurkat E6.1 and GFP-expressing cell line
showed significant haptenation as shown by their SMX-HA/GAPDH ratios. This is
not unexpected as the SMX-HA can bind to several types of am
chains on cellular proteins that would by definition be 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 other Tat constructs. This could probably reflect the fact
that all Tat constructs used in this study contain the cysteine-rich domain. One of th
differences seen is the discrepancy between the doxycycline-induced samples of
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 SMX-HA to react
form of the target protein may be hindered or enhanced by the nucl
three-dimensional protein environment. For instance, a nucleophilic side chain may be

embedded amongst residues with non-polar hydrophobic side chains, making i accessible only 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 reactivity and degree o nucleophilic side chains (Divkovic et al., 2005). The degree of side chain ioniz also be altered depending on the site of haptenation as more acidic than the cytoplasm (Divkovic et al., 2005).

Common to the Tat101 and Tat101GFP cell lines as we is the significant decline in the HLA/GAPDH ratios of the SMX doxycycline 1000 ng/ml induced samples, particularly at 200 μM SMX-HA. This can perhaps be attribu ability of doxycycline, a tetracycline, to bind non-microbial targets such as matrix metalloproteinases (MMPs), facilitating its use in the treatment of oth (Golub et al., 1987; Griffin 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 such as 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+} in comparison to 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 chelate-doxycycline molecule would bind to the nucleophile i the amount available for adduction with the electrophilic SMX-HA. This in turn would mean less SMX-HA-protein complex formation. Hence less protein-SMX-HA complex would be detected by the antibody resulting in lower SMX-HA/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, one of the most susceptible potential targets is the thiol group in proteins with reactive cysteine thiol residues (PS⁻). We have shown previously increasing concentrations of SMX-HA increase ROS activity in the cell here (previous chapter). In the presence of increasing concentrati the oxidation of cysteine residues within a protein can result in a oxidative modifications, among them 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 thiols of other proteins to form glutathionylated prote proteins (protein glutathione mixed disulphides) or protein-protein mixed disulphides where these can be either homodimeric or h Those disulphide bridges are known as intermolecular whereas those between thi the same protein are intramolecular disulphide bonds. 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 the reactive metabolite SMX-HA has on these interactions.

The presence of the reactive cysteine thiol groups in Tat protein as targets for SMX-HA haptenation, which may also provide the chance to form intramolecular disulphides as well as intermolecular protein mixed disulphides. A glutathionylated forms of protein are not resolved by one or 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 residues are indispensable for the transactivation function of HIV protein; indeed, the transcriptionally active form of Tat and reducing agents have been found to strongly inhibit its activity, suggesting that these residues i Tat form intramolecular disulphide bonds required for transcriptional activation (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 this end, we carried out 2D gel electrophoresis experiments to determine their expression in T cells and of its deletion mutant and to characterise any quantitative or qualitative changes in disulphide bonds, and what effect, if any, the a metabolism SMX-HA will have on protein disulphide bonds in Jurkat T cells.

The most ubiquitous and abundant of the prominent spots on the 2D gels were the putative peroxiredoxins. Peroxiredoxins (Prxs) are antioxidant enzymes found in all organisms with the single exception of Borrelia burgdorferi (and other Borrelia species) (Hall et al., 2009). This family of antioxidant enzymes are proteins approximately 20–30 kDa in size and use specialized cysteine residues to decompose perox Prx towards peroxides in combination with their high expression levels make proteins of the Prx family are a primary line of defence against p critical role as cellular antioxidants (Peskin et al., 2007). The physiological importan peroxiredoxins is illustrated by their relative abundance; Prxs are one of 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 Prxs found in T cells, Prx1 being the major cytoplasmic form in Jurkat T cells (Chae et al., 1999). All Prx enzymes contain conserved reactive 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 head-to-tail (Seo et al., 2000). Peroxide detoxication 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 peroxide to a sulphenic acid intermediate, C_P-SOH at the active, reactive cysteine. The resolution step occurs 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 and the C (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 disulphide (Bang et al., 2005; Wagner et al., 2002). This leads to loss of peroxiredoxin activity and the formation of the dead end sulphinic acid in competition with the resolution step of 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 enzyme. However, when exposed to stron oxidative stress, the normal form of Prxs disappears due inactivation by oxidation which effectively acts as a redox indicator the (Rabilloud et al., 2002)

The presence of the Prxs protein in sulphinic form (figure 1a) each of the 2D gels Jurkat cell lysate samples demonstrates that the E6.1 Jurkat were being subjected to mild oxidative stress even before the treatment with SMX-HA. There is an 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 that is oxidized to Cys-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 or al (Cys-SOH) is further oxidized to sulphinic (Cys-SO_2H) or sulphonic acids (Cys-SO_3H) instead of forming a disulphide. The formation of the sulphinic acid (Cys-SO_2H) is reversible by ATP-dependent reduction of Cys-SO_2H by sulphiredoxin (SRX) (D'Autreaux & Toledano, 2007). Additional oxidation of the 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 (pro-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-1 or HIV deletion mutants also showed an apparent decrease in the amount of pro-1 at every concentration of doxycycline both the parental cell line and the HIV-1 infected cell line there seems to apparent decrease in the amount of pro-1 between the uninduced and ma induced cells of the Tat-expressing cell lines. This suggests there was an inactivatio the Prx enzyme to compensate for the further oxidation of peroxiredoxin and that the expression of Tat or deletion mutants resulted in additional ox within the Jurkat T cells.

The addition of SMHA to the different cell lines also lead to further lower 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 disulphide (Figure 4.7). The treatment of the Tat-expressing cell lines with SMHA also resulted in th inactivation of Prx and an apparent decrease of the amount of the protein pro-1 compared to the cells treated with no doxycycline (Figure). For the cell lines expres Tat101, Tat72 and GFP, 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 to be 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 -Lynn et al. (2003). In that study the authors compared gene expression CD8+ T cells of HIV seropositive and bulk CD8+ T cells of seronegative individuals and found tha Prx1 and Prx2 genes were expressed in greater quantity in bulk CD 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, can be -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 cDNA 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 (Masutani et al. 1992; Okamoto et al. 1992)

A report by Mais et al. (2007) corroborated the finding about Prx2 showing significantly elevated serum levels of Prx2 in HIV-1-exposed, uninfected individuals compared to their HIV-infected sexual partners and also comp controls. Furthermore, RT-PCR analysis conducted on activated purified T subsets from each cohort showed that Prx2 transcripts were detected mainly 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 the Tat-expressing cells with SM HA is that the HIV-1 Tat protein is able to suppress the expressio as glutathione, via 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 such as Vpr, CA, NC and PR that can potentially form disulphide bonds compared to the cell lines expressing just the Tat protein. The GFP cells had the fewest protein both in the absence and presence of SMX-HA. This is probably due to the fact that the variant of GFP used in this study is not k thiol groups, although it could be potentially due to any antioxidant properties overexpressed GFP possesses.

The "Tat-GFP" cell line consistently demonstrated 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 in the established cell lines. With the exception of the "Tat-GFP mutant" 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. While the majority of the Tat-GFP construct expressed can be found in the nucleus, the loss of the NLS also means readily detected throughout the cytoplasm. In contrast, the intracellular distribution of SMX-HA as determined by Manda 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 protein binding by SMX-HA could be visualized inside the cell near the cell membrane within five min of the cell incubation with SMX-HA. Sixty minutes after exposure to the metabolite, protein binding was visualized throughout the cytoplasm, concentrated discrete areas, but was not 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 in the effects of exposure to SMX-HA. SMX-HA might accumulate more quickly in the presence of SMX-HA to 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 the 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 2 h, the cellular distribution showing SMX-HA binding to protein only in the cytoplasm may be 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 (Aikoumani et al., 2009).

The nuclear envelope separates the nucleoplasm from cytoplasmic 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 cytoplasm

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 (Ribbeck 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 ~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 relative proteins in 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 protein oxidation is selective or part of more global oxidative changes. They used a proteomic approach to 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 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 SMX-HA treated 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 Bradley et al (2005) are moesin, heat shock protein 90², ubiquitin thiolesterase and elongation factor 2, all of which have estimated MW-116 kDA and 96 according to their positions on the 2D gels. That is the same molecular weight range of proteins p, q and r that were unique to the GFP-Tat-expressing cell line after SMX-HA treatment. These proteins may be important as they are potential targets for GFP-Tat, the only molecular Tat mutant with significant cytoplasmic distribution. Moesin is a protein involved in cell structure, functioning as a cross-linker between the plasma membrane and actin filaments. Heat shock protein 90² and ubiquitin thiolesterase are involved in protein folding and degradation, respectively while elongation factor 2 is used in translation and RNA processing. All four are proteins that primarily reside in the cytoplasm.

Further proteomics research in the interactions between Tat and cellular proteins can lead to increased understanding of the mechanisms behind the development of ADRs. One future direction of this study particularly includes the use of techniques such as mass spectrometry to isolate, purify and identify the metabolite-protein adducts in the context of SMX-HA. That would also be beneficial to determine which proteins are involved in the formation of differences the expression of Tat has on 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 by the reactive metabolite SMX-HA. However, the amount of SMX-HA induced haptenation may be less important than the specific protein or cellular target distribution of the Tat protein may be determining specific protein targets 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.

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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 infection h progressed to clinical AIDS, was tenfold 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; immune reconstitution mean need for prophylactic therapy for opportunistic diseases reduced and subsequently, th problems associated with it have decreased (Pirmohamed, 2007). As well, among patients with well controlled viral loads the rate of A 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 and also the impact they have on the development of medicines (Pirmohamed, 2009). of the developing world, disease prevalence, access to medications, d drug management are markedly different from those of developed countries and thus impact 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 the 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 HIV-infected 60% of these 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 the administration of cotrimoxazole and thus represents an ideal model to investigate mechanisms of

hypersensitivity DRs in HIV-positive patients, 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 analyse the mechanisms after treatment with SMX-HA. The data
in Chapter 2 demonstrated that the expression of the first exon of Tat in 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 concentration. We concluded that the first exon of Tat w
affect the redox state of Jurkat T cells. We also concluded that oxidative str
primary mechanism driving cell death.

After obtaining full-length Tat protein from a different isolate, we
constructed deletion mutants in an effort to determine the
was responsible for the toxicity that was found 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, to some extent with a minor role. Of significance,
the deletion mutants had all the same effect as the full-length Tat

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

Evaluation of the consequences of the oxidizing activity of SMX-HA was studied in Chapter 4 by analyzing changes to the cellular redox proteome. Redox two-dimensional (2D) electrophoresis of lysates of cell lines expressing Tat from the produced numerous spots that arose from proteins with intermolecular disulphide bonds. One of the proteins that was highly expressed was peroxiredoxin, an enzyme, the apparent intensity of which in HIV-infected cell lines was lower, under less oxidative stress than the Tat-expressing cell lines, even with exposure to SMX-HA. Exposure of the cells to SMX-HA led to dramatic increase in oxidation of cysteine thiols in proteins in the redox 2D gels.

A major obstacle to the eradication of HIV-1 is persistence of the virus in reservoirs. In patients that have not been treated with AR 90% of plasma virus is mainly generated by repeated replication of infected activated CD4+ (Coiras et al. 2009). The residues to viral replication from the cellular produces HIV-1 at lower rates in resting T cells, infected dendriti macrophages, which are more resistant to virus-induced cytopathic effects in anatomical sites with low pharmacological activity for ARV the ARVs to penetrate tissues such as the central (Aquaro et al. 2008; Chun et al. 2008; Coiras et al. 2009; Guadalupe 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[n to exit] infected cells and enter, uninfected bystander cells. When Frankel and Pabo (198[8]) demonstrated this unique property of Tat, their results su[ggested that uptake] was followed by translocation into the nucleus in a transcriptionally active form. [They] determined that it took 6 h for approximately 80% of the in[ternalized Tat to accumulate] in the nuclear compartment. This means there is a substantial portio[n of Tat in the] cytoplasm for up to 6 h. Furthermore, the basic domain allows Tat to [enter many tissues] including the heart, liver, lung, spleen, kidney, brain and s[keletal muscle] (Schwarze et al., 1999).

Therapy with SMX produces a system wide distribution and while the drug ... liver is quantitatively the primary site of bioactivation fo[r SMX, bioactivation has] also been shown to occur in other organs, including the lung and cells of the ski[n and] immune system (Cribb et al., 1990; Vyas et al., 2006). The cytoplasmic distributi[on of Tat in] the "Tat-GFP cell line" was likely responsible, at least in part, for the increased sensit[ivity] of the Jurkat CD4+ T cells to SMX-HA treatment (Chapter 3). The 2D electroph[oresis] 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 is unknown. This scenario is one where full-length (wild-type) Tat protein is released from HIV-infected cells, enters uninfected cells and localizes to the cytop[lasm] wherein the presence of SMX-HA, Tat lowers the threshold to apoptosis leading t[o] increased cell death of both the HIV-infected CD4+ T cells and of uninfect[ed]

various types (Figure 5.1). The loss of the CD4+ T cell compartment h immune deficiencies for, but the HIV patient 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 contact allergens, chemicals or haptens 2,4-dinitrofluorobenzene (DNFB) and oxazolone (Ox) (Vocanson et al, 2009). CHS responses are mediated by IFN-γ and IL-17-producing CD8+ T cells primed b dendritic cells and 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 hapten-specific CD8+ T cell expansion for CHS responses (Kish et al, 2005; 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 the protein being in the proximity of SMX-HA, raising the cellular toxicity and increasing the incidence of ADRs. Preliminary in th data from experiments looking at the externalization of apoptosis show that the cell death in the 'GFP' and Tat48GFP cell lin the presence of SMX-HA is mediated via apoptosis. This is in keepin

Figure 5.1. The model for increased incidence of ADRs in HIV pati A fraction of the SMX dose is metabolized to SMX-HA and to 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 infecte of uninfected cells where it changes the disulfide proteom for cell death. In the presence of SMX-HA, this would lead to increased antige presentation to CD8+ T cells and the development of an ADR to SMX.

Figure 5.1

found in Adeetya(2009).

Another consequence of increased apoptosis (Figure 5.1) i 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 presented by professional antigen presenting cells (APCs) via MHC I molecules to CD8+ T cells (Heath & Carbone, 2001). 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 cross-present exogenous antigens into the pathway for presentation to CD8+ T cells is known as cross-priming and th resulting stimulation of CD8+ T cells is termed cross-priming (Rock & Shen, 2005).

It is not clear exactly how apoptotic cells are processed and acqui by the APCs, though there is now evidence that the im presentation to monitor tissues for foreign or tumorous antigens from viruses. There is also evidence that individuals with chronic HIV infecti T cells seem to activate a wide repertoire of CD8+ T cells epitope specificities (Rosenberg et al., 2007). Indeed the magnitude of the CD8+ T directed against apoptotic peptides correlated with the decline CD4+ T cells. Raw (2007) suggests the presence of these CD8+ HIV-infected individuals with CD4+ T cells can be attributed to CD4+ T cell help may be due to their ability to be independently stimula

variety of cells 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, antigens derived from intern apoptotic cells has been shown for mouse skin and pancreas-restricted, antigen cells that support HIV infection and are permissive for Tat expression (Albert, 2004; Fleeton et al., 2004; Mandl et al., 2003). In the model of ADRs in HIV p (Figure 5.1), the metabolism of SMX leads to the formation of SMX-HA and SMX-NO, of S both shown to haptenate to cellular proteins in T cells (Manchanda et al., 2002) and most likely in other cell types. The ability of these haptenated cel to then serve as presentation of SMX antigens results in 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 needed to amplify the cell toxicity of SMX-HA. The HIV-1 Tat used by SMX in that report and the experiments in Chapters 3 and 4 were from two different sources and as a result had amino sequence differences. It would help clarify the cell viability data in Adeyanju et al. (2009) and Chapter 2 and the results describ Chapter 4 if the two Tat variants were subjected to HIV-1-LTR-Luc (HIV long terminal repeat luciferase) reporter assays. The assay would determine the transactivation 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 stable transfection of the Tat protein HIV mutant that retains the protein transduction domain (PTD nuclear localization sequence (NLS) exclusively, to distribu protein in the nucleus. Indeed there was found to be decreased micro experiments suggest that these proteins (Tat101GFP, Tat8 ventured out of the nucleus to impact other organelles s biochemically by these results, we could carry out mitoch subfractionation of cell extracts by differential centrifugation followed and immunoblotting with anti-Tat antibody.

The data in Chapters 2 and 3 showed that stably transfected cell lines showed a decrease in cell viability when treated with HA. However, in contrast, cells transiently expressing Tat101GFP showed a small additive effect on cell viability when treated with HA, but no significant effect with the other expressing Tat constructs, Tat86GFP, Tat72GFP Tat48GFP" GFP (Chapter 3) would be interesting to determine if differences in cell viability between the Tat protein and the deletio they were applied to the T cell culture, both in the presence other words, to see what effect extracellular Tat has on a T c without 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 further research is to evaluate the cytokine profile produced by the incubation of peripheral blood mononuclear c protein first followed by SMX-HA. A similar study by Lee 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 increase in IL-4 production, leading them to conclude PBMC exposed to SMX-HA could lead to a heightened T_H2 immune response. A T_H1 immune response is thought to account for some of the characteris hypersensitivity ADRs such as delayed onset and fever. Tat can 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 Tat protein could lead to a more robust T_H1 response. This might account, at least in part, for the higher incidence of hypersensitivity A 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 systems of developing nations. The mechanisms underlying development of hypersensitivity ADRs are incompletely defined, results of 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 sulphonamide metabolite, SMX-HA.

5.4 References

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APPENDIX

Curriculum Vitae

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Postsecondary Education and Degrees:

Honours Bachelor of Science
Department of Biochemistry
University of Ottawa
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1998-2000

PhD, Microbiology and Immunology
University of Western Ontario
London, Ontario, Canada
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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
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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 PCR and RT-PCR
- Isolation of genomic and plasmid DNA
- Gel electrophoresis
- Restriction enzyme analysis
- Redox-two dimensional 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): 327-32.

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Oral Presentations:

Adeyanju, K. The Contribution of the HIV Tat protein to ... induced by Sulphamethoxazole-hydroxylamine. MICROIMM 540y, Depa... Microbiology and Immunology, The University of Western O... April 2005.

Adeyanju, K. The Contribution of the HIV-1 Tat protein to HIV Adverse Drug R
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Adeyanju, K. The Contribution of the HIV-1 Tat protein to HIV Adverse Drug R
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Adeyanju, K. The HIV Tat protein and Adverse Drug Re
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November 20, 2006.

Adeyanju K. Understanding the mechanism behind adver
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The University of Western Ontario, London, Ontario. October, 2008.

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Activities and Contributions:

I was a member of the governing council of the Society
representative of the department of Microbiology and Imm
Western Ontario 2003-2005

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 2003-2005

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 an
graduate degree. It can be viewed at http://www.uwo.ca/sogs/WGR/index.h
2008.