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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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THE HIV Tat PROTEIN AND ADVERSE DRUG REACTION
A MODEL SYSTEM UTILIZING JURKAT T CELLS AND
SULPHAMETHOXAZOLE/CO-TRIMOXIMINE

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by

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Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfilment
of the requirements for the degree of
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The School of Graduate and Postdoctoral Studies
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Abstract

In 2009 approximately 2.6 million people became infected with Human Immunodeficiency Virus (HIV). In addition to the estimated 33 million currently living with the virus, this makes HIV/ AIDS an epidemic on a scale in modern times. Treatment of HIV infection requires the use of a number of other drugs such as antimicrobials. Hypersensitivity reactions (ADRs) to a variety of drugs used in the treatment of HIV but antimicrobial Sulphamethoxazole remains a major culprit. Hypersensitivity reactions are a significant morbidity, with the skin and liver most commonly affected. The top causes of death in the developed world. While the mechanism of hypersensitivity in general remains incompletely understood, hypersensitivity to Sulphamethoxazole have been linked to one of the HLA class II antigens. Previous work from our laboratory has shown that the HIV protein Tat plays a role in SMX induced hypersensitivity ADRs. We sought to determine if Tat would have an effect on cellular toxicity. We also wanted to know what the mechanism mediated those effects.

We created fusion proteins of Tat and its deletion mutants and expressed the protein and placed them in an inducible vector which was stably transfected Jurkat T cells. We differentially induced expression and then used in assays for cellular toxicity and apoptosis in the presence of SMX. We found that cellular toxicity was dependent on the amount of Tat used. In the presence of SMX, the expression of the Tat protein augmented T cell death caused by SMX and the addition of SMX death occurred via apoptosis. This cell death took place without alteration of

later experiments demonstrating that variant, strongly phenotypically affected death after HASM treatment. Also, expression of protein was able to cause an increase in ROS generated after treatment of wild type mutants had this

To try to further elucidate the role of HIV-1 Tat in redox set of experiments were carried out to detect the consequences of expression in the presence of HIV-1 Tat. Following SMX expression incubation of the Jurkat cells with either vehicle or SMX in the presence of iodoacetamide and the time course applied electrophoresis the absence of SMX expressing cell lines were under a fair amount of oxidative stress compared to the infected cell line. Also in untreated cells, a small number of oxidized exposure of the resting cells to H₂O₂ in SMX did not increase in thiol protein oxidation.

Keywords: HIV-1 Tat, sulphamethoxazole, pyrazonamide, hypersensitivity adverse drug reactions, Jurkat cells, gel electrophoresis.

Co-Authorship

Chapter 2:V Tat potentiates cell toxicity in a T cell
Sulphamethoxazole induced adverse drug reactions

Drs. Dekaban and Rieder supervised the project and aided in the preparation of the manuscript. Aotiana Kriisova is a co-author having contributed equally to the manuscript. (Figures 2.1A, 2.1B, 2.2A, 2.2B, 2.3, 2.4A and 2.4B)

Chapter 3: Cytoplasmic distribution of sulfhydryl oxidase in Jurkat T cells
Sulphamethoxazole and benzylamine induced toxicity

Drs. Dekaban and Rieder supervised the project and aided in the preparation of the manuscript. DiptBend also aided in the preparation of the manuscript and is responsible for carrying out all the experiments in this Chapter with the assistance of Chan and Chowey.

Chapter 4: Detection of oxidant sensitive thiols by electrophoresis in H₂O₂ treated cells by redox sensitive electrophoresis

Drs. Dekaban and Rieder supervised the project and aided in the preparation of the manuscript. DiptBend also aided in the preparation of the manuscript and is responsible for carrying out all the experiments in this Chapter with the assistance of Mao.

All manuscripts in this thesis were written by K. Adeyemi with the assistance/ comments/ contribution of the other authors.

Dedication

This work is dedicated to my parents who always encourage
of my goals.

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LIST OF ABBREVIATIONS, SYMBOLS, NOMENCLATURE

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

IN	integrase
LG ₀	lethal concentration, 50%
LTR	Long terminal repeat
MA	Matrix
MMP	matrix metalloproteinases
mtDNA	mitochondrial DNA
MTT	(3-(4,5-dimethyl-2-yl)-2-phenyltetrazolium bromide
MW	molecular weight
Ni	Nickel
NF- κ B	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 [•]	superoxide radical
PBMC	peripheral blood mononuclear cells
PCP	Pneumocystis pneumoniae
PI	protease inhibitors
pi	pharmacological interaction
PR	Protease
PSH	protein thiol
PSSP	protein disulphide
PSOH	sulphenic acid
PS ₂ O	sulphinic acid
PS ₃ O	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	sulphyd groups
SMX	Sulphamethoxazole
SMXA	Sulphamethoxazole
SMXHA	Sulphamethoxazole
SMXNO	sulphamethoxazole
SOD	superoxide dismutase
SJS	Stevens-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. Introduction

The human immunodeficiency virus (HIV) is a member of the Retroviridae family of viruses. It is a group of RNA viruses that infect a wide range of animal species and replicate in the host cell using the enzyme reverse transcriptase to convert its RNA genome into DNA. Lentivirus, a subfamily of Retroviridae, is characterized by its ability to cause diseases with long incubation periods (Levy, 1998). They are transmitted as retroviruses, and HIV is the etiologic agent of acquired immunodeficiency syndrome (AIDS), a disease characterized by the progressive depletion of CD4+ T cells from the peripheral blood and other organs, leading to an immunocompromised state that in turn leads to the development of opportunistic infections and tumors (Levy, 2004; Sette et al., 2005).

There are two strains of HIV, HIV-1 and HIV-2, which share approximately 40-50% homology in the envelope protein region (Levy, 2004). HIV-1 primarily infects CD4+ T cells, macrophages, and dendritic cells. The infection eventually leads to the failure of the immune system and susceptibility to opportunistic illnesses and cancers (Levy, 2004). HIV-2 is associated with a clinical spectrum similar to HIV-1 but is less pathogenic and is not transmitted at all stages (Bette et al., 1998; Potts et al., 1999). HIV-2 is associated with a clinical spectrum similar to HIV-1 but is less pathogenic and is not transmitted at all stages (Bette et al., 1998; Potts et al., 1999).

reduced rate in the loss of CD4+ T cells in AIDS patients is not clear at present and after will be defined

1. HIV Genome and Replication

The mature HIV virion has a spherical morphology with a diameter of 100 nm and consists of a lipid bilayer envelope surrounding a nucleocapsid core (Sierb et al 2005). The nucleocapsid contains the genomic RNA, proteins protease (PR), reverse transcriptase (RT), integrase (IN) and some cellular proteins (Sierb et al 2005). The HIV genome consists of two identical single-stranded RNA molecules of 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 the viral glycoprotein receptors. The major receptor is the CD4-HIV gp120 receptor, and the seven transmembrane domain receptors, CXCR4 and CCR5 (Carruthers & Xiang, 2002). Binding to the receptors is followed by the fusion of the viral and cell membranes which leads to the injection of the viral nucleocapsid. Viral uncoating is carried out by cellular factors as well as viral proteins (MA), Nef and Vif. The viral RNA genome is reverse transcribed into double-stranded DNA (dsDNA) by the viral reverse transcriptase (RT) and integrase (IN) (Hartshill & Hooke, 2002). The dsDNA associates with the viral proteins to form a preintegration complex (PIC) in the cytosol of the cell. The PIC then enters the nucleus where the viral proteins integrate the viral genome into the host genome by taking advantage of the nuclear pore complex.

is required for the integration of the viral DNA into the host chromosome (Acosta-Ramos, Haffar & Bukrinsky, 2001; 1998). The initial round of proviral transcription is carried out by cellular RNA polymerase II which leads to the production of the HIV proteins Tat, Rev and Nef. Once a sufficient amount of these proteins is produced, they act as transcription factors and enhance further transcription of HIV genes.

The HIV genome can be transcribed to produce mRNA depending on the pattern of splicing (Schwartz & Baltimore, 1997). There is the 9 kb transcript which is the partially spliced transcript and the 4 kb transcript. Edwards, 1999. Different transcripts predominate at different stages of the replication cycle. In the early phase of the replication cycle, the 9 kb transcript predominates and encodes the regulatory proteins Tat, Rev and Nef.

When the accumulation of Rev is sufficient, Rev binds to the Rev Response Element (RRE) present in the 9 kb transcript and facilitates their transport through the nuclear pore (Dillard & Malim, 1998). The HIV-1 Gag polyprotein is translated from the 9 kb transcript. The polyprotein is translated from the 9 kb transcript and is cleaved into the proteins gp160, p17, p24, p15, p12, p9, p7, p6, p5, p4, p3, p2, p1, p10, p11, p12, p13, p14, p15, p16, p17, p18, p19, p20, p21, p22, p23, p24, p25, p26, p27, p28, p29, p30, p31, p32, p33, p34, p35, p36, p37, p38, p39, p40, p41, p42, p43, p44, p45, p46, p47, p48, p49, p50, p51, p52, p53, p54, p55, p56, p57, p58, p59, p60, p61, p62, p63, p64, p65, p66, p67, p68, p69, p70, p71, p72, p73, p74, p75, p76, p77, p78, p79, p80, p81, p82, p83, p84, p85, p86, p87, p88, p89, p90, p91, p92, p93, p94, p95, p96, p97, p98, p99, p100. After translation the polyprotein inserts into the membrane and viral assembly takes place (Derdovska, 2004; Seftal, 2000) complex consisting of

length genomic RNA, viral enzymes and cellular compounds immature core and this complex buds through the plasma membrane to form an immature virion. Budding of the HIV-1 virion is autocatalytic and involves the cleavage of the Gag and Gag-Pol polyproteins into mature structural proteins and enzymes. The individual proteins undergo maturation in the immature virion that is able to infect another cell.

1.3 Transactivator of Transcription

Once integrated, the HIV proviral genome is not only regulated by the host cell, but is also able to regulate its own transcription (Karn, 1999; Stoltz, 1985). This is in contrast to murine and avian retroviruses where transcription from the viral long terminal repeat requires permissive cells and does not require a viral protein (Karrar, 1999; Stoltz & Karn, 1985). Tat is synthesized at both early and late stages of viral replication. The protein is encoded by two exons. The first exon of Tat encodes a protein while the second codes for the remainder of the amino acid sequence. The protein has a molecular weight of 21 kDa and exists predominantly in two isoforms: a long form (115 amino acids) and a short form (86 amino acids). The long form is the predominant form of the protein in clinical isolates of HIV-1, except subtype B which has a synonymous single nucleotide polymorphism creating a stop codon in the second exon and an alternative reading frame (Campbell & Loric, 2009). Laboratory virus strains including NL4.3 and a truncated variant of NL4.3 (NL4.3 Δ 10) (Jiang et al., 1999) has been suggested that a premature termination

arose artificially during tissue culture passage (Nguyen et al. 1996) this form of Tat that has heretofore been by most used (Campbell & Lorentz 2009) the product of a double splice of the viral transcript. Two forms of Tat are generated in vivo depending on the number of times splice occurs. One form of viral transcript produces a protein of 101 amino acids form evident after the onset of infection. Some down singly splice viral transcripts to the cytoplasm to be translated into proteins. Rev means a protein of 67 amino acids encoded by the first 72 amino acids in the gag gene (Dougherty & Peterlin, 1994; Miller) in vitro, variants are able to transactivate LTR efficiently but infect cells differently.

Transcription of the HIV-1 promoter is characterized by an independent and dependent phase. In the presence of Tat, transcription of Tat is increased several hundred fold. The HIV-1 LTR is composed of U3, R and U5 regions with U3 divided into the modulatory and promoter regions (Fiegler et al. 2009). The modulatory region is the binding site for a wide variety of proteins that either enhance or repress transcription. The promoter has two TATA boxes, while the promoter has three Sp1 binding sites. Downstream of the promoter is the TAR element that is recognized by the Tat protein. The TAR element is the one which contains the Tat activation site (Brignone et al. 2003). The LTR contains several additional DNA binding domains.

Figure 11. The structure of the HIV-1 genome. A detailed structure of the genome is shown, highlighting the numerous regulatory sites that are involved in the regulation of HIV-1 transcription. Also in the LTR are two binding sites for the NF- κ B transcription factor, a tandem repeat of Sp1 sites and a TATA box. The Tat protein is encoded by two exons. The first exon codes for the protein that is sufficient for viral transactivation while the second exon gives rise to the p7 protein. Tat recruits transcription factors to the LTR to upregulate HIV-1 gene expression. Adapted from Roman et al. 2010.

Figure 1

cellular transcription factors, including NF- κ B (Wang & Greene, 2007). The NF- κ B and Sp1 binding sites are required for HIV-1 transcription. While other sites enhance transcription, they are not essential (Mullins & Greene, 2007). Members of the NF- κ B family can have both positive and negative effects on transcription depending on which subunit is present in the LTR complex (Brigati et al. 2003; Williams & Greene 2007). c-Rel and p50 homodimers bind the LTR and promote recruitment of transcription factors and histone deacetylase (Mason et al. 1996; Williams & Greene, 2007) in response to cellular stimulation, except during infection or cytokine production. IL-1 β and TNF- α , Sp1 and NF- κ B proteins bind the LTR cooperatively to initiate transcription synergistically (Prinzie et al. 1999; Petri et al. 1994; Popik & Pitha, 1999; Prinzie et al. 1999; Sune-Blaizis et al. 1998). The RelA/p50 heterodimer can displace the p50 homodimers and also recruit the cellular transcription factor CREB-binding protein (CBP) to the complex. In addition, RelA directs recruitment of cellular RNA polymerase II including TFIIB (positive transcriptional elongation factor 2) for transcription (Williams & Greene, 2007).

In the absence of Tat, RNA polymerase II in complex with transcription factors such as TFIID (transcription factor IID) initiates transcription and is able to cleave the promoter to transcribe TAR which has a characteristic RNA stem loop structure that binds to RNA polymerase II. The RNA stem loop structure is located at the 5' end of all nascent HIV-1 RNA. Tat, however, controls the complex is defective for elongation.

production of abortive short-lived transcripts in the presence of Tat, the binding of RNA polymerase II to the Tat transcription complex where it also binds the TAR with the TEF (Dingwall 1989). TEF is composed of cyclin kinase 9 (CDK9) which is the catalytic subunit and cyclin 9 (CYC9) which is the regulatory subunit. The binding of Tat complex with TAR RNA is believed to transcriptional elongation through the phosphorylation of CDK9 subunit-TEF (Plaxada & Roeder, 1996) and this phosphorylation is thought to overcome the action of act to repress transcriptional elongation thus TARs as an attenuator of viral transcription.

There are four functional domains in the Tat protein (Figure 1) is the N-terminal or acidic domain (1-21) which contains several acidic acids and is predicted to be a single residue changes in this domain (Jeang et al 1999). The second domain (37-71) is a cysteine domain consisting of a highly conserved cluster of seven cysteines involved in intramolecular disulphide bonds (Gadea et al 1988; Ruben 1989). Changes in six of the seven cysteine residues will affect function (Jeang et al 1999). Next is the core domain (74-103) which contains a conserved VCR motif involved in tubulin binding and apoptosis and the RKR motif. HIV2 and SIV Tat, though still functional (Glover et al 2002). Perhaps the best studied region of Tat is the fourth domain (106-126) which contains a basic RKKRRQRRR motif that is important for nuclear

Figure 1. Functional domains of the protein. Conserved functional domains have been identified in the protein. The domains are: (1) N-terminal, (2) hydrophobic, (3) core (4) basic (5) glutamine (6) C-terminal domains.

Figure 1.2

protein is the sequence that allows it to be taken up by an uninfected cell and taken up by other (Erickson et al 1989; Fentress et al 1993; Frankel & Pabo, 1988; 1989). The glutamine domain (amino acids 72,) in combination with the basic domain, confers TAR RNA binding (Dingwall et al 1989; Roy 1990; Weeks & Crothers 1988). The terminal domain contains the RGD (arg, gly, asp) sequence and the highly conserved ESKKKVE motif. This binding site is a target for integrins expressed by activated endothelial cells, macrophages and the significance of the latter has been demonstrated (Barnes et al 1993; Dingwall 1990).

In addition to its regulatory role, Tat has been shown to act as a transactivator of numerous cellular genes including the expression of cytokines and chemokines as well as the expression of adhesion molecules (Verma et al 1997; Deegan et al 1997; Taylor et al 1992; Vaccaro 1994; Westendorp et al 1997). The transactivating effects of Tat in macrophages and T cells due to a unique property allows the protein to be internalized and act in the absence of a signal peptide (Brigante et al 2003). Tat is able to enter uninfected cells through interactions with heparan sulfate glycosaminoglycans displayed on the surface of most mammalian cell types and translocate into the nucleus (Frankel & Pabo, 1988; 2001). In uninfected cells, Tat can transactivate cellular genes such as tumor necrosis factor- α (Sury et al 1999) and has also been shown to down regulate several genes involved in HIV pathogenesis.

l thereby providing a mechanism for the virus to evade the
 result in the establishment of persistent infection (Howcroft et al 1993; Vetha et al
 1998; Weiss et al 1998)

1. HIV Infection and Pathogenesis

The Joint United Nations Program on HIV and AIDS (UNAIDS) estimates that HIV infection has killed approximately 25 million people around the world since its first description in 1981. There were approximately 33 million people living with HIV in 2000 with more than 50,000 (UNAIDS, 2001) dying each year. The course of HIV infection can be divided into three phases beginning with a primary cytotoxic lymphocyte (CTL) response within the first four weeks after infection in the majority of individuals and is associated with a high viremia and a sharp drop in peripheral blood CD4⁺ T cell counts. The establishment of a reservoir of latently infected CD4⁺ T cells (D'Aquila et al 1991; Data et al 1991; Henrard et al 1995) This is followed by the specific cellular and humoral immune responses that lead to the decline of plasma viremia and the establishment of the set point (Bozzette et al 1994; Kotler et al 1994; Rosen et al 1997) The chronic or asymptomatic phase is accompanied by persistent viral replication in other viral reservoirs, a rapid turnover of plasma virions and a gradual decline in peripheral blood CD4⁺ T cell counts. The viremia usually eventually results in a CD4⁺ T cell count in peripheral blood that is less than 200 cells/mm³; at this stage the total number of CD4⁺ T cells in the body is reduced by at least 50% (D'Aquila et al 2003) As a consequence the patient's immune

incapable of controlling pathogenic pathogens and cancers that
 final symptoms of HIV (Dubeck et al 2003)

The discovery of a persistent correlation between a high viral
 load and an increase in the rate of disease progression has led to
 more complete suppression of viral replication as an effective
 strategy to control HIV infection. This led to the introduction of antiretroviral therapy
 currently conducted using a combination of at least three antiretroviral
 drugs from at least two different classes. This has dramatically increased the
 lifespan of HIV patients. There are currently four main classes of antiretroviral
 drugs; nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside
 reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), and integrase
 inhibitors (SI) (Sierata et al 2005). The combination of HIV patients ART resulted in reduced
 viremia to undetectable levels and increased CD4+ T cell counts
 for years (Sierata et al 2005). However, the success of ART in controlling viral
 loads is mitigated by resistance development, pharmacokinetic interactions,
 and the cost of the drugs.

1.5 HIV and Adverse Drug Reactions

The use of antiretroviral therapy has had a significant impact on the course of
 HIV infection and on the morbidity and mortality of HIV. However, the
 advantages of ART come with a marked increase in the number of adverse
 drug reactions (ADRs) in this patient population. These include both minor and
 major reactions. In fact, within the first year of ART, the incidence of ADRs is

treatment failure that are the most common reasons for antiretroviral (Barnes et al 2008). Up to 80% of HIV patients experience an ADR at some point during the course of the immune dysregulation or altered drug metabolism due to polypharmacy used to treat the disease (Barnes et al 2008; Maetz et al 2007).

Adverse drug reactions are unintended reactions to a standard dose for diagnosis, prophylaxis (Pitman et al 1998). They are classified pharmacologically into two major types (Rawlins & Thorne 1975). Type A reactions are predictable due to known pharmacological or toxicological actions of the drug. Approximately 80% of ADRs are of this type. For example, the use of minocycline can cause a dermatological condition in the skin known as hyperpigmentation. Such side effects are sometimes relatively common in users of these drugs and are usually delayed by weeks or months. Type B reactions are unpredictable or idiosyncratic, beginning shortly after the introduction of the medication, which are usually within two days (Rieder, 2009; Roujeau, 2006). This (Roujeau, 2006) suggests sensitization to immunological memory rather than direct toxicity which shows a specific reaction threshold. These idiosyncratic reactions are hypersensitivity reactions that start with fever and, over the course of a few days, a cutaneous eruption develops and is sometimes accompanied by hypotension (Sullivan & Shea, 2001). These reactions usually occur shortly after the start of therapy while the most severe reactions often be

(Roujeau, 2006) hypersensitizations can be divided into two main types or delayed immune-mediated reactions. These hypersensitization reactions are mediated by specific IgE or IgG antibodies (e.g. by penicillin)-specific T cells (Rajchoudhury & Svensson, 2000). Factors that make an individual susceptible to these idiosyncratic reactions are speculative and the pathophysiology of these reactions is multifactorial.

Though the use of ART often leads to good control of HIV, HIV diagnosis often means that the CD4 count is very low (CD4 count < 200 cells/ μ l) and a co-infection with tuberculosis at the beginning of antiretroviral therapy is common. These factors may lead to a variety of adverse drug effects such as rashes, hyperpigmentation, hypersensitivity reactions such as exanthema multiforme, Stevens-Johnson syndrome (SJS) or epidermal necrolysis (TEN) (Bosch & Emswiler, 2000). Overall, HIV-infected patients have a higher risk of developing cutaneous reactions to drugs (Hernandez et al., 2006; Lazzari, 1998). Cutaneous ADRs have been reported for all antiretroviral drugs used in the management of HIV infection, as well as for zidovudine and anticonvulsants, which are not only complications of infection but also of HIV-positive individuals. The severity of cutaneous reactions in patients with AIDS is up to 100 times higher risk of developing cutaneous reactions such as SJS and TEN than those in the general population (Rizley et al., 1998). Exactly why a particular individual is more or less susceptible to developing cutaneous drug reactions is unknown, but is thought to be related to genetic background and concomitant medication use (Marrim et al., 2006).

One of the first opportunistic diseases encountered by HIV 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 leading AIDS illness for up to 60% of patients (Morris et al 2004). Although prophylactic and ART therapy have reduced the incidence of PCP in the HIV infected population, trends over the last several years show that the incidence of PCP has plateaued rather than declined. PCP remains the most common serious opportunistic infection in the United States. Studies have shown that when a patient dies from an AIDS related illness, the mortality rate for PCP is 38% for those not receiving ART, compared to 15% for those receiving ART (Jain et al 2003; Kaplan 2000). The situation in the developing world is even worse with prevalence rates of up to 51% in various age groups (Morris et al 2004).

Sulphamethoxazole-trimethoprim (SMX-TMP) is a combination of two drugs used for the first line treatment and prophylaxis of PCP. The main advantage of the combination is that it does not require the synthesis of folic acid while humans obtain folic acid through their diet. Folic acid is essential for the synthesis of thymidine and other purines and bacterial DNA synthesis. Sulphamethoxazole inhibits the bacterial synthesis of tetrahydrofolic acid, the active form of folic acid (Masterson et al 2003). Sulphamethoxazole is a structural analogue of para-aminobenzoic acid and inhibits the synthesis of the intermediate dihydrofolic acid. Trimethoprim is a structural analogue of

dihydrofolic acid and competitively inhibits dihydrofolate reductase, the enzyme responsible for the synthesis of tetrahydrofolic acid. This sequential blockade of two enzymes in a single pathway synergistically and ably prevents the development of bacterial resistance (Mastichak, 2003).

Sulphamethoxazole-trimethoprim is effective against Enterobacteriaceae which include *Escherichia coli* and *Klebsiella pneumoniae* why it is widely used to treat urinary tract infections (UTI). The combination drug is commonly required upper and lower tract infections, including for prophylaxis and treatment of HIV. However, the use of SMX has been associated with very high incidence of hypersensitivity reactions, both in infected patients (Gordon et al. 1984; Heaney et al. 1995; Jeffers 1983; van der Vliet et al. 1994) and in non-infected patients (Blumenthal et al. 1986; West et al. 1971). Clinical manifestations of hypersensitivity to SMX in infected and uninfected patients include severe reactions such as SJS and TEN in a few patients (Vilatchikina et al. 2003).

1.6 Proposed Mechanism of Drug Reaction

Mechanism(s) to explain how drugs produce what clinical immunological reaction remain(s) unclear and to some extent controversial. Numerous lines of evidence suggest that the drug component enters the immune system in the pathogenesis of (Riedel, 2009) cutaneous reactions. There are currently two major hypotheses that are put forward to explain

Figure 1.3

first is that every other molecule is chemically reactive and drug metabolites happens when they bind to proteins or peptides processed by an antigen presenting cell (APC) and as a result modified peptides of Figure 4. These small molecular weight compounds are believed to be able to elicit an immune response. However, very few drug compounds are chemically reactive. Most must be metabolized to a reactive species prior to haptening (Riordan & Spentzi 2005). A survey of drugs associated with cutaneous ADRs revealed either metabolized or degraded (Riordan & Spentzi 2005). For SMX the major route of metabolism is in Figure 15. It is a detoxification. A small fraction of a SMX molecule undergoes cytochrome P450 biotransformation to 4-hydroxylamine (SMX-HA) metabolite sufficiently stable to circulate and be excreted (2001). Further autooxidation gives rise to nitroso (SMX) believed by some to be the ultimate (Gierbera & Pechl 2006) in the liver is the primary bioactivation enzyme, cytochrome P450. Other drug metabolizing enzymes have been found to be in other cells and (Riordan & Spentzi 2005) metabolite can be detoxified by the antioxidant glutathione (GSH), converting it to the subsequently, the parent drug (SMX) (This 2007) comes important in the context of HIV infection as the virus exerts a degree of cellular glutathione content, leading to a decrease in the conversion of nitroso metabolite to its parent compound and thus increases

Figure 1 Proposed mechanism of the haptens and their metabolism in drug reactions. Metabolism of S-Mepacrine chemically reacts with drug metabolites, -SMX and SMOX. These haptens then bind covalently to peptides or proteins thus producing adduct which are subsequently taken up by an antigen presenting cell and presented as modified peptides by neoantigenase adapted to MHC class II. (Saford et al, 2006).

Figure 1.4

Figure - The metabolism of Sulphamethoxazole. The majority is acetylated. The rest is bioactivated by cytochrome P450 to sulphamethoxyhydrazine that is subsequently bioactivated to sulphamethoxazole. Both metabolites can be reduced back to the parent compound by glutathione. Image adapted from Sanderson et al. (2006)

Figure 1.5

hypersensitivity to SMX (Simpson & Pichler 2007) hepatic tissues, many of the drugs are inactive metabolites are less active than in the liver.

Another set of factors that may determine individual hypersensitivity ADRs to SMX are the known polymorphisms in enzyme deficiency of NAT10 both the genotypic and phenotypic have been reported to be associated with ADRs (Riemshamed & Park, 2001; Riedl 2001; Zietz 1998) contrast to other acetylation transferase gene polymorphisms were shown to be major predisposing factors not predict individual susceptibility (Riemshamed 2000) Functionally significant polymorphisms in the other genes in SMX metabolism were also investigated using polymerase chain reaction (PCR) Restriction fragment length polymorphism analysis to the CYP2C9 and CYP2C19 genotypes were evaluated in the hypersensitivity group not statistically significant. Furthermore, polymorphisms in the glutathione transferase genes (GSTM1) not associated with SMX (Riemshamed 2000; Wolkenstein 2005)

There have been a number of observations that support these findings. These include the finding that SMX metabolites haptenate mononuclear cells (PBMC) in vitro (Grimm et al 1996; Naitoh 1999) SMX haptenated proteins have also been detected in patient antibodies in patients (Biechert et al 2000; Gruchalla 1998; Naitoh

2003) Reactive SMX metabolites can stimulate T cell proliferation or cytotoxicity in estrimHC (Schneyder et al. 1997a; Schneyder et al. 1997b) In the last decade the specificity of drug hypersensitivity has been supported by the establishment of T cell clones from the blood lymphocytes or skin lesions of patients who react to a drug (Muller et al. 1995; Schneyder et al. 2000; Schneyder et al. 1997a) cell clones could be derived from patients with a drug ingestion history of cutaneous drug reactions (Rogge et al. 2005) TCC were obtained from already sensitized patients and in the case of a new drug, they may be involved in re-sensitization and/or re-sensitization.

Despite this evidence, there is still controversy about the validity of the hypothesis. For example, for several drugs self-activation reactions appear too fast for any involvement of APCs (Schneyder et al. 1997a) In the presence of APC, activation of T cells almost immediately and sustained intracellular signaling is observed (Schneyder et al. 1997a) This timing cannot be reconciled with an intracellular processing that requires at least an hour (Schneyder et al. 1997b) In addition, the bioactivation of a drug is a key requirement for the initiation of an immune response. In many studies have provided evidence against processing of drugs. Many different, chemically inert drugs were found to be able to activate TCR in an MHC independent manner including SMX, lidocaine, carbamazepine (Schneyder et al. 1997a) Specific TCCs reacted to the parent drug

even when ~~Chw~~ APC fixed by glutaraldehyde which excludes metabolism and processing.

In contrast, unlike typical haptens, certain drugs do not. After pulsing APC with the drug for an hour ~~cells, in the~~ was no stimulation ~~by the~~ ~~Trugs~~ lidocaine, carbamazepine, SMX (Piche ~~et al~~ 2006; Z ~~and~~ 1998) however, the reactive-metabolite NO was able to stimulate ~~with~~ T cells capable of covalently modifying MHC peptide ~~complex~~ (S ~~and~~ 1997) Many studies ~~of~~ skin ADARs using immunolabelling of skin biopsies have found infiltration of a predominance of CD4+ T cells in the dermis and CD8+ (Miy ~~and~~ 1991; V ~~and~~ 1992)

In an effort to account for the above observations, P ~~proposed~~ a second model to explain drug ~~induced~~ cutaneous pharmacological interaction with ~~immune~~ receptors. According to ~~the~~ authors, this hypothesis is not intended to replace or ~~contrast~~ rather to complement ~~the~~ ~~idea~~ that states that certain drugs can bind reversibly to the highly-specific TCR ~~and~~ ~~by~~ binding the MHC peptide complex (Fig ~~1~~ 6), leading to an immune response directed drug ~~action~~ (P ~~et al~~ 2006) Such a ~~TCR~~ ~~binding~~ would be ~~metabolism~~ ~~independent~~ but the MHC peptide complex would still be necessary for cell activation and would in fact mimic drug ~~mechanism~~ ~~of~~ ~~action~~ ~~in~~ ~~the~~ ~~immune~~ ~~system~~ This model was elucidated ~~by~~ ~~the~~ ~~TCR~~ ~~and~~ ~~Bs~~ ~~possibility~~ ~~that~~ ~~both~~ ~~cell~~ ~~receptors~~ also activated in a ~~similar~~ ~~mechanism~~

situation seen in the case of divalent nickel ions (Ni), which happens even though they do not bind to proteins but rather form coordination complexes (2005) identified and characterized a promiscuous site on the TCR where Ni interacts simultaneously with Tyr29 and Tyr94 in CDR1 of the TCR. Thus, much like a bridge between both receptors though requiring idiotypic recognition.

The hypothesis would explain some of the aspects of cutaneous drug reactions, namely the fact that drug reactions can occur within a few hours after administration of the drug. This is the case of contrast agents used to improve the visibility of internal body imaging techniques such as CT (computed tomography), or MRI (2002; Christa et al. 2007). The kinetics of the RCM reaction is much faster than expected, how else could RCM or other inert drugs stimulate the immune response usually required to induce an immune response?

To explain these phenomena, the authors proposed that by bypassing the innate immune system, the memory T cells have a lower threshold of reactivity compared to naive T cells. In addition, a secondary immune response is generally faster and more robust than a primary immune reaction within the time frame observed for some A

Figure 1A6 comparison of the mechanism behind the hapten concept in stimulating T cells. The solid lines indicate covalent binding of antigens and the MHC while the dashed lines depict noncovalent cases, as they also bind covalently to the TCR from Pichler 2006.

Figure 1.6

would explain the higher incidence of ADRs during infections. The concept implies that ADRs are actively of up to 10% of memory T cells to certain drugs. In line with this notion, EBV or CMV infection there is a general stimulation of T cells, a risk factor for ADRs. The incidence of ADRs to SMX is 2% but increases to 50% in the HIV population, while ADRs to amoxicillin increase from 5% in the general population to 90% during an active EBV infection.

While some clinical and laboratory evidence exists in support of this hypothesis, I believe that the preponderance of evidence points to the primary pathway for the development of hypersensitivity reactions associated with ADRs have known reactive metabolites, but I note that the absence of known reactive metabolites does not rule out drug bioactivation. The detection of reactive metabolites is a function of the limits of currently available analytical methods. Our ability to detect reactive metabolites is thus very limited (Leaver et al., 2008). Although quantitative drug metabolism occurs in the liver, the reactive metabolites are unlikely to be stable in other organs unless the metabolite is able to avoid detoxification. It is stable to clinical studies over the past decade have shown the presence of drug-metabolizing enzymes in immune cells as well as skin cells including keratinocytes, mast cells, and dendritic cells (Roychowdhury & Svensson, 2015). These skin cells express less of the metabolizing enzymes than hepatocytes.

counterbalanced by the larger weight and surface area of the skin. This helps to explain why the skin is a primary target for drug-induced hypersensitivity reactions (Sander et al. 2008). In addition, while most important hepatic CYPs are expressed in the liver, several CYPs that are much more abundant in the skin and the liver (Sander et al. 2008) were noted above, which suggests that the concentrations of many detoxifying enzymes need to deal with the products of drug metabolism, systems which are often much more abundant in the skin. Also, the skin is immunologically privileged, and T cell activation is more likely to result in tolerance than a hyperergic immune response (Sander et al. 2008). Thus, immunologically relevant metabolism may be tissue-specific, with cutaneous metabolism being more relevant than hepatic.

Recently, the generation of T cell clones has allowed knowledge of the ongoing attempts to decipher the mechanisms behind drug-induced hypersensitivity reactions (DRH). Using this methodology, a recent study determined the relative frequency of drug-induced T cells in the peripheral circulation of patients with a history of hypersensitivity ADRs. The authors found that 78% of patients displayed hypersensitivity against SMX metabolites (both SMX and SMXO). They also showed that there was specific T cell stimulation; first, clones that were obtained from patients stimulated by the parent drug, but not clones stimulated by SMX and SMXO metabolites. The authors also showed data that concluded that responsive T cells were stimulated through a hapten mechanism involving an APC.

The strongest evidence came from the finding of association between certain drug hypersensitivity reactions and the HLA-Jon patients with ADRs to abacavir, approximately 19.4% carrying compared to only 1.7% of controls. This is also reported for carbamazepine treatment with the appearance of Stevens-Johnson syndrome carrying the HLAB*1502 allele (Cheng et al 2004). Such strong associations with HLA support to the important role for HLA molecules in ADRs, the hapten hypothesis for at least these drugs. Furthermore, there has been shown evidence that suggest polymorphisms in detoxication enzymes play a role in an individual's susceptibility to ADRs. This was first discovered by a polymorphism in the gene for the enzyme thiopurine methyltransferase (TPMT) in an HIV patient with ADRs to SMX, a polymorphism that caused the enzyme to be inactive (Cheng et al 2004).

1. Oxidative Stress and Redox Proteomics

It is generally accepted that a central pathologic feature of HIV is oxidative stress, leading to apoptosis and depletion of CD4+ T cells. Oxidative stress can be defined as a situation where cellular homeostasis is altered by an excess of reactive oxygen/nitrogen species (ROS/RNS respectively) that overwhelm cellular antioxidant defences, leading to a disruption of redox balance and subsequent damage to cellular or molecular structures. These reactive species target a large number of biological molecules such as proteins, nucleic acid, unsaturated lipids and carbohydrates with the latter being the most common target making up approximately 50% of oxidized molecules (Rimoldi et al 2008). Reactive oxygen/nitrogen species can cause specific, reversible and/or irreversible oxidation of proteins, lipids, nucleic acids and carbohydrates.

proteins that can lead to neurodegeneration (Draetta and Beach 1988; et al 2007)

Reactive oxygen/nitrogen species can target lipid membranes leading to peroxidation and the formation of multiple aldehydes, including 4-hydroxynonenal (HNE) a highly reactive molecule capable of inhibiting DNA synthesis (Sacktor et al 2004). Sphingolipids are a major class of membrane lipids in cells and are particularly abundant in the nervous system. Sphingomyelin is the most abundant sphingolipid in the brain (Sacktor et al 2004). Sphingomyelinase levels have been used as markers of oxidative stress and inflammation. In these two markers are significantly increased in the cerebrospinal fluid of brain patients with subcortical dementia (Hestemal et al 2005). HIV infection in adults infected with HIV develop dementia which is now referred to as HIV-associated dementia in adults younger than 60 years (Peters et al 2005).

Antioxidant enzymes have evolved to counteract the potential consequences of living in an oxygen-rich atmosphere. These include superoxide dismutase, catalase, and peroxidase (Hall et al 2009; Poole et al 2005). In addition, non-protein molecules such as GSH, thioredoxin, vitamin C and E, and flavonoids also act as antioxidants and it is the depletion of these antioxidants that lead to added oxidative stress in patients. The concentration of GSH decreased in the liver and CNS of elderly patients while administered the GSH precursor, N-acetylcysteine (NAC) (Peters et al 2005).

shown to increase mortality (Hertzberg et al. 1997; Poerzgen et al. 2005). The micronutrients selenium, vitamin C and vitamin E are also important for HIV-infected subjects (Ojima et al. 1989; Poerzgen et al. 2005). Selenium supplementation increases glutathione peroxidase activity and reduces HIV replication while supplementation with vitamins C and E reduces infection and produced a downward trend in viral load (AVI study et al., 1998; Ho et al. 1997). Supplementation with vitamins A, C and E decreased the levels of oxidized DNA bases and lipid peroxidation and activity of antioxidant enzymes superoxide dismutase and catalase (Saini et al. 2002).

Oxidative stress induced by HIV is a major cause of neurodegenerative disease. Specifically, some studies suggest that oxidative stress is a contributor to dementia. In the brain the main cell types are microglia, macrophages and astrocytes, so the viral replication is limited in astrocytes (Lipton, 2005; Lipton et al. 1994) but is actively released by these infected cells and is taken up by neurons such as neurons (Mason et al. 2005). In patients infected with HIV, significant dysfunction and neuronal loss occur despite the fact that HIV is not found in the brain (Nath, 2002) is thought to be a cause of neurotoxicity with brain extracts from HIV-infected patients having elevated levels of Tat (Parnich et al., 2005). The Tat sequences from these brain extracts are different substitutions in the Tat sequence (Brenneman et al. 1998; La et al. 2009). These mutations may decrease the ability of Tat to be taken up by cells, thus increasing concentrations of Tat in the brain and possibly neurotoxic (Fay et al. 1993) at

induced neurotoxicity is thought to be mediated by two distinct excitotoxic mechanisms involving glutamate (Matsuda et al 2005) that is capable of depolarizing hippocampal neurons and human cerebellar granule cells. This is followed by mitochondrial calcium uptake, generation of reactive oxygen species (ROS) and apoptosis (Kinsler et al 2006). Tat also activates phospholipase C, which increases levels of free calcium from intracellular stores. This increases the production of inositol trisphosphate (IP3) and diacylglycerol (DAG), which activates protein kinase C, all precursors of the stress response (Saughey et al 1999; Kreutz et al 1998; Sabatini et al 1991). Tat also activates astrocytes and induces the expression of inducible nitric oxide synthase (iNOS), leading to the overproduction of nitric oxide (NO) that can react with superoxide to form neurotoxic peroxynitrite (ONOO-) (Kraut et al 2002; D'Onofrio et al 2002). Tat also induces TNF, which in turn induces iNOS, leading to the increase in HIV in HIV infected macrophages (Bukacinski et al 1995; Li et al 2009).

TNF is known to stimulate HIV replication in infected cells. The mechanism of action of the transcription factor NF- κ B is well known (Vesce et al 1995). We have examined the ability of Tat to mediate a TNF response. We found that Tat expression was able to enhance the response to TNF in stably transfected cells (Wang et al 1995). In Jurkat cells, the addition of Tat protein was added to Jurkat T cells and induced TNF, it protected cells from TNF-induced cell death. As this was inhibited by the addition of a TNF inhibitor, it is suggested to the authors that the activity of Tat is to enhance TNF. It has been further observed that Tat, either produced intracellularly or added to cells, significantly reduced RNA and protein levels of superoxide dismutase (SOD).

dismutase (SOD), an enzyme which serves as the primary de-
 derived free radicals in cells. In another study looking at
 redox state of T cells (2005) showed that Jurkat T cells in
 recombinant Tat revealed an increase in intracellular H_2O_2 . If
 H_2O_2 was due to Tat, the authors treated the cells with these
 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). Under
 nitrosative stress contains sulphur residues, cysteine and methio-
 susceptible to oxidation undergo various reversible and
 alterations in response to ROS and RNS as
 of organic sulphur derivatives characterized by the presence
 at their active site (Dinkler et al 2007; Rinaldi et al 2008) Biological
 thiols can be classified as large molecular mass proteins
 proteins, free thiols and cysteine. All thiols are important
 sensors, as most protein thiols do not react with oxidants
 found in (Eaton, 2006) majority of cytoplasmic proteins con-
 sulphhydryl with a pKa greater than 8.0 and in the reducing
 remain protonated at pH (Dinkler 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).

Cysteiny l thiols can undergo a range of redox reactions which are dependent on the species and concentration of the oxidant. In the presence of increasing ROS concentration and an oxidant, a reactive protein (thiol) is oxidized to a disulphide (PSSP), sulphenic (PSOH₂H) or sulphinic (P(S)OH) species. The first three of these modifications (disulphide, sulphenic and sulphinic) are reversible. Disulphide bonds may form within a protein, intramolecularly, or between two separate protein molecules. In the presence of oxidants, protein thiols may also form intermolecular mass thiolates (S₂-D₂Donat G S2-0071) widely thought that the formation of protein disulphides can protect the protein from oxidative damaging, or (Rdaailoutcail2008)

A number of methods have been developed to study protein disulphide bonds, including diagonal gel electrophoresis which is a non-reducing method for investigating disulphide modifications since disulphide bridged proteins will migrate at high resolution (Fig 17). Proteins are electrophoresed in the first gel under reducing conditions then reduced in the gel and then transferred onto a second gel and electrophoresed in the opposite direction. In the second gel, proteins migrate at angles to their original migration direction. If the proteins are not disulphide bridged, they will fall on the diagonal in the second gel. If the proteins migrated equidistance in both directions during electrophoresis, they will fall on the diagonal line connecting opposite corners of the gel. If the proteins are disulphide bridged, they will fall off the diagonal. The subunits of proteins connected by intermolecular disulphide bonds will fall off the diagonal because the individual units migrate faster than the

Figure 1. Redox-dimensional gel electrophoresis. Electrophoresis in the first dimension is carried out under non-reducing conditions. The relevant spots are excised and placed in a reducing solution and then a second gel is run perpendicular to the original direction. In this second gel, proteins migrate at right angles to their original migration. The majority of cellular proteins will fall on the diagonal, having migrated the same distance in both dimensions. Upon denaturation, subunits of proteins that were held together by intermolecular disulphide bonds will resolve below the diagonal, while those held together by intramolecular disulphide bonds will migrate more slowly above the diagonal. Image adapted from Alberts et al. (2002).

Figure 1.7

the second dimension (2D). Proteins with intramolecular disulfide bonds form a more compact structure that will be unfolded following reduction. Proteins that resolve slowly in 2D and resolving above the diagonal are called protein spots (Rinaldi et al 2008)

1. Research Hypothesis and Objectives

HIV currently infects 33 million people around the world and 70,000 people in Canada. Infection with HIV usually leads to opportunistic diseases and death, often by AIDS. Such as Twenty years after the identification of HIV as the etiological agent, there is no cure for HIV though there is an effective regimen of antimicrobials that can sustain an infected individual for years. However, hypersensitivity adverse drug reactions (ADRs) to many drugs, used to treat HIV, are significantly more common in patients with HIV infection than in uninfected people. Hypersensitivity ADRs to antiretroviral drugs. These ADRs can be fatal; ADRs are one of the leading causes of death in Canada. Despite this, the mechanism(s) leading to these ADRs is not understood. Understanding of the mechanism(s) of ADRs in the context of HIV infection will allow us to better predict the population at risk. It will also offer some insight into the design of safer and more effective therapeutic agents or treatments that would mean a higher quality of life for people with HIV.

Hypothesis: The HIV-1 Tat protein alters the intracellular environment of target cells to increase cellular sensitivity to reactive metabolites.

Specific Objectives:

1.a) Establish T cell lines in which the HIV-1 Tat protein is expressed. Generate Tat mutants (Tat86, Tat72Q, Tat74S) and determine if they differentially induce hypersensitivity. Characterize the expression of the Tat protein.

2. Determine if the differential expression of Tat101 or the differential cellular sensitivity in-HLA presentation of SMX metabolite SMX.
3. Determine the ability of intracellular oxidant to be upregulated and the deletion of the generation of ROS time to reach a high absence of the reactive-HA metabolite, SMX
4. Determine what effect Tat or its deletion might have on the proteome with or without SMX in Jurkat T cell lines.

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CHAPTER 24 Tat potentiates cell toxicity in model Sulphamethoxazole-induced adverse drug reaction

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2.1 Introduction

Human Immunodeficiency Virus (HIV) is the etiologic agent of acquired immune deficiency syndrome (AIDS), a disease characterized by immune dysfunction resulting in opportunistic infections (Huigert al.2004). Though the mechanism(s) underlying the pathogenesis of AIDS is still not completely understood, studies have shown a link between the loss of CD4+ T cells and the appearance of the manifestations of AIDS (Dofu et al. 2003). The link between the disease and the loss of CD4+ T cells suggests there is a contribution made by other factors. The HIV transcriptional activator (Tat) protein is believed to be one of those proteins.

Tat is a 15 kDa protein essential for efficient HIV transcription (Huigert al.2004; Pugliese et al. 2005). The Tat protein is a transcription factor generated by the transcription of multiple spliced transcripts (Bridger et al. 2003; Huigert al.2004). Very early after viral entry into the infected cell, its two main forms as a 101 amino acid protein. Subsequently, the result is the predominant transcription of a single spliced, one-exon form of the tat gene (Huigert al.2004; Malani, 1988).

In addition to its role in HIV transcription, the ability to influence cell phenotype by modulating the expression of cellular factors (Weib et al. 2002a; Weib et al. 2000). The protein has been found to stimulate immunoregulatory cytokines, including the production of interleukin-6, which is implicated in the pathogenesis of AIDS (Malani, 1988).

psoriasis, B cell lymphoma and Kaposi's sarcoma (Al-Korayem et al. 1997). In another study, Tat was able to increase the expression of TNF- α by activating the TNF- α promoter (Sapkota et al. 1990). Tat also inhibits the transcription of the tumor suppressor gene, p53, possibly contributing to the development of cancer (Liet al. 1995). Tat suppresses the expression of manganese superoxide dismutase (SOD), contributing to oxidative stress, a condition exacerbated by a systemic decrease in glutathione levels in infected individuals (Luet al. 1989; Staal 1992b; Walmsley 1997). Tat is also proposed to have a major, but indirect, role in apoptosis, a mechanism thought to contribute to the progressive loss of CD4+ T cells in infected individuals (Blumenthal, 2004). This is thought to occur by mechanisms that reduce the ability to regulate the expression of CD45 in T cells, thereby increasing their susceptibility to apoptosis (Baird & Epstein, 1999; Westendorp 1995a).

The steady decline of CD4+ T cells during the course of HIV infection progresses to a stage where the patient develops multiple opportunistic infections, the most common of these diseases being *Pneumocystis carinii*, which is responsible for the lung infection known as pneumocystis pneumonia (PCP) (Morris et al., 2004). Without treatment, over 75% of people with AIDS die from this infection (Morris et al. 2004). The drug combination trimethoprim-sulphamethoxazole (SMX) (TMP) is the treatment of choice for the prophylaxis of PCP (Morris et al. 2003). SMX is a sulphonamide drug which

associated with hypersensitivity adverse drug reactions (ADRs) in patients with AIDS usually present as a high fever cutaneous eruption (Rutjens & Stern, 1994) for patients with AIDS approximately 50%, significantly more prevalent in a population of 2-5% (Caert et al, 1993). There is a marked increase in risk among people with HIV infection and AIDS compared to both people with other causes of immunodeficiency or primary immunodeficiency (Devics & Sheare, 2008). The reasons for this increase in risk for ADRs appears to be very specific for a broad range of drugs (Lindley, 2006).

Previous data indicates that hypersensitivity ADRs to reactive metabolites of SMX, hypoxanthine, and trimethoprim (SMX) (Devics et al., 1988). Given the increased risk of adverse drug events in infection, alterations in the ability of HIV/AIDS to handle reactive metabolites may be an important element in the pathophysiology of reactions among people with HIV and AIDS.

In a study by Caert et al (1993), the degree of hypersensitivity to SMX was linked to toxicity by showing that primary lymphocytes from hypersensitive HIV patients are significantly more reactive to SMX metabolite than lymphocytes from sensitive controls. We further confirmed this study by showing that CD4+ HIV-infected individuals are more susceptible to toxicity by SMX than the uninfected population (Rieder et al, 1995). The metabolite produced depends on the toxicity in individuals with HIV.

human T lymphoblasts, a result significantly greater than cells. HIV infected cells also had substantially lower glutathione content than uninfected cells. There was a dose-dependent decrease in glutathione content when both cell types were transfected with the HIV regulatory proteins. These results are consistent with the findings of other studies using lymphocyte cell lines transfected with the HIV regulatory proteins. These results are analogous to those reported in other cell models. These results suggested that there was a relationship between glutathione and HIV Tat protein.

The relationship between Tat expression and HIV infection is a significant increase in the viral load of a patient at the late stage of infection indicative of a similarly considerable overall increase in viral load extracellularly. This increase in viral load might have an effect on the hypersensitivity and its metabolism. In this study we sought to determine if differential expression of Tat protein is associated with differential HIV infection. Thus, we conducted this study to investigate how expression changes the ability of cells to detoxify reactive

2. Materials and Method

2.2 Cell lines

The human T lymphocyte cell line J152k was obtained from the American Type Culture Collection. The Jurkat and HIV₁ obtained through the AIDS Research and Reference Reagents Program, National Institute of Allergy and Infectious Diseases. The cell lines were maintained in RPMI 1640 medium (Invitrogen) supplemented with 20% fetal calf serum, 1mM sodium pyruvate and 100units/ml of penicillin and streptomycin.

Construction of plasmids and cell lines

The Tat gene was amplified from the plasmid pSV2tat72. This construct encodes the first exon of the protein and was obtained through the AIDS Research and Reference Program, Division of AIDS, National Institute of Health. A 1.9kb fragment was cloned into the plasmid pEGFP1 (Clontech), resulting in a TatGFP fusion gene. This gene was excised from pEGFP1 with the I/Not I fragment and inserted into the digested plasmid pBIG2i, an inducible expression vector (Stratagene, 1999). The result was pBIG TatGFP. For control, pEGFP1 was also cloned into pBIG2i to create pBIG GFP.

pBIG TatGFP or pBIG GFP were transfected into Jurkat cells by electroporation (BioRad Gene Pulser II) according to the manufacturer's protocol to generate stable Jurkat pBIG TatGFP and JurkatpBIG GFP cell lines.

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

Selective media

Post transfection, positive cells were selected by growing in a concentration of 0.8mg/ml. Stable clones were derived by limiting dilution into 96 wells and the resulting cell lines were grown in complete RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, 100units/ml of penicillin and streptomycin.

2.2 Confocal microscopy

Confocal microscopy was performed with a BioRad RSC 1024 scanning microscope. Jurkat pBIG GFP cells were treated with 500ng/ml of doxycycline for 72hrs, fixed with 1% paraformaldehyde at 4°C, then centrifuged onto a microscope slide using a cytochrome c kit. Cells were cover slipped with Vectashield mounting medium containing Dapi (Vector Laboratories). The images were processed using LaserSharp (Cybernetics) and Adobe Photoshop 6.0 (Adobe Systems, Inc.).

2.2 Real time PCR analyses of Tat/GAPDH mRNA levels

RNA was isolated from Jurkat T cells as well as cell line following doxycycline induction. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) by following the manufacturer's protocol. Total RNA was reverse transcribed using the High Capacity cDNA

EDTA pH 8, with the addition of a protease inhibitor (Diagnostica). The cytosolic fractions of the 15% SDS-PAGE gels. The proteins were transferred to PVDF membrane and probed with anti-IL-6 antibody (Santa Cruz Biotechnology) or rabbit monoclonal antibody (Cell Signaling Technology). The bound antibodies were subsequently detected using horseradish peroxidase (HRP) conjugated secondary antibody (HRP conjugated secondary antibody respectively (Jackson ImmunoResearch Laboratories) and chemiluminescence (Amersham). The consistency of protein loading was confirmed by actin monoclonal antibody.

2.2 MTT Cell Viability Assay

Drug toxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay. Cell viability was quantified by colorimetric conversion of MTT, a methylene blue derivative (Mosmann, 1983). Jurkat E6.1 (control), maximally induced Jurkat E6.1 (control) and differentially induced Jurkat pBIGW6 (GFP control) were seeded into 96-well plates and incubated with 100 µM SMX (parent drug) (Sigma) or 258 µM of S-MX (active drug metabolite) for 2 hours. After 2 hours, the cells were resuspended in media and allowed to rest for 4 hours. Next, 1mg/ml MTT was added to the cells for another 4 hours. The reaction product was solubilized overnight at room temperature.

dimethylformamide/ 20% SDS solution and the percentage of TatGFP cells was determined by an ELISA plate reader at 590nm.

2.3 Flow Cytometry analysis of GFP expression and apoptosis

Dose response experiments were carried out after incubation of Jurkat pBIG GFP-1000 cells with 0 doxycycline for 38hrs and analyzing them using a FACScalibur (flow cytometry) calibrated with FITC-anti-CD45 (Becton Dickinson). Time course experiments were conducted with TatGFP cells 50000/ml of doxycycline for up to 72hrs, processing samples through the flow cytometer. At least 10,000 cells were measured in each sample. Flow cytometry data was analyzed using FlowJo software. Appropriate, calcein acetate cap (10⁶) was used as a positive control. In each sample, events collected were gated for GFP fluorescence.

Alternatively, caspase-3 and GFP fluorescence was used to measure HA toxicity and Tat expression. Jurkat pBIG TatGFP cells were treated with concentrations of doxycycline for 24hrs and then treated with 0, 100 and 200 nM 8hrs. The cells were then washed in PBS, fixed with formaldehyde, permeabilized with cold 70% ethanol and stored at 4^o, in the dark. After removing the ethanol, the cells were stained with phycoerythrin (PE) and rabbit anti-caspase-3 monoclonal antibody (BD Biosciences) for 1hr. GFP and caspase-3 were then analyzed by flow cytometry.

2.2 Statistics

MTT cell viability data were analyzed in SPSS Version 20.0. Standard deviations (SD) were used to report variability. Tukey's post hoc procedure was used to compare means from different concentration levels. To account for multiple comparisons, only differences considered statistically significant. GraphPad InStat program (San Diego, CA) were used to calculate the mean and standard deviation. The EC_{50} values were calculated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). The X values (concentration) were transformed and the Y values (% cell viability) were normalized to define 0% and 100% as the endpoints. This was followed by a linear regression (variable slope).

2. Results

2.3 Characterization of Inducible Cell Lines

Our goal was to test whether the HIV Tat protein could be induced in a Jurkat T cell model for sulphamethoxazole resistance (SMR). At one end, the 5' portion of the Tat coding region was amplified by PCR and fragments were inserted into an EGFP plasmid to create the fusion construct. This product was then inserted into a doxycycline-inducible pBIG2i vector (1999). The control vector consisted of the GFP fragment of the pBIG2i vector. After transfection and selection of Jurkat E6.1 cells, single cell clones were isolated by limiting dilution. Confocal microscopy and western blotting were utilized to assess fusion protein and GFP in their corresponding cell lines. Jurkat pBIG TatGFP transfected Jurkat cells with 500 ng/ml of doxycycline resulted in the respective induction of GFP and TatGFP. Confocal microscopy images showed GFP fluorescence throughout the cell. Jurkat pBIG GFP and Jurkat pBIG TatGFP (Figure 2A and 2B, respectively) showed GFP fluorescence throughout the cell. Immunoblotting 38 h of induction with 1000 ng/ml of doxycycline (Figure 2C) and anti-Tat (Figure 2D) antibodies showed that Jurkat pBIG TatGFP cells not induced with doxycycline did not produce the Tat protein (Figure 2C and 2D).

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

Figure 2. Expression of GFP and the Tat-GFP fusion protein in Jurkat pBIG GFP (A) and Jurkat pBIG TatGFP (B) cells, treated with 500 ng/ml of doxycycline. The images were captured with a laser scanning confocal microscope with a 488 nm diode laser. Nuclear staining with DAPI and GFP staining. Cell lysates from Jurkat pBIG GFP cells (lane 1) and Jurkat pBIG TatGFP cells (lane 2) were treated with 1000 ng/ml doxycycline (lane 3) were processed by SDS-PAGE and western blot analysis. The membranes were probed with anti-GFP antibody (C) or a mouse anti-Tat antibody (D).

Figure 1

determine the time course of induction, Jurkat pBIG TatGFP cells were treated with varying concentrations of doxycycline (0, 1, 10, 100, 1000 ng/ml) and analyzed. As expected, the level of induction is time dependent and the effect plateaus after 38 hours (Figure 2A). The viability of the cell population was consistently over 95% throughout the duration of the experiment.

To determine the ability of TatGFP to be differentially induced, Jurkat pBIG GFP cells were treated with increasing concentrations of doxycycline (0, 1, 10, 100, 1000 ng/ml) and analyzed after 38 hrs. Western blots probed with Tat antibodies showed the gradual increase in the level of Tat protein expression with increasing doxycycline concentration (Figure 2B). Densitometric examination of these blots revealed that TatGFP expression was maximal at 1000 ng/ml of doxycycline. These results not only indicate that TatGFP is induced in the absence of doxycycline, but also that the level of TatGFP expression directly correlates to the amount of GFP fluorescence when the cell population is treated with doxycycline. In parallel, similar results were obtained for GFP cells (not shown).

We have previously demonstrated that H9 and Jurkat cells show increased cellular proliferation in the presence of doxycycline (Apostol et al 2005; Reiter et al 1995). It is, therefore, important to determine the doxycycline concentration that induces physiologically relevant proliferation. We chose to do this by PCR analysis. It was not possible to quantify the amount of Tat protein expressed in the H9 cell line. The amount of Tat protein expressed in the H9 cell line was quantified by the amount of Tat protein in the culture medium. The concentration of doxycycline was 1000 ng/ml.

Figure 2n2 increases in the concentration of doxycycline result in increased TatGFP expression.

(A) Time course of TatGFP induction upon exposure of Jurkat cells to doxycycline. Jurkat pBIG T_{at}GFP cells were treated with 0, 15, 31, 62, 125, 250 or 1000ng/ml of doxycycline. Flow cytometry was used to determine the level of induction at 0, 12, 24, 36, 48 and 72hrs. Data are from three independent experiments carried out in triplicate.

(B) Differential expression of TatGFP protein. Cell lysates obtained from Jurkat TatGFP cells treated with 1000ng/ml of doxycycline were probed with anti-TatGFP or anti-actin antibodies. Data are representative of three independent experiments.

(C) Total RNA was isolated and subjected to RT-PCR. RT-PCR was performed using specific primers and mRNA expression of Tat was quantified by real-time PCR. GAPDH was used as a housekeeping gene. The assay was performed on three separate occasions.

Figure 2.2

mRNA in HIV-infected Jurkat and H9 cells. The results showed that the levels of Tat mRNA (250ng/ml) of doxycycline induction are well within the range of HIV-infected Jurkat (Figure 12C).

2.3. Cell viability is further decreased in the presence of Tat following treatment with SMX

Earlier studies demonstrated that HIV-1 is cytotoxic to CD4⁺ T cells in the presence of Tat (Arpa et al 2005) to determine if there was a relationship between increasing levels of Tat and cytotoxicity, SMX exposed cell populations of Jurkat pBIG-TatGFP cells expressing different levels of Tat were treated with concentrations of SMX then assessed cell viability using an MTT assay. The assay confirmed the cytotoxicity of the parent drug (SMX) (data not shown) in all cell lines. There was, with respect to Tat-induced TatGFP expression, a decrease in cell viability with increasing concentrations of SMX (Figure 12D). The assays confirmed that Jurkat cells differentially induced for TatGFP expression were sensitive to the effects of SMX either of the control cell lines, Jurkat pBIG-GFP cells and also significantly in the uninduced Jurkat pBIG-TatGFP (Jurkat pBIG-0TatGFP) (Figure 12E). Furthermore, MTT assays were carried out with Jurkat E6.1 cells pretreated with 500ng/ml of doxycycline and with various concentrations of SMX. There was no significant difference in cell viability between Jurkat E6.1 cells pretreated with doxycycline and cells that

Figure 2. TatGFP expression decreases cell viability upon exposure to SMX. The MTT cell viability assay shows a dose-dependent decrease in cell viability of Jurkat E6.1 cells exposed to increasing concentrations of SMX. Data are representative of three independent experiments performed in triplicate. *P<0.01, Jurkat E6.1 vs. TatGFP: 0ng/ml dox; #P<0.01, TatGFP: 0ng/ml dox vs. all other groups; &P<0.01, Jurkat E6.1 vs. TatGFP: 31ng/ml dox; §P<0.01, TatGFP: 31ng/ml dox vs. all other groups. TatGFP: 0ng/ml dox vs. TatGFP: 31ng/ml dox.

Figure 2.3

EC_{50} , the median effective concentration, or HA_{50} at concentration which 50% of the cell population is dead, was determined using GraphPad Prism, v4.0 (GraphPad Software, Inc., San Diego, CA). These values, shown in Table 2, confirm our interpretation of the results of Figure 1. Namely, that in our model TatGFP expression and cell viability are inversely related: higher the TatGFP expression, the lower the cell viability (p < 0.05). The induction of GFP expression with 500 ng/ml of TatGFP in the cell line did not have a statistically significant effect (Table 2).

2.3 Apoptosis is a mechanism of SMX resistance

We have previously shown, using flow cytometric analysis, that SMX induced cell death as detected by Annexin V staining. In a treatment of cells with SMX in a dose dependent fashion, following induction of TatGFP expression (Arp et al 2005), cells not treated with SMX were highly viable and the initial stages of apoptosis were not observed. SMX 1000 again confirmed the effect of the parent compound (Arp et al 2005). These previous experiments demonstrated that at SMX concentrations of 100 and 1200 cell populations that was more pronounced in cell populations expressing higher levels of TatGFP.

The relationship between SMX resistance and Tat expression was further extended here by examining whether key participants in the apoptosis pathway are activated as assessed by a tyrosine phosphorylation assay. Cytochrome c is a protein normally confined to the intermembrane space of mitochondria. Upon activation, it translocates to the cytosol where it binds to Apaf-1 and procaspase-9, leading to the activation of caspase-3, which is the executioner of apoptosis.

Table 1-2 Expression of Tat-GFP in Vero Cells were obtained after a
 MTT data using the GraphPad Prism 4 software. of three inde
 experimental pts.: doxycycline. *P<0.01 #P<0.01 vs. pBIG GFP:
 500ng/ml dox. &P<0.01 vs. pBIG TatGFP. &P<0.01 vs. pBIG TatGFP
 31ng/ml dox.

Table 12

Cell Population	EC ₅₀ (uM)	95% Confidence Intervals
Jurkat E6.1	175.3	16.00 1920
pBIG GFP: 500ng/	146.0 *	13.0 15.64
pBIG TatGFP: 0n	143.0 *	13.5 15.0
pBIG TatGFP: 31	68.0 ^{##} 2	63.2 07 322
pBIG TatGFP: 62	580 ^{##} &	53.7 4 6 26 4
pBIG TatGFP: 12	51.5 ^{##} &	47.1 6 5 6 2 7
pBIG TatGFP: 50	525 ^{##} &	48.4 6 5 6 8 9

mitochondria (Green, 2005) the event of apoptotic cell death, it is in the cytosol where it functions to activate caspases (Green, 2005) and a cascade of this cascade is the activation of caspase-3. In order to study the induction of Tat expression and drug treatment, cytosolic fractions were prepared from each of the various cell lines and loaded onto SDS

None of the Jurkat pBIG TatGFP cell lines or Jurkat cells contained cytochrome c (Figure 2A). In non-induced Jurkat pBIG TatGFP cells, 200 nM of SMX was required to cause the translocation of cytochrome c to the cytoplasm (Figure 2A). As higher levels of TatGFP are induced with increasing concentrations of doxycycline, cytosolic cytochrome c increases (Figure 2A). The level of cytochrome c expression doubles in the Jurkat pBIG TatGFP cells as the concentration of doxycycline increases from 3 nM to 300 nM exposure to 500 nM SMX as assessed by Western blot analysis. The results demonstrate that there is a direct connection between the amount of cytochrome c released from the mitochondria, with the level of TatGFP expression associated with a more pronounced release of cytochrome c from 200 nM of SMX.

Western blot analysis of caspase-3 expression also confirmed our previous annexin V flow cytometry data and SMX in TatGFP treated cells. Jurkat pBIG TatGFP cells were treated with SMX for 2h then left to recover for 18h, after which the cytosolic lysates analyzed by SDS-PAGE shows that caspase-3 is not activated in the absence of TatGFP expression (0 ng/ml dox). Jurkat pBIG TatGFP cells

Figure 2. TatGFP expression in combination with S-MX in the induction of apoptosis.

(A) TatGFP expression increases in Jurkat cells upon treatment with S-MX. Jurkat pBIG TatGFP cells upon treatment with S-MX (0ng/ml doxycycline) and cells induced with 500ng/ml of doxycycline were each exposed to 200nM of S-MX for 2hrs, followed by a recovery period of 18hrs. Populations were harvested and the cytosolic fractions isolated. Blotting for actin was analyzed for consistency of protein loading.

(B) Increases in TatGFP expression result in higher induction of apoptosis. Jurkat pBIG TatGFP cells and cells induced to express two different levels of TatGFP (50 or 500ng/ml of doxycycline) were treated with 200nM of S-MX for 2hrs, followed by a recovery period of 18hrs. Populations were harvested and total protein was analyzed by Western blot. Blot data are representative of three independent experiments.

(C) The rise in TatGFP expression is comparable with the increase in apoptosis upon treatment of Jurkat pBIG TatGFP cells. Jurkat pBIG TatGFP cells were induced with 0, 100 and 500ng/ml of doxycycline then treated with 200nM of S-MX for 8hrs. The cells were fixed, stained with anti-TatGFP antibody and then run through the flow cytometer. Data are representative of three independent experiments.

Inset is the same experiment carried out with Jurkat pBIG TatGFP cells induced with 500ng/ml of doxycycline, treated with S-MX for 8hrs and processed as above.

Figure 2.4

cells expressing increasing levels of TatGFP.

2. Discussion

The advent of effective treatments for HIV infection challenges. The treatment regime requires a complex combination to combat the primary viremia as well as the many opportunistic infections. However, HIV infection is associated with an increased incidence of hypersensitivity ADRs to many medications, with SMX rank 1st (BISIMX rank 1st) et al (1992; Davis & Shearman & Sterling, 2000) are several factors that influence the susceptibility to ADRs in HIV-infected individuals. These include immune hyperactivation, perturbation of drug metabolism and altered drug pharmacokinetics. In cells, these ADRs usually manifest as asymptomatic HIV infection (AIDS) (Rieder & Dekaban, 2000) suggests that risk for the development of ADRs is linked with expression of a factor unique to HIV. We have shown that expression of HIV Tat correlates with sensitivity to SMX (Arp et al 2005) though it is unclear if the expression level of HIV Tat is a predictor of progression from HIV infection to AIDS and if this is the mechanism of ADRs, there is evidence that maintaining control of viral load appears to lower the risk for drug-induced ADRs (Chay et al 2002). In this study, we tested whether a differential level of Tat expression could modulate cellular sensitivity to SMX in a dependent manner in a T cell model of HA induced ADR. To do this, we used a system to differentially induce HIV Tat and then assess cell death. Upon exposure to SMX

The inducible pBIG vector developed by Saito et al (1999) enabled us to differentially express a TatGFP fusion protein

directly by resonance methods. The TatGFP fusion protein doxycycline induction as can be seen in the confocal image using flow cytometry and western blots illustrated the induction system.

Our laboratory has previously demonstrated that constitutive produces a cellular sensitivity to the active drug similar to that seen in the cell viability experiments. The MTT assay demonstrated no toxicity to the parental cells, a significant decrease in cell viability was seen in the presence of the active metabolite. SMX dependent cell death associated with SMX seen in all cell populations, even those expressing TatGFP. In the case of cells expressing TatGFP, a decrease in cell viability was evident as the expression of the physiologically relevant levels of Tat seen in HIV infected cells. TatGFP had a cooperative effect, SMX effect was dependent on the level of Tat expression. Of importance, cell death occurred at levels of Tat but also in the absence of SMX, likely to be seen in tissues of patients undergoing treatment [15].

There is limited data on the relative contribution of cell death in the cells of patients who have sustained hyperkeratocytosis from lesions of patients with drug-induced epidermal necrolysis (TEN) and Stevens-Johnson syndrome, two potentially threatening diseases resulting from ADRs, have been shown

apoptosis (Aislet et al 2003; Paquet & Pierard, 2002). In a study suggested a potential mechanism for the sensitivity of Jurkat cells to SMX. HA was able to induce apoptosis in cells lacking TatGFP, but not in cells co-expressing TatGFP. Apoptosis was potentiated as TatGFP expression increased. The role of a specific TatGFP domain in this process is not clear. Data showing the release of cytochrome c and the presence of the activated caspase-3 in a manner dependent on increasing TatGFP expression support a mechanism for this that may involve Tat's known ability to suppress antioxidant defenses. In models of traumatic brain injury, reductions in MnSOD and glutathione peroxidase activity have been shown to be associated with increased apoptosis of cytochrome c (Newent et al. 2001; Sullivan 2002). However, further experiments are needed to establish this. The synergistic effect of HA and SMX on Tat protein that potentiates apoptosis in Jurkat cells. The appropriate effects of Tat, which have been found to include such as those described by Li et al (1995) and Park et al (2001) could contribute to the increased incidence of AIDS in HIV infection, which is potentially an example of apoptosis deregulation, which has been observed in other conditions such as neurodegeneration (Jarratt et al 2004; Li et al 2004; Rego & Oliveira, 2003).

Tat protein expression has been linked to an increase in oxidative stress and mitochondrial dysfunction (Chen et al 2000; Rea et al 2002). Specifically, in the association between HA and SMX established by Park et al (2005), cells stably transfected with Tat were significantly more sensitive to SMX and this sensitivity correlated with decreased total GSH levels. From these observations, we hypothesized that expressing Tat in cells

to S-MXA would be related to a perturbation in the GSH redox cycle. The GSSG/GSH ratio expression increased. However, the concentration of oxidized forms of GSH in the cells not expressing TatGFP was similar to those expressing increasing levels of TatGFP (data not shown). In contrast to the truncated 72 amino acid (single cysteine) form of Tat in our model. Westenberg et al. (1995) showed that total GSH content of cells exposed to a 10⁶ Tat protein was significantly lower than the control cells. This same study also demonstrated that cells exposed to the same concentration of GSH had a lower content of those exposed to the truncated 72 amino acid form of Tat (Choi et al. 2000). Choi et al. (2000) showed that the expression of full length transgenic mice was associated with decreased GSH synthesis (Choi et al. 2000). The fact that the GSH content of the cell populations was similar irrespective of the level of the 72 amino acid form of Tat suggests that there are other factors involved in the pathogenesis of hypersensitivity to Tat expression and HIV infection. Additional studies similar to those using full length and 72 amino acid forms of Tat will help to clarify this.

Furthermore, the role of other HIV proteins such as gp120 and gp130 in mitochondria needs to be determined (Mehrad et al. 2003; Ye et al. 2005). Our data suggests that Tat, in addition to conferring increased sensitivity to the intrinsic activation of macrophages, also confers an increased sensitivity to the intrinsic activation of macrophages (Hsieh et al. 2004; Yang et al. 2003). The data presented here constitutes an important step in understanding the mechanism of hypersensitivity ADRs during HIV infection.

in Tat expression during the progression of AIDS. The role of HIV in the development of the infected individual to hypersensitivity ADRs related to the use of drugs is an important in the care of patients living with HIV infection.

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CHAPTER 3: Cytoplasmic distribution of H2L6/3
Jurkat T cells. Sulfamethoxazole-induced
toxicity.

3.1 Introduction

One consequence of HIV infection is the gradual decline of the immune system which allows for vulnerability to opportunistic infections. Pneumocystis carinii takes advantage of this immunodeficiency to cause pneumonia, a lung infection that is commonly treated with cotrimoxazole (SMX-TMP). This combination antimicrobial is used to treat urinary tract infections. The SMX moiety of the drug is associated with a 50% or more severe drug reaction (ADR) in HIV infected patients, but only 25% in patients not infected with HIV. Adverse drug reactions can be defined as a response to unintended drug doses normally used for therapy (Riephly, 2009). The most serious is the hypersensitivity which usually presents as a delayed reaction characterized by the onset of fever, the development of a skin rash, Stevens-Johnson syndrome or toxic epidermal necrolysis. The liver and kidneys may be involved, presenting clinical features of hepatitis interstitial (Riephly, 2009).

The mechanism of hypersensitivity to cotrimoxazole is not clear, however the hapten hypothesis of bioactivation and haptent formation of the drug is critical in initiating an immune response. Sulphamethoxazole is a substrate of the cytochrome P450 enzyme CYP2C9, which is oxidized to N-SMX. Both metabolites are thought to be toxic to cells, however this cytotoxicity is mitigated with the addition of a thione

(GSH) antioxidant, its precursors, and its synthesis (Reinherz et al. 1988) deplete in vitro toxicity was the basis for the diagnosis of hypersensitivity A peripheral blood lymphocytes from patients with a history of allergic rhinitis showed significantly increased toxicity to a concentration of 10⁻⁵ M SAMX compared to those from controls and of a type of sensitivity with a similar reaction (Reinherz et al. 1988). Similar findings were reported in other sensitive patients where SAMX cytotoxicity was greater in peripheral blood lymphocytes (Gardiner et al. 1993). Enhanced lymphocyte sensitivity to SAMX related with expression of 17β-estradiol and activator of transcription-1 was associated with reduced intracellular GSH concentrations (Srinivasan et al. 2005).

Tat is a small functional transcriptional regulator essential for the replication of human immunodeficiency virus type 1 (HIV-1). Tat binds to the specific sequences of TAR (Tat activation region), a stable RNA hairpin that binds all nascent viral transcripts. This binding recruits and potentiates RNA polymerase II to synthesize full-length HIV transcripts (Campbell et al. 2010).

The coding sequence of the Tat gene is located on two exons and the resulting protein has a molecular weight of 10.1 kDa. It is made up of 101 amino acid residues encoded by the first exon and the second exon. The 101 amino acid Tat protein is found predominantly in clinical isolates of all HIV subtypes except subtype B, a synonymous single nucleotide polymorphism creating a stop codon in the second exon and a truncated protein (Campbell & Lothrop 2009). A few laboratory strains that express

amino acid form of Tat, which represents an artificially truncated protein. However, this 86 amino acid Tat has frequently been shown to possess properties of the protein. The protein is divided into six domains. They are: the N-terminal domain (1-21), a cysteine-rich domain (22-47), a core domain (48-60), a basic domain (61-72), a zinc finger domain (73-101) (Campbell & Loret, 2009; Derse et al, 1991; Reman, 2010). The basic domain, also known as the transduction domain, contains the nuclear localization signal and confers on the protein the ability to enter uninfected target cells. Ultimately, extracellular Tat facilitates the spread of HIV infection. The basic domain includes a region that confers TAR RNA binding properties to Tat. The protein, which consists of three domains, represents the combination of the active regions that is required for functional HIV-1 transactivating activity.

In this study we sought to determine which domain of the protein was responsible for modulating the cellular toxicity of Tat and SMX. Furthermore, to determine if the same protein was responsible for modulating the cellular

3.2 Materials and Methods

3.2.1 Cell line

The human T lymphocyte cell line HL60 (ATCC) was obtained from the American Type Culture Collection. The cell line was maintained in Complete RPMI 1640 medium (Invitrogen) supplemented with fetal calf serum (100 units/ml of penicillin and 100 units/ml of streptomycin) (ATCC CRL 1651) and keyhole limpet hemocyanin (Miles) in Modified Eagle's (DMEM) medium with 10% fetal calf serum and 100 units/ml penicillin and streptomycin obtained from ATCC.

Construction of plasmid lines

The Tat gene was amplified from the plasmid pSVTat. Tat encodes the full length Tat gene and was a kind gift from Dr. KT J. of Molecular Microbiology, the Molecular Virology Section. The full length Tat gene (Tat101), with or without stop codons, and Tat72, Tat48 (Sato et al., 1999) were cloned into the pEGFP-N1 (Clontech), resulting in Tat101, Tat101GFP, Tat86GFP, Tat72GFP, Tat48GFP respectively. The fusion genes were excised from pEGFP-N1 fragments and inserted into the Nhe I/Not I digested pEGFP-N1 doxycycline responsive expression vector (Sato et al., 1999). The results were the following plasmid constructs; pBIG1GFP, pBIG1GFP, pBIG1GFP, Tat86GFP, Tat72GFP, pBIG Tat48GFP. As a control, the GFP gene pEGFP1 was also cloned into pBIG2i to create pBIG GFP.

constructs or pBIG GFP were transfected into Jurkat cells by nucleofection (Lonza, Amaxa Nucleofector) according to the manufacturer's protocol. Stable cell lines of Jurkat pBIG TatGFP and Jurkat pBIG GFP were also transfected with the pBIG TatGFP constructs for control. The expression of TatGFP fusion protein in Jurkat cells was induced by incubating the pBIG2⁺ Jurkat cells with doxycycline (Sigma).

Selective media

Post-nucleofection, Jurkat cells were selected in the presence of hygromycin B (Invitrogen) at a concentration of 500 µg/ml. Jurkat cells derived from single cells following bulk selection were also used. The stably selected transfected cells were also sorted and used. The resulting cell lines were maintained in complete RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, streptomycin and 0.5 mg/ml of hygromycin B.

3.2 Confocal microscopy

Jurkat pBIG TatGFP cells were incubated with 500 ng/ml of Hoechst 33258 and Jurkat pBIG GFP cells were incubated with 6 µM MitoTracker Far Red (Invitrogen) for 30 min. The images were taken with LSM 510 META confocal microscope. The images were processed using ImageJ (Media Cybernetics) and Adobe Photoshop (Adobe Systems, Inc.).

3.2 Dose response and doxycycline induction

The concentration of doxycycline required to induce expression of fusion proteins was determined in experiments where cells from different cell lines were treated with doxycycline for 40 hours. The cells were analyzed on a FACScan (Matter Science) instrument (Becton Dickinson). Time course experiments were done with cells with 1000 ng/ml of doxycycline for 72 hours, with aliquots taken and analyzed by flow cytometry on a FACScan. Both the dose response and time course experiments were done in triplicate. Flow cytometry data were analyzed and the percentage of GFP+ cells determined using the FlowJo program (TreeStar).

3.2 Real time PCR analyses of Tat/GAPDH mRNA levels

To compare the level of Tat mRNA present in the stable HIV-infected cells, cells from each of the stable pB1G TatGFP cell lines were treated with 0, 2400, 600, 800 and 1000 ng/ml doxycycline for 72 hours. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) by following the manufacturer's instructions. RNA was also isolated from uninfected control cells. Total RNA (10 µg) was reverse transcribed using the High Capacity cDNA Archival Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR reactions were performed in the absence of cDNA template using a control housekeeping gene as a reference. Real-time PCR was performed using the PRISM 7900HT Sequence Detection System (Applied Biosystems).

Relative expression levels were determined by the comparison of the expression levels of the target gene to those of a housekeeping gene (Wong & Reed, 2005)

3.2 Immunoblot analysis

To characterize the expression of the TatGFP fusion lines, cells from each cell line were differentially induced with doxycycline, which the cells were washed twice with PBS with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 1 mM EDTA and complete mini protease inhibitor cocktail) and lysates were sonicated for 2x20 sec at 3000 rpm for 5 min at 4°C. Supernatants were collected and the protein concentration was determined by BioRad DC Protein Assay that is a modification of the Lowry assay. BSA was used as the standard in the protein assay.

The proteins were resolved on 4-20% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were probed with anti-Tat antibody (Advanced Biotechnologies Inc.). Bound antibody was probed with secondary antibody and detected by chemiluminescence (Amersham Pharmacia Biotech) (was used as a control for protein loading).

3.2 MTT Cell viability assay

Drug toxicity was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay. Cell viability was quantified by colorimetric conversion of MTT, a method described by Mosmann (1983). Briefly, Jurkat GFP cells were differentiated with doxycycline

for 40 washed twice with PBS (17.5 x 10⁶ wells plate 96 and incubated in buffer 210 SMX (S-Agri) - 80005 of SMX HA for 2 h. After the drug was removed the cells were incubated at 37°C for 18 hrs. Next, 1 mg/ml MTT was added to the 37°C for another 4 h. The reaction product that precipitated was solubilized at room temperature by the addition of 1 ml of 10% SDS (5/0 20% SDS) to the wells. The following morning the absorbance was determined by an ELISA plate reader at 590 nm and equated to cell viability with the help of a Ttiss assay. This assay was performed on three occasions for each cell line.

3.2 Detection of reactive species ROS

Following the 40 h cyclophosphamide induction, cells were washed twice in PBS and resuspended in HBSS (Hank's buffered saline solution) 1 x 10⁶ cells/ml. Suspensions (100 µl) were placed in 96 well plate, centrifuged at 1500 rpm for 5 min and the supernatant decanted. 20 µM DCFDA (2',7'-dichlorofluorescein diacetate, Sigma) was added and the plate incubated for 30 min. The plate was centrifuged at 1500 rpm for 5 min and the supernatant decanted. The cells were washed with HBSS and background fluorescence measured at 485 nm excitation and 527 nm emission. SMX was added to the wells in quadruplicate and the fluorescence readings taken at 30 min. This assay was performed on three separate occasions for each cell line.

3.2 Statistics

MTT cell viability data were analyzed using GraphPad Software Inc., San Diego, CA. ANOVA with a post hoc procedure was used to compare mean differences between concentrations. The IC_{50} values were calculated using the GraphPad IC_{50} values (concentration) were transformed and the Y values (viability) were normalized to define 0% and 100% as the respectively. This was significant slope (variance slope) GraphPad (GraphPad Software Inc., San Diego, CA) calculate mean and standard error. PCR data are real time

3.3 Results

3.3.1 TatGFP constructs

The full length HIV Tat protein (Tat101) was PCR amplified from the terminal domain of a GFP plasmid to create the fusion protein. A number of deletion mutants of the Tat protein were similar to the TatGFP fusion protein constructs; Tat72GFP, which encodes the first three domains of the Tat protein; Tat86GFP, which encodes the first exon of the Tat protein; Tat48GFP expresses the first three domains of the Tat protein (domain 1, 2, and 3). The fusion constructs were inserted into a vector pBIG2i and the different plasmids were subsequently transfected into T cells. After a period of Hygromycin B selection, limiting dilution was used to obtain single cell clones that were used to establish stable cell lines.

3.3.1.1 Intracellular localization of TatGFP constructs

Confocal microscopy was used to determine the intracellular localization of the different Tat constructs in Cos 7 cells. The intracellular localization study was carried out in Cos 7 cells. This study is of imaging analysis of the small and dark cells that have minimal cytoplasm. Studies have previously shown that protein HIV transport to mitochondria (Draha Gaizo & Payne, 2003). In order to confirm this, the cells were treated with 500 ng/ml doxycycline and 200 nM of mitochondrial specific MitoTracker Deep Red 3.3.3.3. The results for D

The confocal images showed that the GFP-Tat104GFP, Tat86GFP, Tat72GFP constructs localized with the Hoechst (Figure 3.1A-C). The mitochondria were found to be evenly distributed throughout the cytoplasm. In contrast, the Tat48GFP constructs showed fluorescence in the entire distribution of the mitochondria in the cytoplasm of the Tat48GFP cells similar to that of the other constructs (Figure 3.1D). This appearance of mitochondrial aggregation around the nucleus in the cells expressing GFP-Tat48GFP (Figure 3.1E) on the punctate/diffuse nature of the GFP fluorescence in Tat48GFP expressing cells suggests colocalization between the GFP-tagged mitochondrial proteins (Figure 3.1D) and the commonly accepted method of visualizing colocalization, to present results as a simple overlay of channels. For example, a pair of green and red images will give spots where the two molecules of interest are present in the same area. There are limits to this rather elementary method as it simply encodes a physical definition of colocalization. More types of fluorescent molecules occupy the same pixel (Zink & Girard, 2009). However, biological colocalization means that two or more different molecules to the same structure. The method of choice to quantify colocalization on subcellular structures relies on their fluorescence profiles (Bolte & Cordelières, 2006). The profiles for the images of TatGFP and Tat48GFP expressing cells showed that GFP colocalized with mitochondria.

Figure 3. Confocal images showing localization of the various Tat proteins from (A) Tat101GFP, (B) Tat86GFP, (C) Tat72GFP (D) Tat66GFP. Cell lines were treated with doxycycline for 40 h, stained with DAPI and analyzed with a Zeiss LSM 510 META confocal microscope. Scale bar represents 20 μ m.

Figure 3.1

3.3.3 Characterization of the TatGFP fusion constructs

The next set of experiments involved the characterization of the TatGFP fusion constructs in different cell lines. Time course experiments were carried out to determine the kinetics of induction of protein expression by doxycycline in different cell lines, which the cell lines expressed the TatGFP fusion constructs. The different cell lines were treated with doxycycline and taken at various time points and analyzed by flow cytometry. In the Tat101GFP cell line, doxycycline induced GFP expression (Fig. 3.2A). At 12 hours after induction, the percentage of GFP-expressing cells was approximately 80%. At 24 and 36 hours, the percentage of GFP-expressing cells increased to approximately 90%. At 48 hours, the percentage of GFP-expressing cells was approximately 90%. At 72 hours, the percentage of GFP-expressing cells was approximately 90%. The other cell lines also demonstrated similar kinetics of GFP induction. In all the cell lines, increasing the concentration of doxycycline resulted in an increase in the percentage of GFP-expressing cells at every time point after the start of induction.

The other cell lines also demonstrated similar kinetics of GFP induction. In all the cell lines, increasing the concentration of doxycycline resulted in an increase in the percentage of GFP-expressing cells at every time point after the start of induction.

Figure 3 Time course of TatGFP expression following different induction. Cells from (A) Tat101GFP, (B) Tat86GFP, (C) Tat61GFP, (D) Tat41GFP, (E) Tat21GFP lines were treated with various concentrations of doxycycline and incubated for 72 h. Aliquots of cells were analyzed by flow cytometry. Data are representative of three independent experiments.

Figure 3.2

incubated (Figure 3.2B). The exact percentage of GFP⁺ cells at each concentration of doxycycline tested differed slightly for instance, at 24 h and 250 ng/ml doxycycline, the Tat101GFP 50% GFP⁺ expressing cells compared to 55% for the Tat72GFP and Tat86GFP cell lines respectively. Furthermore, the maximal GFP expression for the Tat101GFP and Tat86GFP cell lines is maximal at 500 ng/ml Dox for the both cell lines. In addition, GFP expression generally begins to decrease at the 36 h time point and is maintained for at least the 72 h period in these experiments. In four of the five cell lines, there were no cells expressing GFP in the absence of doxycycline (0 ng/ml) by flow cytometry. The exception was the Tat48GFP cell line, which had approximately 6% of cells expressing GFP in the absence of doxycycline (0 ng/ml). Approximately 85% of the Tat101GFP cells expressed GFP at the maximal level of induction by 1000 ng/ml doxycycline. Tat72GFP cell line (Figure 3.2E), but somewhat lower than that of the other cell lines.

Each of the stably transfected Jurkat T cell lines was analyzed by western blotting to determine that the correct fusion protein was present. The data in Figure 3.2 detected GFP fluorescence, but does not explicitly confirm that the Tat portion of the fusion protein is present. To this end, Jurkat pBIG Tat cells were incubated with doxycycline identical to that used in the GFP expression experiments (1000 ng/ml) then the cells were lysed and protein extracts prepared.

The use of specific antibodies showed the presence of the protein in each cell line. The western blots showed that the protein is expressed even in the absence of doxycycline in the Tat48GFP cell line. The expression of the protein increased with increasing concentration of doxycycline. Significant expression of the protein was not detected by western blotting when doxycycline was used although trace of the protein could be detected. The other cell lines showed a similar trend, with significant expression of the protein only when doxycycline was used. The western blots were confirmed the expected protein expression for Tat86GFP (Figure 3.3B) and Tat72GFP (Figure 3.3C). Significant expression of the protein was not detected by western blotting when doxycycline was used although trace of the protein could be detected. The other cell lines showed a similar trend, with significant expression of the protein only when doxycycline was used. The western blots were confirmed the expected protein expression for Tat86GFP (Figure 3.3B) and Tat72GFP (Figure 3.3C). An exception to the general trend is the Tat48GFP cell line. Tat48GFP expression even in the absence of doxycycline is significant. The expression of protein is comparable in the lower range of doxycycline concentrations. Tat48GFP only shows significant increases when concentrations of 250ng/ml and 500ng/ml were used. The western blots were confirmed the expected protein expression for each construct in each case (Figure 3.3).

3.3.4 Quantitative PCR of HIV Tat and TatGFP specific mRNA

In an effort to determine the expression level of the engineered HIV Tat in the cell lines, quantitative PCR was performed. This was necessary as the Tat protein in the cell lines was undetectable via western blots. Using primers specific for HIV Tat, quantitative PCR was performed on all the cell lines expressing HIV Tat proteins as well as the control cell lines.

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

Figure 3.3



The Tat101 cell line showed Tat mRNA in the absence (0 ng/ml Dox, Fig. 3A) 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 at exposure concentrations of approximately 400 ng/ml Dox that the Tat101 cell line reached the level of HIV cells. The Tat101 GFP cells showed a four-fold lower amount of mRNA at 0 ng/ml Dox compared to the HIV cells. It showed a much steeper increase in Tat mRNA in response to increasing doxycycline. Furthermore, the Tat101 GFP line had a higher amount of Tat mRNA overall compared to the Tat100 GFP line. Dox was sufficient to reach the level of Tat mRNA seen in HIV cells.

In the Tat86 GFP cells, there was a small, but detectable amount of Tat mRNA at 0 ng/ml Dox, approximately 10% of that seen in HIV cells. At a concentration of 200 ng/ml Dox, the Tat86 GFP cells showed amounts similar to those of HIV cells (Fig. 3C). The Tat86 GFP cell line showed a clear increase in Tat mRNA with the increase in doxycycline with the maximal amount occurring with 800 ng/ml Dox as a subsequent decrease of the mRNA levels at 1000 ng/ml Dox. The cell line showed a gradual increase in Tat mRNA as the doxycycline increased with a maximal amount at the highest dose tested (Fig. 3D). Like the previous cell line, the Tat72 GFP cells also showed a similar amount of Tat mRNA at 200 ng/ml Dox which is comparable to that seen in HIV cells.

Figure 3 Quantification of Tat mRNA in the various cell lines. Cell lines Tat101, (B) Tat101GFP, (C) Tat86GFP, (D) Tat72GFP (E) were treated with doxycycline for 40 h then RNA was reverse transcribed and used as template for RT-PCR. GAPDH served as the housekeeping gene. Tat mRNA was detected specifically. Data are mean of three independent experiments.

Figure 3.4

The Tat48GFP cell line expressed approximately 100 ng/ml Tat mRNA at 0 ng/ml Dox (Figure 3.4E) in comparison to other cell lines. In addition, there is an increase in Tat mRNA levels as doxycycline concentration is increased. The cell line also had considerably less Tat mRNA than the other cell lines, needing 1000 ng/ml Dox to reach the levels of the other cell lines. The Tat48GFP cell line follows the general trend of Tat mRNA with an increase in tracking with increases in doxycycline concentration (Figure 3.4E). Tat mRNA levels coincide with the level of Tat protein at 200 and 400 ng/ml Dox. PCR analysis was also carried out using RNA from untreated cells as well as Jurkat T cells expressing only GFP and did not show an increase in amount of Tat mRNA (data not shown).

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

Earlier studies have consistently shown the toxicity of SMX in Jurkat T cells after incubation with cells with a history of hypersensitivity to SMX. Studies have also shown that this effect on viability is dependent on the amount of the Tat protein and is also influenced by the amount of SMX (Wang et al., 2005). To determine which region of the Tat protein is involved in the toxicity, the full length protein and deletion constructs were evaluated. These cell lines were employed in MTT assays to determine the sensitivity to SMX and its reactivity to SMX. The assays were carried out using untreated cells (control) and cells treated with concentrations of doxycycline to induce different amounts of Tat protein, or induced to yield the amount of Tat-equivalent

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

Previous studies have shown that SMX has a significant viability of Jurkat cells (Figure 3.5). To determine the viability of Jurkat cells transfected with each of the Tat constructs assayed by the MTT assay, Jurkat cells were then used to assay the viability of differentially induced Tat101 Jurkat E6.1 T cells in the presence of various concentrations of five meta-SMXs. Indeed, cell viability remained largely unchanged in the cell populations until the addition of SMX. At 200 μ M SMX stably transfected Jurkat cells treated with 1000 ng/ml Dox showed a trend towards lower cell viability compared to Dox and the non-transfected Jurkat E6.1 T cells. The difference in cell viability of the transfected cell line E6.1 was significant at 400 μ M of SMX. At this concentration there was no significant difference in the differentially induced Tat101 cells.

For the Tat101GFP cell line doxycycline at 500 ng/ml concentration were tested in the presence of SMX. DMSO (0 μ M SMX) had no discernable effect on cell viability of the Jurkat cells. However, there was a dose-dependent decrease in cell viability with the increasing concentration of SMX in the Jurkat E6.1 cells treated with doxycycline at 100 ng/ml. The toxicity of the Jurkat E6.1 cells was significantly different from the Jurkat E6.1 cells treated with 100 ng/ml doxycycline with 100 μ M SMX. At 50 μ M of SMX, the decrease in cell viability induced with 100 ng/ml doxycycline began to be significantly different from the Jurkat E6.1 cells not induced for Tat101.

expressed 0 ng/ml Dox (Figure 3.5B). At the higher concentration of dox, however, this decline was observed at 200 μ M Dox. There is a significant difference between Dox and 100 ng/ml Dox cell populations and this was also observed in the Jurkat E6.1 cells induced with 500 ng/ml doxycycline followed the general trend of decreasing viability with increasing concentration. However, the differences in cell viability that were significantly different from doxycycline 0 ng/ml Dox).

In the Tat86GFP cell line, the effect of increasing concentration was determined using three cell populations induced at 0, 200, and 400 ng/ml Dox (Figure 3.5C). Between 25 and 100 μ M Dox, there was a slight increase in proliferation of cells treated with 200 ng/ml Dox in comparison to the 0 ng/ml Dox experiment. In fact cells induced with 200 ng/ml Dox had a significant increase in cell proliferation compared to Jurkat E6.1 cells. However, there was also a significant decrease in viability of the Tat86GFP cells at 400 and 800 μ M Dox. In the Tat72GFP cell line, there was a significant decrease in cell viability at 200 ng/ml Dox and the 800 ng/ml Dox cell population. However, the result was not significant in the Tat72GFP cell line. In the Jurkat E6.1 cell line, there was a concentration dependent decrease in cell viability due to the increasing concentration of Dox. There was a significant difference between Jurkat E6.1 cells and the cells of the Tat72GFP cell line at 400 μ M Dox. However, the differential in cell viability of Tat72GFP had no significant effect on cell viability at a

Figure 3.5

The viability of the Jurkat E6.1 cells treated at 48 h was unchanged when treated (Fig. 3). However, a significant decrease in viability of the Tat18GFP cell line compared to Jurkat E6.1 cells was observed at 200 and 400 μ M SMX. At these concentrations, a significant decrease in viability of doxycycline-induced cell populations was observed. At a lower exposure than seen with the cell lines above, at 100 μ M SMX, the viability of the 0 ng/ml population was approximately 40%. At this concentration, the cells induced with 200 ng/ml Dox showed 30% viability. When the cells were induced with 100 ng/ml Dox, but were not significantly different from the 0 ng/ml Dox. Cell viability continued to decrease at the higher concentrations of SMX. No significant difference in the differentiation of the cells was observed. No significant difference between the differentiation of the Tat18GFP and Tat101GFP cell lines (Fig. 3). The main difference between this cell line and the Jurkat E6.1 cell line above is the general sensitivity to SMX. The IC₅₀ of SMX for the Jurkat E6.1 cell line is approximately 70% and 30% cell viability with 50 μ M SMX and 100 μ M SMX, respectively, concentrations which the cell lines expressing Tat101, Tat101GFP, Tat18GFP, and Tat72GFP displayed cell viabilities of 90% and above.

Comparing the IC₅₀ (the concentration, 50%) of each of the cell lines, it was found that both the Tat18GFP and Tat101GFP cell lines are particularly sensitive to SMX. As shown in Table 3, the IC₅₀ of SMX for the Jurkat E6.1, Tat101, Tat101GFP, and Tat72GFP cell lines were in the range of 100-300 μ M SMX, while the IC₅₀ of SMX for the Tat18GFP cell line was lower, at 10-60 μ M SMX.

Table 3.1 shows the IC_{50} values of the various cell lines used in the MTT cell line sensitivity assay. The cells used in the experiment were established cell lines with the exception of the two cell lines given the pooled mean IC_{50} values. The cell lines were generated by hygromycin B selection post transfection. Multiple IC_{50} values were calculated using GraphPad Prism v.5.0 using the methods.

Table 3.

Cell Line	0ng/Ddx	400ng/ml	1000ng/Ddx
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
TatGFP	59	52	57
Pooled Tat48	161	162	137
Pooled GFP	149	146	138

SMXHA respectively. effort to determine if this specificity, on the pBIG Tat48GFP and pBIG Tat48GFP BNGA Twaas used to transfect Jurkat the pool of stably selected transfectants rather than single clones. The IC₅₀ of SMX was 370 μM and 419 μM respectively. While in both cases this is higher than the IC₅₀ of SMX using specific cell lines, much lower than the IC₅₀ of SMX in Jurkat cells. The selectivity of SMX in two cell lines.

3.3.6 Generation of ROS by Jurkat cell lines

To evaluate one possible cause for the cell death observed in the presence of oxidant effects of Doxycycline and its deletion mutants we performed a series of experiments. The cells from the various cell lines were treated with the same doxycycline concentrations as in the MTT assays in the presence of SMXHA or SMXDCFH, a cell permeable dye whose fluorescence is dependent on the presence of ROS. Incubation with SMX generated fluorescence amounts similar to the fluorescence seen when the cells were treated with DMSO used to facilitate dissolution of SMX. Significant ROS production was only evident upon the addition of SMX to the cell lines (Figure 3.6A) and resulted in a dose-dependent increase in ROS production at concentrations of 50 and 200 μM, respectively. Furthermore, increasing doxycycline concentration lead to increased ROS production. Cells induced with 400 ng/ml Dox, produced a small, but significant amount of ROS compared to the control (doxycycline untreated) cell population. Similarly, cells induced with 1000 ng/ml Dox, generated

Figure 3. Reactive oxygen species generated in the presence of various Tat constructs. After induction with doxycycline, cells expressing (B) Tat86GFP, (C) Tat72GFP, (D) GFP, or (E) GFP were treated at a concentration of 1×10^8 cells/ml and incubated with $100 \mu\text{M}$ H₂O₂ for 30 min. The cells followed an incubation with different concentrations of H_2O_2 (0, 10, 20, 50, 100, 200 μM). The fluorescence was determined by a plate reader (excitation 488 nm, emission 520 nm). # $P < 0.05$ vs 200 μM H_2O_2 . Data are mean of three independent experiments.

Figure 3.6

increase in ROS compared with the uninduced HA cells and when this was significant at 500 nM and 2000 nM, the higher concentration of doxycycline did not result in a production of more ROS and from the amount of ROS produced by the cells induced with SMXHA.

The relationship between the HA cell line and the Tat101 cell line with SMX production and the addition of SMX in 4-iron-Biolcan increases in the amount of ROS produced. ROS production from T101 cells shows a biphasic response to the doxycycline concentration as initially, then decreases and is subsequently followed by a decrease in ROS production. The initial increase in ROS production with 100 nM doxycycline produced significantly more ROS than was seen from the 50 μ M SMX. This is followed by a decrease in ROS production with 200 nM doxycycline and 500 nM SMX. Finally, cells induced with 500 nM doxycycline produced significantly less ROS than uninduced cells, but significantly lower than ROS from cells induced with 200 nM SMXHA respectively.

Production of ROS from the rest of the HA cells further produced significantly more ROS than the parent HA cell line. Expression of Tat360 (Fig 3.6) and Tat720 (Fig 3.7) lead to a slight, but not significant increase in ROS production compared to the the fusion proteins. The effect of SMX on ROS production is evident in

Tat48 GFP_u(βe6) and Tat48 GFP_i(guβe6) cell lines expression of t
consist had no significant effect on ROS production.

3.4 Discussion

HIV1 currently infects 33 million people worldwide. The use of antiretroviral therapy (ART) has dramatically improved the quality of life of individuals infected with HIV. There are several classes of antiretrovirals based on the mechanism of action. The primary function of antiretrovirals is (ARV) suppression of HIV replication. ART is most effectively when combined. However, these drugs are associated with serious adverse effects. The primary target of ART is CD4+ T cells and one of the major effects of HIV1 infection is the rapid and massive depletion of CD4+ T cells. This leads to a state of immune deficiency that allows the propagation of opportunistic infections that also require HIV1 infection. It is associated with an increased risk of AIDS. ARV that vary by drug class and mechanism of action with other drugs. One of the most common adverse effects of HIV1 infection is the development of immunodeficiency. This is manifested as HIV1 infection progresses to AIDS. A key factor in the development of AIDS is the depletion of CD4+ T cells. Previous research has shown that the depletion of CD4+ T cells is a key factor in the development of AIDS. The expression of the protein correlates with the concentration of CD4+ T cells (Adey et al. 2005). We have also demonstrated the differential expression of this protein in response to SMX (Adey et al. 2009). In this study we sought to determine the region of the Tat protein that is responsible for this effect. To this end, deletion mutants were created that were tested for their ability to support HIV1 replication upon treatment with SMX.

The initial experiments conducted established the distribution of the full length Tat-GFP fusion protein as well as the localization of Tat protein. As expected, the full length Tat protein localized to the nucleus. The GFP alone was detected in the cytoplasm with the Tat-GFP cell. The deletion mutants Tat78GFP and Tat48GFP showed the same cellular distribution as GFP, and Tat-GFP fusion proteins were distributed throughout the cell, likely due to the loss of the NLS fusion of the proteins into the nucleus. This overall distribution however did not include colocalization of the GFP with the mitochondria. The difference in the pattern of GFP and Tat-GFP proteins at the mitochondria under the lack of colocalization of the GFP fluorescence with the mitochondria in Tat-GFP and Tat48GFP cells as observed with the missing the NLS which also prevents the proteins to pass through the mitochondrial membrane. The localization of Tat-GFP and Tat48GFP cells is most likely due to the Tat proteins to members of the CREB binding protein family. Similar observations were obtained in the co-transfected Jurkat cell lines (data not shown).

The apparent mitochondrial localization of GFP in Tat-GFP expressing cell could be due to the early stages of apoptosis. Mitochondria play an important role in apoptotic signal transduction by releasing cytochrome c and activating caspases. Studies have shown that mitochondrial dynamics have a significant role in regulating apoptosis.

showed quantitatively by laser scanning cytometry that mitochondrial aggregation precedes cytochrome c release and that cytochrome c release is dependent on mitochondrial aggregation". GFP secretion, in the absence of any other stimuli, is able to prime the cell.

The characterization of the cell line was done by immunofluorescence as well as western blotting and quantitative time course experiments were necessary to show that doxycycline dependent induction of GFP RNA expression showed a dose response time course. These experiments were instrumental in determining the time course of the induction of the protein. The RT-PCR experiments proved a relatively easy method to measure the difference in Tat RNA produced each of the cell lines. This was particularly true for the HIV infected cell line. The RT-PCR method is a sensitive blotting protocol able to detect Tat protein from other cell lines. It is not possible to detect very faint bands in the RT-PCR. The lack of sensitivity of the western blot in this instance was due to the fact that Tat protein leaves the nucleus through the nuclear pore complex by passive diffusion. This is in contrast to the other proteins of the protein transduction domains which are transported into the cell. The use of RT-PCR allowed for a comparison of the Tat RNA in different doxycycline concentrations. The RT-PCR showed that Tat RNA in the HIV infected cells is equivalent to the concentration of doxycycline concentrations used. The GFP RNA in the HIV infected cells is approximately the same as in the HIV infected cells.

Results from the MTT assay have demonstrated significant decreases in cell viability in the presence of increasing concentrations of SMX. These observations are incubated with the parent compound, SMX. The conditions constitutive expression of SMX mediated toxicity an effect similar to that seen in the differential expression of Tat cooperative effect. SMX treatment, significantly decreases viability the expression of the TatGFAP (p=0.001). These observations were the basis of the current project. Tat protein is responsible for these results. For this several proteins were generated and tested in cell lines that were not affected by SMX. The results show that the differential expression of Tat at physiological concentrations had no additive effect on cell toxicity in the presence of the highest concentration of SMX. As the concentration of SMX and the amount of Tat present in the degree of toxicity was correlated. Neither SMX nor Tat at a concentration of 200 μ M concentrations of SMX (100 μ M) used in this study seems to be a growth inhibitor at the lower concentrations of SMX in concordance with previous data (Hess et al 1997). This is a significant finding at 200 μ M of SMX where Tat mRNA levels equivalent to or higher than HIV infected. At 100 μ M and 200 μ M the cell toxicity of the three doxycycline treated cell populations significantly different from

other, possibly due to the fact that this is a moderate amount of Tat101 mRNA at 0 Dox (Figure 4) an amount only slightly less than 400ng/Dox. While doxycycline treated Tat101 cell populations are significantly different from control and these are significantly different from that of Jurkat treated with 200µM SMX.

The viability of the Tat101GFP cell line is not significantly different to control populations of 0ng/ml and 100ng/ml doxycycline. As the concentration of doxycycline increased, the viability of the 100ng/ml Dox cell population was significantly different from the 0ng/ml Dox population. At the 500ng/ml Dox population remained largely unchanged at the level of control. SMX (100µM) and was not significantly different from the control at any of the concentrations tested. SMX, a bacteriostatic antibiotic, has been shown to affect the viability of the Tat101GFP cell line where low expression of Tat101GFP results in a significant effect. This is a phenomenon that typically occurs in immunology assays where antibody bind all the receptor sites, leaving nothing available for the next step. This is quite different from the results seen in the Tat101GFP cell line where differences in the amount of Tat101GFP mRNA are compensated in the associated protein levels by the Tat101 protein. Besides the fact that the Tat101 protein is highly expressed in the Tat101GFP cell line, the increase in doxycycline concentration as that found in the Tat101GFP cell line (100ng/ml Dox, 400ng/ml Dox, and 1000ng/ml Dox) only resulted in a 1.5 and 3.5 fold increase respectively than that seen in control cells. In comparison, the amount of Tat101GFP mRNA at

400 ng/ml is a long seat while the concentration is 2160 greater than that at 10 ng/ml. There is a much wider range of concentrations that allow for the observance of the prozone effect.

Another possible explanation for the discrepancy between the results from the Tat101 cell line and GFPs is that the fusion of GFP to Tat101 has an effect on the fluorescence of GFP. As widely used *in vitro* well-known as a molecular marker, although GFP is found in several of marine invertebrates, GFP traditionally refers to the protein from jellyfish. GFP has also been identified in a variety of corals where the protein is bioluminescent. The presence of GFP in fluorescent jellyfish and the coral culture system (Beauchamp & Abdalrhahman 2006) where the fluorescent chromophore originates from the Ser-Tyr-Gly sequence that is partially modified in the presence of oxygen to form a fluorescent ring structure that is maximal at 475 nm and emits at 515 nm (Beauchamp & Abdalrhahman 2006). While molecular oxygen is required for the translation of the protein, its introduction in GFP in corals where hyperoxia and reactive oxygen species (ROS) from photosynthetic activity (Beauchamp & Abdalrhahman 2006) recent study on the distribution and function of GFP in *Carillibeyae* GFPs from the hydrothermal vent system have the ability to scavenge reactive radicals and exhibit SOD activity by competing with cytochrome c reaction with the authors found that the activity of GFP does not affect the fluorescence of the protein and can provide supplementary

protectio... What is actively potentially have a profound outcome of the various described... First, the authors used wild type GFP in their experiments... deletion mutants were fused to EGFP (resistant to proteolysis) mutant that has been optimized for brighter expression in mammalian cells to reduce clustering... (Gustafsson, 1997) since the purpose of GFP in nature is still being debated... EGFP mutation in relation to the original... GFP generated... (Chen et al., 2006) suggests that wild GFP has the ability to specifically... however a study (Gulow et al., 2005) showed that there was no detectable... cell line... incubation with... significant... even if... GFP is maintained in the... mutant, by Gulow et al. (2005) the EGFP Tat and its deletion mutant would have a minimal... that could lead to oxidative stress and cell death.

Therefore an effort to determine if the... a significant... experiments were carried out with... line expressing... the expected... decrease in cell viability in the presence of... other... effect on... GFP expression, even at the concentration of... the pattern was seen in the cell... Tat86 GFP and Tat72 GFP were... nearly visible, but that

The failure of Tat72GFP expression to add SMX-mediated resistance in contrast to previously (Adulyasri et al. 2009; Arpa 2005) this could be due to the fact that the protein used in the previous study was an amino acid pair to the protein used in the current study. Tat variants may have basic Tat properties in this case because differences in the Tat previously confer a greater ability to activate SMX. The substitution of P58 for P57 in Tat72GFP is not expected (Adulyasri et al. 2009; Arpa 2005) in the constructs used in this chapter. The structure of the Tat protein at position 58 could affect protein translocation into the nucleus. The basic Tat protein is known to be more effective than the basic Tat protein. Pelopon et al. (1999) showed that differences in Tat could have an effect on the ability of chemically synthesized six different Tat proteins from HIV-1 found in different parts of the world to bind to the TAR region as well as to transactivate the HIV-1 promoter. They found that all six variants were able to bind to the TAR region and to transactivate the HIV-1 promoter. The experiments showed that the high Tat variants from Africa and South America were more effective than the low Tat variants. Then a number of publications have demonstrated specific Tat protein variants with different transactivation potentials in experiments using conventional reporter genes. These studies have shown that the sequential differences in Tat protein variants are important.

leading differentially expressed genes including those TNF- α , CD63, IL-10 (Desfoes et al. 2005; Gattalini et al. 2009; Samak et al. 2009; Wong et al. 2010) to my knowledge there has not been a study looking for differentially expressed genes that could be affected by those involved in redox regulation for instance glutathione S-transferase, two genes known to be repressed by Tat

The Tat48 GFP "GFP" cell lines are relatively more sensitive to metabolic stress than both the parent cell line Jurkat E6.1 and the Tat48 GFP "GFP" cell lines have in common that they are both missing the protein tyrosine phosphatase SH-PT62, infection, cells infected with the virus produce Tat intracellularly and eventually able to leave the cell and extracellularly. In HeLa cells of Tat to translocate across the mammalian cell membrane via the PTD which also contains the PTD thus the Tat48 GFP and "GFP" fusion proteins escape the nuclear envelope in the cytoplasm. The best study of the Tat protein is a study that shows that the viral genome is able to replicate in various cell lines and that those genes are the cytoskeleton which have been found to be regulated by the Tat protein (Carpino et al. 2006) additionally extracellularly has been found to interact with cytoskeletal proteins (Chen et al. 2002; de Marcellis et al. 2005) the link between microtubule polymerization and protein was first suggested a few years ago and recently has been shown that Tat interacts directly with microtubules and polymerized microtubules.

the formation of abnormally stable pre-erotic microtubules depolymerization (Cahill et al 2002; Huo L, et al 2010). Ability of Tat requires integrity of four amino acid residues served Tat core domain residues 36-39. Experiments by de Alencar et al (2005) showed that the length of Tat protein is not necessary to enhance tubulin polymerization. The central region had the same properties as the full length. This demonstrated that the N-terminal residues are not necessary for interaction with tubulin. In contrast, the C-terminal residues of the dynamic property of the microtubules is discussed by Bisignani et al (2005). A relationship between Tat and mitochondria (Giacca et al 2005; Matarrese & Malorni, 2005) is corroborated in preliminary experiments. Incubation of Tat48 GFP GFP fusion protein with MHA leading to apoptotic cell death.

Both extra and intracellular Tat display the same structure when synthesized inside the cell. The difference is in the release of Tat into the extracellular space. This difference is in the modification of the host cell cytoskeletal structure. The key task of Tat is involved in cell proliferation and intracellular trafficking including the trafficking of HIV-1. The cytoskeleton is also involved in membrane fusion and viral assembly (Lopez de la Cruz et al 2010). In this context, intracellular Tat has been found to regulate several cytoskeletal proteins and tubulin. While binding of extracellular Tat to cytoskeletal proteins is crucial in activating the apoptotic pathway, the

of these proteins by intracellular Tat appears to be independent of apoptosis despite the high expression of these proteins in Jurkat cells. Tat plays a role in the modification of the cytoskeleton structure in HIV infected cells (Correia et al 2006). Further more, study looking at the effect of Jurkat cells stably expressing either Tat or intracellular Tat72 on cytoskeleton related functions such as cell morphology, proliferation and actin polymerization (Horie et al 2010) found that modifications of these functions were dependent on the sequence. A possible explanation for differences in the behaviour of extracellular Tat infected cells is that Tat localizes predominantly in the nucleus but remains largely cytoplasmic after being endocytosed by the cell. It is retained through the binding to cytoskeletal proteins such as p130Cas leading to cellular apoptosis (Chapman et al 2002; Epa et al 2005).

Neither case nor extracellular Tat completely represent the Tat48 GFP and Tat48 GFP GFP cell. Tat48 GFP GFP attract both synthesis and cell death thus capable of regulating skeletal genes, absence of the loss of these constructs and as they the nuclear and cytoplasmic are able to move freely between the two. The higher concentration of these Tat mutants in the cytoplasm bind to cytoskeletal proteins in a manner similar to that seen in the case for extracellular Tat, the concentration of Tat in the cytoplasm is insensitive to apoptosis rather than direct apoptosis. The fact that Tat in has a high concentration

may still be able to alter its basic functions, making it more cellular than the Tat48GFP cell line

The ROS experiments mirrored the cell viability data difference in the production of ROS was generated by the Tat101 and Tat101 cell lines. The effect of ROS exposure on bacteria can often lead to consequences such as oxidative damage to mitochondria mtDNA, which encodes proteins of the electron transport chain and mitochondrial ATP production that ultimately leads to apoptosis (Houten et al. 2006). ROS are also known to be triggers of the intricate interactions with components of the mitochondrial permeability transition pore spanning the inner mitochondrial membrane and its modifications, including cyclophilin D, voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT) impacting mitochondrial anion fluxes, leading to mitochondrial depolarization and eventually collapse of the mitochondrial membrane potential (Barnes et al. 2007; Kokoszka et al. 2004; Seltzer et al. 2005)

Another possible consequence of heightened cellular intracellular signalling and regulation of ROS is the p38 MAPK signalling pathway is associated with a number of processes, the response to oxidative stress is mediated by p38 MAPK kinase, ASK1 whose activity is inhibited by proteins such as thioredoxin (Trx1) and heat shock proteins (Saito et al. 1998). Only reduced Trx1 binds ASK1 regulating its functions as a redox switch that senses cellular ROS and

condition (Fujieda et al., 2007). To initiate Trx dissociation from the signalosome through oxidation of its active cysteine thiol, AKS1 is thus activated and able to signal downstream JNK apoptosis either via the mitochondria or through a pro-apoptotic signal (Chigcu & Aw., 2010).

Given the ability of Tat to modulate redox balance, the role this plays in apoptosis is likely beneficial to experimental contribution of the HIV-1 transcriptional mechanism to the formation of protein thiols as a mechanism of oxidative regulation of peroxiredoxins that occur at this site (Chen et al., 2010). Chapter 4 would be one suitable environment for studies of this type. This should further the understanding of the role of Tat in HIV-1 infection.

3.5 References

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CHAPTER 4: Detection of oxidant sensitive t
HIV-1 Tat expression by redox sensitive
electrophoresis.

4.1 Introduction

During HIV-1 infection, the progression of the disease is associated with an increase in oxidative stress. A number of studies have shown that intracellular levels of glutathione in peripheral blood T cells tend to be markedly decreased at advanced stages of the disease. Several studies have demonstrated decreased levels of glutathione in HIV-1 infected individuals (Cid et al. 1995; Droge & Holmet 1999; Saal et al. 1992). Antioxidant depletion is associated with decreased immune function as cells of the immune system generally require a higher level of glutathione to retain redox integrity and function (Dunster et al. 2002b; Stehbehns, 2004). In addition, oxidative stress plays a role in responsiveness, apoptosis and depletion of CD4+ T cells. There is also an association between decreased survival and low levels of glutathione in serum and CD4+ T cells (Baltzer et al., 2009).

This oxidative stress response is due to the fact that HIV-1 is a strong activator of transcription factor-1 (Tat). Besides being a viral gene, Tat is secreted from HIV-1 infected cells and can infect uninfected cells in a biologically active form. Tat regulates the expression of a number of genes (Higginbotham et al. 2004; Resman et al. 2010). There are several ways for Tat to influence the cellular redox state. One way is by decreasing antioxidant concentrations, thus increasing the oxidative capacity of Tat. Another way is by decreasing antioxidant concentrations is linked to its ability to decrease the expression of manganese superoxide dismutase (MnSOD), a mitochondrial enzyme that is part of the cellular antioxidant defense system.

streptococci (Flores et al 1993; Wesendahl et al 1995). The HIF protein is also able to directly decrease cellular G-SH and glutathione reductase required for synthesis, glutathione peroxidase (GSH-Px) (Cohen et al 2000). It is also able to induce the production of reactive oxygen species (ROS) in a number of different cell types (Gulow et al 2005; Priele 2006; Tuckwell et al 2009). Furthermore, TGF- β activation of astrocytes leads to the expression of inducible nitric oxide synthase (iNOS) leading to the overproduction of nitric oxide (NO) (Percoror et al 2005).

Reactive oxygen species can function as second messengers in signal transduction, modulating a variety of biological responses such as cell proliferation and apoptosis. Transduction of an oxidant signal in response to oxidative stress can be mediated by the oxidation of a thiol group on the side chain of the amino acid cysteine is particularly important and has been established as a redox signaling mechanism. Most protein thiols do not react with oxidants under normal cellular conditions, however some thiols are particularly reactive when they are ionized at physiological pH and undergo deprotonation. Cysteine thiols can sometimes form transient catalytic intermediates with oxidants of many enzymes. This allows oxidants to alter the activity of enzymes by modifying the redox state of functionally important cysteine thiols. This serves as a signal transduction mechanism that couples redox signaling to functional activity. Many regulatory proteins contain cysteine residues that can react with a variety of oxidants, to form covalent modifications. These include intermolecular disulfide bonds and

mixed disulphide and with a GS-H major mechanism by which proteins be controlled or protected during (Exitart j.v. 2006) ess

HIV1 Tat is a protein made up of 101 amino acids, divided into three domains. One of those domains contains a cysteine residue which is highly conserved among strains of HIV-1. The redox state of this domain is essential for its function, a study confirming the presence of a cysteine thiol in this domain. We have examined the protein disulphide isomerase activity of HIV-1 thiol in the presence of SMX (sulphamethoxazole) and SMMA (sulphamethoxazole) by employing a redox -dimensional electrophoresis. The objective was to establish what effect the Tat protein has on SMX or in combination, had on the activity of highly expressed thiol proteins.

4.2 Materials and Methods

4.2.1 Cell lines

The human T lymphocyte cell line -J52k atwE6.0b (ATCC TIB 1640) American Type Culture Collection was maintained in RPMI 1640 medium (Invitrogen) supplemented with 20% fetal calf serum, sodium pyruvate and 100 units/ml of penicillin and streptomycin.

Construction of plasmids and stable cell lines

The Tat gene was amplified from the pSV-Tat₁₀₁ plasmid which encoded full length Tat gene and was a kind gift from Dr. Keith Jean Section, NIAID, NIH. The Tat₁₀₁ gene (Tat₁₀₁) with two stop deletion mutants (Tat₁₀₁^{Δ1-52}) and Tat₁₀₁^{Δ1-57} were cloned into the pBluescript (Clontech Inc.), the Tat₁₀₁ gene, Tat₁₀₁GFP, Tat₁₀₁GFP^{Δ1-52} and Tat₁₀₁GFP^{Δ1-57} respectively. The fusion genes were excised as a HindIII fragment and inserted into the pBIG2i, an inducible doxycycline responsive expression vector (Sionthea, 1999). The result was pBIG2i-Tat₁₀₁, pBIG2i-GFP, pBIG2i-Tat₁₀₁GFP, pBIG2i-Tat₁₀₁GFP^{Δ1-52} and pBIG2i-Tat₁₀₁GFP^{Δ1-57}. As a control, the GFP gene from pN1 was also cloned into pBIG2i to create pBIG2i-GFP.

pBIG2i-Tat constructs or pBIG2i-GFP were transfected into Jurkat cells by nucleofection (Lonza, Amaxa Nucleofector) according to the protocol to generate stable cell lines of Jurkat pBIG2i-Tat₁₀₁GFP and Jurkat pBIG2i-Tat₁₀₁GFP^{Δ1-52}. The expression of both TatGFP and TatGFP^{Δ1-52} was induced by incubating pBIG2i-transfected cells with doxycycline (Sigma). Positive control

were selected using hygromycin B (Invitrogen) at a concentration of 100 µg/ml. Clones were derived from single cells following 1966 and the resulting cell lines were maintained in complete RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin and 0.5 mg/ml of hygromycin B.

4.2 Purification of anti-SMHA antibodies serum of SMHL immunized rabbits

The polyclonal SMHA antibody was prepared by Dr. Jane M. Rieder in her laboratory at Dr. Michael Rieder at Robarts Research Institute, University of Toronto. Immunization serum from New Zealand white rabbits was conjugated to Keyhole Limpet Hemocyanin (KLH) and stored at 4°C. The immunization serum samples (10 ml) were dialyzed overnight at 4°C with continuous stirring, against 2 L PBS (pH 7.4) and dialysis membrane (1.8 cm/ml) (Spectra and Texaco) was purified using a Protein G Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech) to selectively capture all IgG antibody subtypes. Concentrations of each sample were determined by protein estimation assay (BioRad) and samples with protein concentrations greater than 1 mg/ml were dialyzed overnight at 4°C with continuous stirring, against 1 L PBS (pH 7.4) until needed.

4.2 Slot blot

Cells from the different cell lines were incubated with SMHA and treated with varying concentrations of SMHA. The cells were collected

and analysed RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA (pH 8.0), and protease inhibitor cocktail 1 (Roche). Total lysates were sonicated twice for 20 s each then centrifuged at 13,000 rpm for 15 min. The supernatant was collected. Samples from all the treatment conditions of every cell line were generated on three separate occasions and subsequently blotted through

1 µg protein resuspended in cold PBS was applied to slot-blot microfiltration devices (Apparatus, according to the manufacturer's instructions). Briefly, after a slot-blot apparatus with the polypropylene membrane, the apparatuses were set up. The flow valve was adjusted to allow the entire sample to filter through. A gentle vacuum then each well was washed with TBST (Tris 7.6, 150 mM NaCl), again by pulling the wash liquid through the wells. When completely drained, the membrane was removed.

The blots were first blocked with TBST (1% Tween 20) containing dry milk at room temperature for 1 h and then probed overnight at 4°C with anti-SMA rabbit polyclonal antibody (1:1000) in TBST. The incubation with the primary antibody was followed by washing for 10 min each time with TBST. A secondary anti-rabbit IgG goat anti-rabbit antibody diluted to 1:5000 in TBST was then treated with chemiluminescent agent (Thermo Scientific Pierce ECL Western Substrate) and visualized with a Fluorchem imager (Amersham Pharmacia Biotech). The blots

then stripped and probed with a monoclonal antibody (1:1000) (Sigma) followed by a donkey anti-mouse IgG conjugated to HRP at 1:2000. Bands were determined by the AlphaView software (Santa Clara, CA).

4.2 Sample preparation for 2D gel electrophoresis

Jurkat pBIG TatGF- β cells (2.5 $\times 10^6$) were induced with 0, 200, or 1000 ng/ml of doxycycline for 4 h. The cells were washed with phosphate buffered saline (PBS) by centrifugation at 1500 rpm and resuspended in HEPES buffer and seeded at 1 $\times 10^6$ cells/ml. The cells were then treated with 200 μ M vehicle (DMSO) at a concentration of 0.5% for 4 h. The cells were subsequently collected and lysed in RIPA buffer (1% NP-40, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, complete mini protease inhibitor cocktail (Roche cat. # 1836170) and 150 mM NaCl). The cell lysates were sonicated on ice for 20 min at 1300 rpm for 15 s. The supernatant was stored at -80°C for future analysis.

4.3 Protein assay

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

4.2 Non-reducing 2D SDS electrophoresis

The diagonal SDS procedure was a modified version of Sommer and Traut (1974). Briefly, protein samples were dialyzed free of reducing agents and then deposited on a 1.0mm thick, 10% polyacrylamide gel by subjecting the gel to electrophoresis at a constant current of 125mA. After electrophoresis, the gel strip was excised from the gel and immersed in SDS sample buffer containing 100mM DTT (Dithiothreitol) at room temperature with gentle rocking. The gel strip was then immersed in running buffer then immersed in SDS sample buffer at room temperature with gentle rocking. After another wash, the gel strip was applied horizontally to a 10% polyacrylamide gel (23cm(L) x 20cm(W)) in the second dimension at constant current of 175mA. Each treatment of every cell line was subjected to the above redox 2D electrophoresis 3 times.

4.2 Silver staining analysis of 2D gels

Each set of 2D gels was stained simultaneously and then washed with freshly prepared 50% methanol, the gels were sensitized with 0.2% sodium thiosulfate with deionized water. The sensitized gels were then stained with silver nitrate solution (0.25% silver nitrate and 0.05% formaldehyde).

washing with deionized water for an additional 20 seconds. The gel was then washed in a solution of 6% sodium carbonate, 0.012% sodium formaldehyde until the desired gel resolutions were obtained. The gel was then incubated in a solution of 5% acetone and fixed for 5 minutes. The gel was then scanned by a scanner (Epson).

4.2.8 Statistics

Slot data were analyzed by means and standard deviation. ANOVA with Tukey's post-hoc procedure was used to determine differences between groups for concentration of each line.

4. Results

4.3. Quantification of mixed haptenation in Jurkat T cells

Our current belief is that the mechanism of drug hyperreactivity based on initial hapten formation may or may not lead to hypersensitivity in cases of pharmacologically metabolized to chemically reactive -SMX and electrophilic haptens. It is hypothesized that covalent conjugates of cellular proteins and antigen processing and presentation, immune response will be in state of hapten protein conjugates. Previous data from our laboratory already shown that SMXHA at concentrations of 100 µg/ml reacts with cellular proteins (Mariano and et al 2002) determined expression of protein Tat on its deletion in Jurkat T cells affect the SMXHA induced haptenation in Jurkat cells. blot experiments were carried out.

The various cell lines were cultured for concentrations of doxycycline to facilitate expression, followed by treatment with various concentrations of the reactant SMXHA. The protein isolations were subjected to SDS and blotted with an SMXHA antibody to visualize the haptenation pattern. The qualitative differences between the haptenation patterns were deciphered on the western blots with the use of a 125I-labeled amount of different protein samples were blots and the membrane probed with SMXHA antibody. The results are shown in the

Figure 4R Representative film and slot-blot analysis of SMX in Jurkat E6.1 cells. (A) Image from a western blot of Jurkat E6.1 cells treated with SMX for 2 h, protein extracted and resolved on a 12.5% SDS-PAGE gel, transferred onto a nitrocellulose membrane which was probed with anti-SMX antibody. Lanes 1, 4, 7: Jurkat E6.1; lanes 2, 5, 8: Tat101GFP; lanes 3, 6, 9: Tat72GFP. (B) Slot-blot analysis of Jurkat E6.1 cells treated with SMX for 2 h, protein extracted and resolved on a nitrocellulose membrane via slot-blotting. The membrane was probed with anti-SMX antibody, then stripped and probed with anti-GAPDH antibody.

Figure 4.1

SMX specific polyclonal antibody preparation means that bands that could be detected on the membrane for SMX the same HA (solvent control) or 200 μ M SMX (negative control), membranes were then stripped with GAPDH antibody and the intensity of the bands determined to determine the degree of loading. The ratio of the band intensities were used to generate the graphs in Figure 2.

All the blots had a SMX/HA/GAPDH ratio of approximately 0.1 in the absence of SMX (Figure 2). The ratio decreased 2.5 fold when each of the cell lines were treated with SMX and further increases in the concentration of SMX increased the ratio. The parent line showed a significant increase in SMX/HA/GAPDH with the addition of the 50 μ M SMX metabolite. While the ratios increased with the higher concentrations of SMX, the ratios were significantly different from the effect with 50 μ M SMX. Substitution of the cells with 100 ng/ml treatment with SMX resulted in a loss of SMX/HA/GAPDH 100 and 200 μ M, specifically 34% and 26% (Figure 2).

Induction of Tat10 expression approximating those seen in infected Jurkat cells was not significantly increased in SMX treated cells. The HA/GAPDH ratio at 50 μ M SMX compared to the control was an increase of 1.0. With 100 ng/ml Dox treatment the ratio increased approximately 2 fold with a doubling of the concentration of SMX 100 μ M, but there was no significant difference at various doxycycline concentrations at 1000 ng/ml. Similarly, the ratio at 0 Dox was 1.0 and 4.0 ng/ml

2-fold, respectively and the titre of 200 μ M SMX the ratio from the 1000 ng Dox treated samples at 200 μ M SMX HA was about the same as observed at 50 μ M SMX HA, this was still lower than the 1 and 400 ng Dox samples. At 200 μ M SMX again, the higher concentration of doxycycline seemed to have a protective effect on the SMX HA as the ratio was different than at 100 μ M SMX.

The Tat101 GFP cell line showed a similar trend in that the SMX HA/GAPDH ratio with increasing concentration of SMX HA (Figure 4.2). There was, however, no significant difference between the various doxycycline concentrations with the Jurkat and Tat 101 cell lines from the Dox samples at 200 μ M SMX. The ratio was unchanged for 100 μ M SMX and lower than observed for the 0 and 200 ng/ml Dox seen at 200 μ M SMX.

For the Tat72 GFP cell line a concentration dependent increase of SMX HA/GAPDH ratio at each concentration of SMX HA for the 0 ng/ml and 1000 ng Dox samples, though there were no significant differences between the various doxycycline concentrations (Figure 4.2). There is also a concentration dependent increase of the SMX HA/GAPDH ratio at 50 and 100 μ M SMX. At 200 ng Dox sample which approximates the amount of infected cells the ratio decreases to the 50 μ M level. At 100 μ M SMX 100 μ M SMX 200 ng Dox sample was significantly higher than samples seen for 0 and 1000 ng Dox also significantly higher than the 200 ng/ml Dox sample at 100 μ M SMX (Figure 4.2). The protective effect of 1000 ng/ml doxycycline was observed at 200 μ M SMX concentration and was maintained at 200 μ M.

Figure 4S ~~SMHA~~ induced haptentation in Jurkat T cell lines. doxycycline, cells from 6th day ((A)) Jurkat GFP, (C) Tat101, (D) (E) GFP or (F) GFP cell lines were treated with SM protein was isolated. The protein samples were applied onto membrane and then blotted for GAPDH bands. SM intensities were measured and the ratio of treated mean of three independent experiments. 400, 1000, 2000 μ M SMHA, respectively. P < 0.05, for 400, 1000, 2000 Dox at 0.5 μ M SMHA, respectively. P < 0.05, for 2000 μ M SMHA

Figure 4.2

Results from GFP cell line were somewhat different. A concentration dependent increase of the ratio-HA to actin at 100 μ M doxycycline samples was not significantly different, there was no significant difference in the ratio for the 400 ng/ml Dox compared to the 1000 ng/ml Dox at 100 μ M SMX (Figure 4E). At 200 μ M SMX, the ratios from the 0 ng/ml Dox samples were almost identical to the 400 ng/ml Dox samples which approximates the amount of Tat in the cells. However, that was higher than that of the 1000 ng/ml Dox samples (Figure 4E). The ratios from the GFP expressing cell did not show any differences between the concentrations (Figure 4E). Thus, taken together it does not appear that Tat or its mutants causes significant differences in the hapten attachment to the protein. Doxycycline at the concentration of 1000 ng/ml did have a small to moderate protective effect against haptenation.

4.3 Detection of redox sensitive thiol proteins

While Tat did not affect the expression of SMX, expression is known to alter the overall pattern of gene expression therefore may alter a set of genes to make the cell more susceptible to protein haptenation. To explore this possibility, a 2D SDS-PAGE electrophoresis assay was employed to identify proteins that are redox regulated via oxidation of cysteine residues through formation of protein disulfide bonds that contain reduced protein thiols (residues being exposed to the first dimension) and on the diagonal the gel is run in the second dimension.

are not further reduced by DTT. Protein mixed disulfides are reduced by treatment with DTT in the first dimension gel matrix, after electrophoresis run, so that the thiolst from mixed disulfide bonds are resolved below the diagonal line in the second dimension. However, proteins with disulfide bonds are not reduced in the second dimension with DTT.

In the present study, the primary protein was identified as disulfide bonds in the HIV-1 Tat protein deletion mutants more specifically to document oxidative changes (i.e. endogenous protein mixed disulfides) as a result of treatment with DTT. Each treatment condition was analyzed at least three times and recorded only if present in at least two of the three gels.

A summary of the protein spots identified on the gels. The first set of images shows 2D gels of protein samples from control cell lines, untreated Jurkat T cells and Jurkat T cells treated with 1000 ng/ml doxycycline (Fig. 4A and B, respectively), Jurkat T cells infected with HIV-1 (Fig. 4C) and the stably transfected Jurkat T cells only (Fig. 4D). Fig. 4E shows 2D gels of control, untreated Jurkat T cells (Fig. 4A) and HIV infected Jurkat T cells (Fig. 4B) as well as gels from vehicle control induced cells (0 ng/ml Dox) of different cell lines. The reducing/reducing 2D gel system revealed a number of

Table - 5. Summary of protein spots seen in the redox 2D gels. The presence of the corresponding protein spot in that cell line is indicated by the presence of the corresponding protein spot in that cell line. 200M SMXA.

J-E6.1 + dox: Jurkat E6.1 incubated with 1000ng/ml doxycycline. E6.1 cells infected with HIV. *Concentrations of doxycycline are TatGFP mRNA equivalent to that used in Jurkat cells. Tat150 GFP Ph and Tat72 GFP cell lines were used, which were used for the rest of the cell lines.

Table 4.1

Figure 4R. 2D dox-dimensions of thiol proteins from various cell lines; (A) Jurkat E6.1, (B) JFIP 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 lines were treated with DMSO for 2hrs, collected and isolated. 85µg of each protein was loaded onto the first dimension gel and run for 3hrs followed by a second dimension gel. On the left side of each gel, molecular weight standards were enumerated on both sides of the gels so that the panels A and B are duplicated and used as a reference in

Figure 4.3

intermolecular disulphide bonds (between the spots), the most just below 30 kDa (and above) which are putative peroxidase proteins (high abundance proteins in this specific molecular weight range) identified by assisted laser desorption/ionization (MALDI) MS peptide digests performed at the London Regional Proteomics Centre cell peroxidase and peroxidase 2 and in HEK 293 cells (peroxidase peroxidase 2 and peroxidase 3) at Dr. J. Beckwith University of Western Ontario, London, Ontario, Canada, MSc Thesis, 2004, shown in Figure 4.3, all the samples from the different cell lines of induction displayed the same protein patterns of the digests indicated that they were within most of the spots resolved between 9.75 kDa but differences in the number of resolved spots of the control samples in Figure 4.3, the spots showing the most protein spots (18 and 173 Figure 4.3B), indicative of oxidative stress, with the majority having intermolecular disulphide bonds along the diagonal line. The parental cell line Jurkat E6.1 (Figure 4.3C) shows only five proteins (Figure 4.3D). Jurkat cells incubated with doxycycline shows only five proteins (Figure 4.3D). Jurkat cells incubated with doxycycline show only spots 1 and 7 (Figure 4.3C). Tat72GFP cell line (Figure 4.3E) and Tat101GFP cell line (Figure 4.3F) show only spots 1 and 7, respectively) disappear in the presence of disulphide bonds. With the five of the six proteins resolved on the gel. Both cell lines had proteins 1, 2 (Figure 4.3C and 4.3E) and Tat101GFP cell line (Figure 4.4D) had proteins 1, 2, 5, 7,

Figure 4 Redox-dimensioned 2D PAGE of thiol proteins from (A) Ju-101, (B) Ju-Hk1a/t, (C) t101, (D) t101 GFP, (E) Tat72 GFP Panel (F) /Tat72 GFP. redox 2D gels in panels A and B are replicas. 4.3. Cells from each of the various lines were then treated with doxycycline. Cells were then collected and the protein was isolated. 85µg of each protein was separated on a first dimension gel and run for 3hrs followed by an overnight run on a second dimension gel. On the left side of the image are molecular weight standards and on the right side are enumerated on both sides of the image.

Figure 4.4

Figure 4.5 shows two-dimensional PAGE analysis of thiol proteins from (A) E6.1, (B) HIV-1 (Cat101, Tat101 GFP), (C) Tat72 GFP, (D) HIV-1 Tat panels F. Cells were induced for 40 hrs with 400 ng/ml doxycycline (C and D) or 0.05% DMSO (A and B) for 2 hrs, collected and the protein was isolated. The sample was loaded on the first dimension gel and run for 3 hrs followed by a run of the second dimension gel. The gels were stained with Coomassie Brilliant Blue G250. Molecular weight protein standards are indicated on both sides of the gels.

Figure 4.5

Figure 4. Redox-dimensional PAGE of thiol proteins from (A) Ju-
 (B) Ju-Hlat, (C) Cat101, (D) Cat101 GFP, (E) Tat72 GFP and (F) Tat
 panels E and F induced for 40hrs with 1000ng/ml doxycycline prior
 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
 and run for 3hrs followed by an overnight run on the left side
 side of the diagonal on each gel are molecular weight
 enumerated on both sides of the image.

Figure 4.6

protein only seen in the HIV infected cells as at this point the TatGFP sample had only two proteins (1 and 7,

I first examined the different cell lines treated with doxycycline that would induce the expression of Tat or TatGFP mRNA to approximate the level of Tat mRNA that occur in HIV infected cells. Induction of Tat expression with 400ng/ml doxycycline (Dox) did not change the protein pattern on the gels similar to that of the vehicle control (0ng/ml Dox) samples. Induced cells did not produce the induction of Tat101GFP. 200ng/ml doxycycline did not change the protein pattern to resolve away from the diagonal compared to the 0ng/ml Dox sample. The Tat72GFP with 200ng/ml Dox (Figure 4.5E) the disappearance of protein no. 4, one average protein no. 1, 2, 5 and 7 as well as protein (designated p. 2) in patterns similar to that of the GFP cell line induced with 400ng/ml Dox. The appearance of protein below the diagonal protein no. 10 and 11 were found above the diagonal line. These two proteins were not induced by doxycycline (Figure 4.3F), nor on any of the other samples from the GFP cell line. Results tend to indicate that doxycycline responsive proteins 1, 2 and 7 were found in the treatment with at 400ng/ml Dox.

Induction of protein with 1000ng/ml doxycycline (Figure 4.6) resulted in the appearance of proteins 9) seen in gels of lower concentration of doxycycline. In the gel for the Tat72GFP 0.5ng/ml

Dox (Figure 4eB) shows the appearance of protein bands in comparison to the gel of the parental cell line (Figure 4eA). The level of GFP expression induced at 1000 ng/ml doxycycline (Figure 4eB) resulted in a protein pattern almost identical to that induced by 400 ng/ml doxycycline with the exception of the 10 kDa band. The induction of GFP with 1000 ng/ml doxycycline resulted in the disappearance of a protein band (Figure 4eB). The Jurkat E cells showed proteins of 2, 4, 5 and 7 kDa but a loss of 3 kDa protein upon incubation with 1000 ng/ml doxycycline (Figure 4eB) compared to cells grown in the absence of doxycycline (Figure 4eA).

The control cell lines treated with doxycycline showed no obvious differences between the addition of doxycycline and the control. A dramatic reduction in the intensity of the putative protein bands was observed in the Jurkat E cell line treated with doxycycline (Figure 4eB) compared to the control cell line (Figure 4eA). An increase in the number of protein bands was observed in the Jurkat E cell line treated with doxycycline (Figure 4eB) and are numbered. These protein bands are the result of the induction of GFP and are numbered. The protein bands are listed in Table 4.1.

Treatment of the parental cell line Jurkat E with doxycycline resulted in the appearance of protein bands of 2, 4, 5 and 7 kDa (Figure 4eA) to the parental cell line. The only difference between the parental cell line (Figure 4eA) and the Jurkat E cells incubated with doxycycline (Figure 4eB) is the apparent reduction in the intensity of the 3 kDa protein band indicating lower amounts of this protein occur as a result of the induction of GFP.

Figure 4R 7 dox -dimo dimensiona PASES of thiol proteins from vario cell lines; (A) Jurkat -E6V1, (CB) GJUPk and (D) Jurkat E6.1 + dox. The sample from panel D was incubated for 40hrs with to drugtment. Cells from each of the various lines were t SMXHA for 2hrs, collected and the protein was isolated. 8 was loaded onto the first dimension gel and run for 3hrs f the scd dimension gel. The gels from panels A and B are reference in fig 1.0s 4.8 left side of the diagonal on each weight proteintband are enumerated on both sides of the ima

Figure 4.7

protein mixed disulphides) in the Jurkat E6.1 cells treated with HA and SMX. Treatment of infected Jurkat cell-line with HA and SMX resulted in a loss of protein spots 1, 2, 3, 4 and 5 and also dramatically increased the number of protein spots 6, 7, 8, 9, 10 and 11 including proteins unique to this cell line. Redox 2D gel electrophoresis of extracts from cell line infected with HA and SMX showed the resolution of proteins 1, 2, 5, 7 and 10 (Figure 4.8C) in the Tat101GFP cell line.

With minimal Tat protein induction at 0ng/ml Dox, cell lines were treated with HA and SMX. The results are shown in Figure 4.8. The Tat101GFP cell line shows the most protein spots (Figure 4.8A) which are proteins with intermolecular disulphide bonds, resulting in a large number of unresolved proteins on the gel. In the Tat101GFP cell line, HA and SMX treatment of vehicle control cells resulted in the appearance of protein spots 3, 4 and 5 and the resolution of proteins 1 and 2 (Figure 4.8C).

The Tat101GFP cell line saw the resolution of protein spots 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, but there were no changes to the protein spots 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. There was a much more dramatic effect with the addition of SMX to cells of the Tat72GFP cell line and the resolution of protein spots seen in Figure 4.4E, protein spots 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. HA specific proteins a, f and i (Figure 4.8E) were resolved. The cells of GFP Tat1 line at 0ng/ml Dox resulted in the resolution of protein spots 3, 5, 8, 10, 11 and 12 (Figure 4.8F) in the absence of SMX that concentration of doxycycline (Figure 4.8G) and HA specific proteins a, b, c, d, g and i were also resolved in the

The induction of RNA expression in the different cell lines was approximately 10-fold higher in HIV-infected cells. The induction of SMXHA and the results of redox 2D electrophoresis are presented in Figure 4.9. In the Tat101 cell line the intensity of proteins 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 was reduced. In addition proteins 1, 2, 5, 7 and 8 were resolved. Proteins from the Tat101GFP cell line resolved proteins 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. SMXHA specific proteins a, i and m (Figure 4.9D). For the combination of 200ng/ml SMXHA and 200nM Dox resulted in the resolution of proteins 1, 2, 5, 6, 7, 8, 10, 11 and 12 as well as proteins 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. Expression of GFP at 400ng/ml Dox resulted in the resolution of proteins 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 and the resolution of proteins 1, 2, 5, 6, 7, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. SMXHA (Figure 4.9F). The control cell line expressing just GFP saw the resolution of proteins 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. analysis.

Redox 2D electrophoresis of protein samples from Tat101GFP cells treated with 1000ng/ml Dox then treated with SMXHA is shown in Figure 4.10. In the Tat101GFP cell line, proteins 1, 2, 3, 4, 5, 7 and 12, in addition to proteins 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 were resolved (Figure 4.10C) while the Tat72GFP cell line produced proteins 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. f, g, i and s (Figure 4.10E). The Tat101GFP cell line at 1000ng/ml Dox and SMXHA only differed from the untreated cell line by the intensity of proteins 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and the addition of proteins a, f, i and r, the last of which was not resolved (Figure 4.10D). again! GFP control line presented the most proteins resolved. severe oxidative stress conditions of 6 proteins seen in the

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

In summary, the induction of GFP expression with 400ng/ml or Dox produced proteins 10 and 11, two proteins otherwise from the Tat72GFP cell line were. The GFP induction followed by treatment with SMX resulted in protein no. 6 being resolved also seen in the Tat72GFP cell line. Furthermore, the maximum Tat72GFP in cells induced with 1000ng/ml Dox in combination with SMX produced proteins oxidized of any of the experimental a number of proteins unique to each cell line. This can also though there were not as many oxidized proteins produced lines. Different induction of Tat101GFP did not appear to affect spots resolved away from the diagonal. There was however a number of spots seen when doxycycline induction is combined with SMX in this expressing cell line. There were also a number of redox 2D gels of lysates of cells in the absence and presence of HA that were unique to this cell line. Overall the control cell line as the parental E6.1 cell line produced very few proteins in comparison to the other cell lines and the HIV line.

Figure 4.7. Redox-dimensioned 2D gels of thiol proteins from (A) Jurkat, (B) Jurkat IV, (C) Tat101, (D) Tat101 GFP, (E) Tat72 GFP. Panels (C) and (E) were treated with doxycycline. Redox 2D gels in panels A and B are replicates. Cells from each of the various lines were harvested after treatment for 2 hrs, collected and the protein was isolated. 85 µg of each sample was run on the first dimension gel and run for 3 hrs followed by an overnight run on the second dimension gel. The left side of the diagonal on each gel are molecular weight standards and are enumerated on both sides of the image.

Figure 4.8

Figure 4.9

Figure 4 Redox-2D dimension PIASES of thiol proteins from (A) Ju-100, (B) Ju-Hk1/t, (C) Cat101, Tat101 GFP, (E) Tat72 GFP and (F) a Tat. four panels showing samples induced for 40hrs with 1000ng treatment. The redox 2D gels in panels A and B are replicates. Cells from each of the lines were then treated with 200µM SMX collected and the protein was isolated. 85µg of each protein was run on a first dimension gel and run for 3hrs followed by an overnight second dimension. On the left side of each gel are molecular weight standards are enumerated on both sides of the image.

Figure 4.10

4. Discussion

The class of antimicrobials known as sulphonamides are broad spectrum antibacterials. A combination of Sulphamethoxazole and Trimethoprim (SMX-TMP) is used to treat uncomplicated urinary and genital tract infections. It is a first line treatment and Prophylaxis for Pneumonia (PCP), a common opportunistic infection in HIV patients. Although very effective, SMX-TMP is implicated in the development of hypersensitivity reactions in a high percentage of HIV negative population. The mechanisms that lead to the development are still unclear, but are thought to be due to the formation of covalent conjugates with proteins that can be recognized as antigens. This states that the immunogenicity is due to the metabolic activation of the drug, in which case of both SMX and TMP, the hydroxylation of SMX to SMX-OH, a reactive intermediate, is further oxidized to nitro derivatives (SMX-O₂). These reactive metabolites or haptens form covalent conjugates with proteins that can be recognized as antigens. Responses may be directed against the individual haptens of the conjugate. Machanda (2002) showed that a concentration of 250 µM SMX binds to cellular proteins. The high incidence of hypersensitivity reactions in the population led to the expression of a HIV protein, which was shown to increase the level of SMX haptens in Jurkat T cells.

Our data confirm that protein haptens are formed when exposed to SMX at concentrations as low as 50 µM. The percentage of haptens is a

the concentration of the reactive (Figure 4) Platelet is increased
 dose of trimoxazole appears to be a plasma concentration of 1.5
 SMX-HA concentration of 0.2% of these plasma concentrations
 and SMX accounts for approximately 2% (Mantel et al 2002) the
 concentrations of SMX in this study are within the therapeutic
 range attained.

Our data also showed that the induced levels of HA are
 comparable to those in infected cells increased HA level
 happens after (Figure 2) The reactions of drugs with proteins
 formation of bonds of different strengths including hydrogen
 considered weak interactions and a bivalent bond is considered
 bonds. The interaction between a hapten and a protein results
 covalent bonds, allowing the formation of a complex of SMX
 reactive electrophilic sites on the ring susceptible to protease
 haptenation nucleophilic substitution reactions of small molecules
 electrophilic sites as SMX amide chains with nucleophilic
 such as the cysteine sulfhydryl group, amino groups, imidazole
 imidazole (Drivkovic et al 2005) proteomic studies have shown that
 containing reactive cysteine thiols are susceptible to oxidation
 ionization of the cysteine thiols for the formation of disulfide
 (Liebler, 2008; Wong & Difeo, 2008) if a protein has a cysteine
 rich domain (amino acids 237) that would make it a very attractive
 electrophilic metal-binding site. HA-FITC-SMX analysis by deletion and point

in the cysteine domain has revealed that six of the seven Cys for Tat transcription (Kralancic et al. 2009) however, the structural basis for this activity is still not well understood, though the transcription factor has been shown to be a monomer, and reducing agents dramatically increase activity suggesting that Cys residues form two intramolecular disulfide bonds for transcription (Kralancic et al. 2009). Thus given that of the seven cysteine residues in the domain form a disulfide bond for Tat activity, free thiols that can be oxidized to disulfide bonds (Kralancic et al. 2008). Both control cell lines E6.1 and 3T3 cells showed significant haptentation as measured by SMX/HA/GAPDH ratios. This is not unexpected as the HLA class II chains on cellular proteins that would be covalently modified by disulfide bonds in cell lines.

Whereas the expression of Tat in the different cell lines was similar, SMX/HA/GAPDH ratios, the cell line that was consistently different from the others was 3T3. This is probably due to the fact that all constructs used in this study had the cysteine domain. One of the differences seen is the discrepancy in the size of the Tat101 and Tat101GFP proteins. The discrepancy in size of the Tat101 and Tat101GFP proteins may be due at least in part to the fact that the latter adds 25kDa to the size of the protein. The haptentation of the target protein may be hindered or enhanced by the nuclear localization signal. For insect cells, the nuclear localization signal may be

embedded amongst residues with hydrophobic amino acids making it inaccessible to hydrophilic chemicals. Similarly, the surrounding amino acids at the pH of the local microenvironment are often different from the surrounding medium, thus greatly influencing the reactivity of nucleophilic sites (Devkota et al 2005). The degree of side chain ionization also be altered depending on the site of haptenation as more acidic than the surrounding medium (Devkota et al 2005).

Common to the Tat101 and Tat101GFP cell lines as well as is the significant decrease in the activity of doxycycline at 100 µg/ml in all samples, particularly in HA 200 µM SAM X perhaps be attributable to the ability of doxycycline to bind to metalloproteinases as matrix metalloproteinases (MMPs) and its use in the treatment of osteoarthritis (Goleb et al 1987; Gertler et al 2010). The oxygen atom in the doxycycline molecule is critical for binding and interference with this region of the metalloproteinase active site, thus affecting the effectiveness of the drug. This region is also important for the binding of doxycycline to proteins may be greatly enhanced by the formation of a complex with divalent metal ions such as Zn²⁺ (Mishra et al 1986). The binding of doxycycline to MMPs is thought to be mediated by the zinc atom and structural changes within the metalloproteinase active site (Mishra et al 2007). The relative affinity of doxycycline for a given metal ion is highly dependent on pH and the presence of other metal ions (Mishra et al 1985; Letombe et al 1984). The relative superiority of doxycycline as a metalloproteinase inhibitor is due to its increased affinity for Zn²⁺ over other divalent metal ions.

minocycline, another chemically (Burdick et al. 1990) tetracycline can form coordinate bonds with nucleophilic centres in the protein comparable in strength to covalent bonds. In essence the chelating tetracycline molecule would bind to the nucleophile in the amount available in the cell. The electrophile in turn would mean less -S-MX-A in complex formation. Hence per se -S-MX-A complex would directly affect the activity of GAPDH.

Another consequence of -S-MX-A in the cell is the ability of reactive metabolite to induce oxidative stress by generation of oxidative stress susceptible potential target group in protein with reactive thiol residue (-S). We have shown previously increasing concentration of -S-MX-A increases ROS activity in the cell here (previous chapter). Presence of increasing concentration of oxidative stress in a protein can result in a oxidative modification among them formation of sulphenic (-PSOH) and subsequent oxidation to disulphide (-PS-S-PS) (Daly and Donnet al. 2009). The protein disulphides are able to react enzymatically with glutathione or with thioglutarate to form mixed disulphides where these can be either homodimeric or heterodimeric disulphide bridges as is known in the cell. Whereas those between the same protein are intramolecular disulphides. The redox 2D electrophoresis system is an effective way to analyze the

disulphide bonds formed in the HIV Tat protein in the active metabolite SMX on these interactions.

The presence of active cysteine in Tat is not necessary for SMX haptenation and may also provide the chromophore of a molecular disulphide as well as intermolecular mixed disulphides. A glutathionylated protein is not resolved by SDS gel because of the small molecular weight from the addition of to the protein. However, formation of either Tat intermolecular disulphide is likely to result in a significant structural or functional change that could be a contributing factor in the progression of HIV infection. The primary structure of Tat is divided into functional domains. The domain contains seven highly conserved cysteine residues. Mutagenesis studies have shown that these residues are indispensable for transcriptional activation of HIV. Tat is transcriptionally active from Tat and its analogues have been found to inhibit transcription, suggesting that these residues in Tat form intramolecular disulphide bonds for transcriptional activation (Kabatani, 1994). So, increased transcription activity was seen in Tat was subjected to refolding, which allows for the formation of disulphide bonds (Kabatani, 2008). These suggest that the redox state of these residues affects the biological function of Tat. Furthermore, Kalantari (2008) showed that three of the seven cysteine residues in free thiol form and can form disulphide bonds independently.

to maintain TAToathis site driven 2 Dugel electrop experiments to determine if expres T admbfits deletion andeantheactive or qualitative n asurphide bonds, and what effect, if any, the a metabo SMH will have on the sulphide bond at T cells

The most ubiquitous and pnomline the spsthe pproative peroxire ppxite in Peroxire (Prx) are antioxidant founz d m e s all organisms with the sing b berexica ptu ngal of e Brer resipaeies) (Habt al2009) his family of antioxidant a pproteins 30 kDya 2in size and se specialized cysteine residues to decompose perox Prx towards peroxides in combinatio haw it xpt e s s i o n i g m e a e proteins Prx t h e family are a primary line of defence against p critical role as cellu (B esleithia, 2007) the physiological important peroxiredoxins is illustrated by their Prx relative abundance as abundant proteins in e h a t m o g y o e s n a f w e t h a w i d e c e l l u l a r d i f f e r e n t i s i o n f o r m e s c y t o s o l , n u c l e u s , m i t o c h o n d r i a , e n d o p l a s m a m e m b r a n e a n d m a l i a n c e l l s c o n t a i n a t l e a s t s i x d i f f e r e n t P r x e n z y m e s (P r x 1 - P r x 6) w i t h a l l s i x P r x e n z y m e s f o u n d i n c l e a u s e P r x 1 b e i n g t h e m a j o r c y t o p l a s m i c f o r m (C h a n k a l 1 9 9 8) P r x e n z y m e s c o n t a i n c o n s e r v e d c y s t e i n e r e s i d u e (d e s i g n a t e d t h e p e r o x i d a t i c c y s t e i n e) P r x 1 - P r x 4 c o n t a i n a n a d d i t i o n a l c o n s e r v e d c y s t e i n e r e s i d u e (c o n s e r v e d c y s t e i n e) T h e P r x e n z y m e s t h a t c o n t a i n t h u s r e f e r t o C y s P r x a s w h i l e P r x e n z y m e s w i t h o c o n s e r v e d c y s t e i n e r e s i d u e s a r e r e f e r r e d t o C y s P (Z h o e t a l . 2 0 1 0 ; H t a l a l . 2 0 0 9) T h e - C y s P r x s e x i s t a s c o v a l e n t l y l i n k e d

homodimers, with the two monomers (Section 2.0.1) and a disulfide bond (Figure 4.1). The first step of the catalytic cycle is the selective oxidation of the active site cysteine residue to a sulfenic acid (Cys-SOH) at the active site. The second monomer then reacts with Cys-SOH to form a disulfide-linked intermolecular dimer and produce water. The catalytic cycle is completed when the disulfide bond is subsequently reduced, typically by thioredoxin, recycling the free thiol form (Cys-SH) (Cetani et al 2010; Sedgwick et al 2000). Peroxiredoxins can also be oxidized to sulfenic acid or sulphonic acids when a fraction of the sulphenic acid is converted to a catalytic cycle rather than forming a disulfide bond (Bray et al 2005; Waite et al 2002). This leads to the formation of a dead end sulphinate species with the resolution step of the catalytic cycle (Woolley et al 2003; Yan et al 2002). Under mild oxidative conditions, a small amount of inactivation caused by peroxiredoxin oxidation is observed by the synthesis of the native protein. However, when exposed to strong oxidative stress, the normal form of Prxs disappears due to inactivation by oxidation which effectively inactivates the enzyme (Rabilloud et al 2002).

The presence of Prxs in Jurkat cell lysates (Figure 4.1) demonstrates that Prxs are being subjected to mild oxidative stress even before the appearance of SMC release.

Figure 4. The catalytic cycle of peroxidase enzymes. Peroxidases are homodimers that reduce H_2O_2 in three main steps: peroxidation, resolution and reduction. H_2O_2 is first reduced with a peroxidatic cysteine (Cys₁) to the SOH then condenses with the resolving Cys of the other subunit to form a disulfide bond. The disulfide bond is subsequently reduced by the reducing cysteine (Cys₂) to regenerate the active site. The homodimeric enzyme can become overoxidized (4), when a fraction of the SOHs are further oxidized to cysteine sulphonic acid (Cys-SO₃H) instead of forming a disulfide bond. The formation of the sulphonic acid is irreversible and is dependent on the reduction of Cys-SO₃H by a reductant (D'Autreux & Toledano, 2007). Additional oxidation of the sulphonic acid leads to the formation of the sulphinate (Cys-SO₂H) and the irreversible inactivation of the enzyme (Figure adapted from D'Autreux & Toledano, 2007).

Figure 4.11

the Prx protein disulphide (protective) is inhibited in the HIV-1 infected cell line is under enhanced oxidative stress compared to the long established consequence (Bull 1998, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, 633, 634, 635, 636, 637, 638, 639, 640, 641, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716, 717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 827, 828, 829, 830, 831, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 848, 849, 850, 851, 852, 853, 854, 855, 856, 857, 858, 859, 860, 861, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 873, 874, 875, 876, 877, 878, 879, 880, 881, 882, 883, 884, 885, 886, 887, 888, 889, 890, 891, 892, 893, 894, 895, 896, 897, 898, 899, 900, 901, 902, 903, 904, 905, 906, 907, 908, 909, 910, 911, 912, 913, 914, 915, 916, 917, 918, 919, 920, 921, 922, 923, 924, 925, 926, 927, 928, 929, 930, 931, 932, 933, 934, 935, 936, 937, 938, 939, 940, 941, 942, 943, 944, 945, 946, 947, 948, 949, 950, 951, 952, 953, 954, 955, 956, 957, 958, 959, 960, 961, 962, 963, 964, 965, 966, 967, 968, 969, 970, 971, 972, 973, 974, 975, 976, 977, 978, 979, 980, 981, 982, 983, 984, 985, 986, 987, 988, 989, 990, 991, 992, 993, 994, 995, 996, 997, 998, 999, 1000)

The addition of SMX to different cell lines also lead to lower oxidation and inactivation of peroxiredoxin in the cell lines. In comparison between the parent cell line, Jurkat E6.1, and an apparent lower amount of the peroxiredoxin (Figure 4e). The treatment of the cell lines with SMX also resulted in the inactivation of an apparent decrease in the peroxiredoxin to the cells treated with the parent cell line. The cell lines expressing Tat101, Tat72 GFP and HA looked to be more pronounced than the parent cell line perhaps roughly equivalent to that seen in the parent cell line (Figure 1). This was observed at every concentration of doxycycline. The HIV infected cells do not compensate for the oxidative stress.

SMHA than the cell lines expressing T_H1 and T_H2 GFP probably due to two distinct factors; the first is the ability of HIV infection to upregulate the expression of the Prx1 protein in accordance with a study by Dyantcheva et al. (2003). In that study the authors compared the expression of HIV seropositive CD4⁺ T cells of seronegative individuals and found that Prx1 and Prx2 were expressed in greater quantities in HIV⁺ CD4⁺ infected individuals. Additionally, they showed that these amounts in blood plasma of HIV⁺ (not HIV⁻) but not in persons treated with ART or untreated persons at these genes, not regulated via oxidative stress as indicated by stimulation with anti-CD3 and anti-CD28. As chronic immune activation of cells a consequence of untreated HIV infection, this could account for the elevated levels of Prx1 and Prx2 in HIV⁺ individuals (Dyantcheva & Silverstein 2005). In Jurkat T cells treated with HIV-1, results in elevated levels of Prx2 protein that inhibit HIV replication as previously shown that some Prxs have antiviral activity by decreasing HIV free radical concentration and their antioxidant enzyme function in a different way, thereby blocking replication (Hirata et al. 1992; Okamoto 1992).

A report by Ma (2007) corroborated the finding about Prx2 showing significantly elevated serum levels of Prx2 in HIV⁺ individuals compared to their HIV⁻ partners and also compared to controls. Further analysis conducted on a defined subset from each cohort showed that Prx2 transcription levels were elevated

to a lesser extent in T0 D4 with the reason for the apparent decrease in amount of the Prx disulphide after exposure to the SMXHA is that the Hsp70 protein is able to suppress the expression of Prx as glutathione reduction in glutathione S-transferase that would normally counteract the oxidative stress (Fares et al 2003 with Westendahl 1995b)

The Hsp70 infected cells showed a greater number of protein spots which anticipate this cell line as a conditionally immortalized cell line such as Vpr, CA, NC that can potentially disulphide compounds in cell lines expressing just the GFP protein had the fewest spots in the absence and presence of SMXHA. This is probably due to the fact that the variant of GFP used in this study is not a thiol glutathione but would be potentially an antioxidant property overexpressed GFP possesses

The GFP cell line consistently displays more spots on the gel than the other expressing cell lines may be because of the distribution of the Tat construct so far as the basic region with the exception of the afore mentioned one which is missing the basic region (and thus the nuclear localization signal) and thus the nuclear localization signal is not expressed in this study have a predominantly nuclear localization. The GFP construct expressed can contribute much less of the total protein readily detected in the cytoplasm. In contrast, the distribution of SMXHA as determined by Mandala (2002) using labelling and

confocal microscopy that showed the active metabolite was distributed in the cell cytoplasm. The SMX antibody staining demonstrated by at least SMX-HA could be visualized inside the cell, the vesicle membrane within five minutes of cell incubation with SMX. Sixty minutes after exposure to the metabolite, binding was visualized in the cytoplasm as concentrated discrete foci. This cytoplasmic localization of SMX-HA and GFP suggests that GFP constructs might have a temporal advantage over the other constructs if these cells SMX-HA might accumulate more quickly in the same compartment. In addition, the relative nature of SMX suggests the majority of the introduced protein would be bound to other proteins in the cytoplasm before it is able to reach the nucleus.

The report by Ma et al (2002) evaluated the cellular distribution of HA only at two time points, one of which was 4 hours after treatment with SMX-HA in this system. It was not clear for a period of 2 hours why SMX-HA binding to protein in the cytoplasm was not observed. Indeed there have been studies showing that nuclear accumulation of proteins can take up to several hours and, depending on the size of the protein (Aikawa et al 2009).

The nuclear envelope separates the nuclear and cytoplasmic compartments, thereby necessitating nucleocytoplasmic transport. All proteins imported from their site of synthesis in the cytoplasm while in the nucleus such as mRNA, need to be exported to the cytoplasm.

Cumming et al 2004; Fata et al 2002). Given that ERD has a molecular weight of 36 kDa, there is no protein in the sample that matches this weight. It could be a reflection of the oxidizing used in the experiment as well as the concentration of oxidant treatment. Other interestingly identified by (2005) in heat shock protein 90², ubiquitin thiolesterase of 61 kDa. We have estimated MW-11.6 kDa according to their positions. That is a molecular weight of proteins prepared in vitro to the ER. Expressing cellular SMX treatment may be important as they are potential targets of ER. The molecular weight of a mutant with significant plasmic distribution. A protein involved in cell structure, function, and the plasma membrane filaments. Heat shock protein 90² and ubiquitin thiolesterase are proteins that primarily reside in the ER. Further proteomics research in the interactions between cellular proteins can lead to increased understanding of development. One future direction includes the use of techniques such as mass spectrometry to isolate, purify and the metabolite SMX. The context of that expression can be beneficial to determine which proteins are involved in the formation of differences in the expression of a sample. The expression

of Tat lead to mitochondrial protein synthesis of mixed disulphide bonds?

In summary, the results from these experiments suggest that expression of the HIV Tat protein can have an effect on the amount of mixed disulphide bonds formed by the reactive metallothionein. However, the amount of mixed disulphide bonds formed is less important than the specificity of the target distribution of the Tat protein. It may be that the specificity of the target distribution of the Tat protein may contribute to cellular sensitivity. At the end of the experiments confirmed that the oxidizing agent that is used in the study is about the formation of mixed disulphides, bonds that could be formed between proteins required for cellular homeostasis.

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CHAPTER 5: Discussion and Conclusion

5.1 Discussion and Conclusions

Prior to 1980, antiretroviral drugs were unavailable and HIV infection therapy consisted of treating opportunistic diseases arising from immunodeficiency (Fiscella et al. 1987). In that environment, antimicrobials used for treatment or prophylaxis against the most attention-worthy drugs responsible for hypersensitivity reactions in AIDS patients (Crater & Cooper, 1995; Crater, 1995). Itraconazole, used for treatment of cryptococcal pneumonia, was the most commonly cited drug, but a significant incidence of hypersensitivity was observed with other sulconazole antimicrobials (Crater & Cooper, 1995; Crater, 1995). For unclear reasons, the rate of ADRs among people with HIV infection progressed to clinical AIDS was higher than that of the general population.

The advent of antiretroviral therapy (ART) almost dramatically changed the course of HIV infection in America from a rapidly fatal disease to one which is now a chronic condition (Pirmohamed et al. 2007). The use of ART has also resulted in a change in the epidemiology of ADRs as the need for prophylactic therapy for opportunistic diseases has decreased. Problems associated with the development of resistance (Pirmohamed et al. 2007). As well, among patients with controlled viral loads, the rate of ADRs is much closer to that of the general population. However, ADRs to antimicrobial drugs have been replaced by ADRs to antiretroviral agents including protease inhibitors, anticonvulsants, and other drugs used for the treatment of HIV-associated dementia (Crater & Cooper, 2000; Pirmohamed & Park, 2000).

In the general population, ADRs are responsible for 3% of all hospitalizations and occur in 20% of hospitalized patients (Lipman et al., 1998). They are a major public health problem due to their frequency, potential for major morbidity, and also the impact they have on the development (Priederand, 2009) of the developing world. In addition, due to disease prevalence, access to medications, and drug management strategies that differ from those of developed countries, the impact on the incidence and nature of ADRs in these countries of the first studies determine incidence and nature of ADRs is still unclear. During the HIV/AIDS pandemic, a 12% increase in the incidence of ADRs in a study population in South Africa has been reported (Holtz et al., 2008). In contrast, a study in South Africa has shown that the incidence of ADRs in HIV-infected individuals is higher than in the general population with approximately 12% of the total population infected and 60% of those infected on ART. In their study, 20,000 patients were observed that ADRs to antiretroviral drugs were more frequently reported than in developed countries and severely-infected HIV individuals, who were more frequently treated with frequently developed ADRs to protease inhibitors.

Cotrimoxazole is associated with a 10% increase in the incidence of ADRs in the HIV negative population (Vermant, 1991). In HIV positive patients, ADRs occur in 30% of patients at prophylactic doses and 50% of patients at therapeutic doses (Cooper, 1995). Cotrimoxazole is a combination agent composed of sulphamethoxazole and pyrimethamine (SMX/MPX). It is believed to be the aetiological factor for the hypersensitivity ADRs that lead to the discontinuation of cotrimoxazole and thus represents an ideal model to investigate the mechanisms of

hypersensitivity. HIV positive patients our considerable knowledge its disposition and metabolism (Lima et al., 2007)

The research embodied in this thesis raises the contribution of HIV-1 Tat to Jurkat cell toxicity induced by phenanthroline. To study these issues we used a vector system that by inducing expression of Tat and the antibiotic resistance gene SMX^R in Jurkat cells demonstrated that the expression of the first exon of Tat in Jurkat T cells is a cooperative effect. At a low, 5 nM, effect dependent on the level of Tat expression demonstrated that a potential primary mechanism of cell death that may be associated with Tat expression is cellular GSH/GSSG conversion. We also concluded that the first exon of Tat may affect the redox state. We also concluded that oxidative stress is a primary mechanism driving cell death.

After obtaining the full length Tat protein in a purified form we also constructed deletion mutants in an effort to determine the region that was responsible for the toxicity that was observed. We found that the deletion mutants were able to rescue Jurkat T cells from cell death. Jurkat T cell viability and ROS assays demonstrated that the cell viability assays showed that the expression of the first exon of Tat induced cell toxicity. However, the ROS assay showed that expression of the full length Tat protein increased the ROS generated by Jurkat T cells. We also demonstrated that the first exon of Tat played a role. Of significance, the deletion mutants had a lower percentage of cell death than the full length

protein is required for increased HIV-1 RNA levels. Previous reports have shown that Tat mutants (Tat Δ 1-22 and Tat Δ 86) augment HIV-1 production in cultured cells, as shown by the presence of extracellular HIV-1 in the culture supernatant (Akse et al 2006; Gulaw. 2005; Liu et al 2005)

Evaluation of the effects of the inducible SMXHA was studied in detail by analyzing changes to the redox proteome produced by electrophoresis of lysates of cells expressing the protein. Numerous spots were identified in the 2D gel, and the apparent molecular weights of these spots were determined. One of the spots that showed a significant increase in intensity under oxidative stress conditions was identified as a cysteine thiolase protein. Exposure of the cells to SMXHA led to a dramatic increase in the intensity of this protein in the redox 2D gels.

A major obstacle to the eradication of HIV-1 in reservoirs. In patients that have not achieved a sustained virological response, the virus is mainly generated by repeated cycles of infection of CD4+ T cells (Cohen et al 2009). The result is a reservoir of HIV-1 in resting T cells, infected dendritic cells, macrophages, and other long-lived cells. These cells are more resistant to cytopathic effects of the virus and are found in anatomical sites with low pharmacological activity for ARV. The ARVs to penetrate tissues such as the genital tract (Aguzzi et al 2008; Cehuan et al 2008; Coia et al 2009; Guadalupe et al 2006; Ruz et al 1999)

Ongoing viral replication in CD4+ T cells cannot be fully excluded infection, patients undergoing antiretroviral therapy, Tat protein is continuously produced. The basic domain allows the protein to enter infected cells and enter, uninduced by standard cells (1988). Also demonstrated this unique property of Tat, their results supported was followed by transcriptionally active form determined that it took 6 h for approximately 80% of the protein in the nuclear compartment. There is a substantial portion in the cytoplasm for further. The basic domain allows Tat to translocate, including the heart, liver, lung, spleen, kidney, and brain (1999).

Therapy with AZT produces a system wide distribution and the drug liver is quantitatively the primary site of bioactivation for AZT. It has also been shown to occur in other organs including the skin and immune system (Cremata 1990; Vyas 2007). The cytoplasmic distribution of the Tat protein was responsible for the increased sensitivity of the CD4+ T cells to AZT treatment (C 3). The 2D electrophoresis of Jurkat CD4+ T cells expressing Tat and treated with AZT unique protein spots that are probably due to the destruction of the Tat fusion protein. This is an in vivo effect. The length (wild type) protein is released from HIV cells, enters and localizes to the cytoplasm where the presence of Tat causes the induction of apoptosis leading to increased death of the HIV infected CD4+ T cells and subsequent infection.

various (Figure 5.11) loss of the CD4+ T cell compartment has been associated with immune deficiencies for, that HIV could potentially also lead to ADRs due to the loss of the regulatory CD4+ CD25+ T cell subset. This mirrors the situation described by Koishikawa et al. (2007) for contact hypersensitivity. Contact hypersensitivity is a common inflammatory skin disease, also known as allergic contact dermatitis (ACD), which is a hypersensitivity ADR. It is a common inflammatory skin disease caused by repeated skin exposure to irritants or allergens, or contact with 2,4-dinitrochlorobenzene (DNCB) or oxazolone (Ox) (Cohen et al. 2009). The responses are mediated by Th1-producing CD8+ T cells primed by dendritic cells - particularly Langerhans cells (LC) migrating from skin to the draining lymph nodes (Falkenburg et al. 2007). CD4+ T cells are not required to mediate the CHS response, either as effector cells or as regulatory CD4+CD25+ T cells have been shown to restrict CD8+ T cell expansion for CHS (Stern et al. 2005; Stern et al. 2007). One of the major differences between CHS and hypersensitivity ADRs induced by SMX is the nature of the effects caused by SMX. In CHS, the response is localized to the area of skin that is sensitized by the allergen in CHS. This is in contrast to the severity of the SMX ADR, which is a widespread distribution of the disease. This increases the probability of the response occurring in the area of skin that is sensitized by the allergen in CHS. This is due to the cellular toxicity and increasing the incidence of ADRs. In the data from experiments looking at the externalization of SMX, we show that the cell death observed in the Tat48 GFP cell line is the presence of SMX mediated via apoptosis. This is in keeping with the

Figure 5. The model of increased incidence of AIDS in HIV patients on the SMX dose is metabolized to S-MX which is able to haptenate cellular proteins. The haptenated proteins are processed by an antigen presenting cell (APC) leading to recognition by a T cell and an immune response. During HIV infection, Tat is released by infected cells and enters uninfected cells where it changes the disulfide proteome for cell proteins. In the presence of SMX, Tat binds to proteins, increasing their antigenicity and presentation to CD8⁺ T cells and development of an AIDS response to SMX.

Figure 5.1

found in Adety (2009).

Another consequence of induced apoptosis (Figure 5.1) is that these apoptotic cells can be a source of immunologic information that may influence subsequent responses. It has been shown that antigens derived from phagocytosed dying cells may be taken up by professional antigen presenting cells (APCs) and presented by MHC I molecules to CD8+ T cells (Uhlir et al. 2009). Classically, MHC I molecules present antigens synthesized within the cell to CD8+ T cells, while antigens derived from other cells via endocytic uptake are loaded onto MHC II molecules for presentation to CD4+ T cells. The process by which APCs acquire and export exogenous antigens into the MHC I pathway for presentation to CD8+ T cells is known as cross-presentation, and the resulting stimulation of CD8+ T cells is termed cross-presentation (Rock & Shen, 2005).

It is not clear exactly how apoptotic cells are taken up and acquired by the APCs, though there is now evidence that the immunogenicity of these cells is related to their ability to be taken up for presentation to monitor tissues for foreign antigens from viruses. There is also evidence in individuals with chronic HIV infection that CD8+ T cells seem to activate a wide repertoire of CD8+ T cells against epitopes presented by MHC I (Rawson et al. 2007). Indeed the magnitude of the CD8+ T cell response directed against a particular peptide is correlated with the decline in CD4+ T cells (Rawson et al. 2007). It is suggested that the presence of these CD8+ T cells in HIV-infected individuals with CD4+ T cell depletion and the CD4+ T cell help may be due to their being independently stimulated.

variety of signaling pathways. T cells expressing the CD40 ligand (CD40L) (Petro et al 2004) had or dying used as an antigen for presentation is not limited to CD40L, derived from interdigitating cells has shown for major histocompatibility complex (MHC) class II restricted, antigen presenting cells that support HIV infection for Tat (Alpers, 2004; Fleeton et al 2004; Mantel et al 2004) the model of ADRs in HIV pathogenesis (Figure 4, 5). The metabolism of SMX leads to the formation of S-MX, both shown to haptenate to cellular proteins in T cells (Cohen et al 2002) and most likely in other cell types. These haptenated cells are then presented to T cells as immune response antigens, which clinically as an ADR to SMX.

5. Future studies

One of the major objectives of this work upon the work published by Adey et al (2009), specifically to ascertain the role of Tat in the cell toxicity of SMX in that report and the chapters 3 and 4 from two different sources. The results had a minor sequence differences. It would help clarify the cell viability data in (2009) and the results described in Chapter 4 if the two Tat variants (1-WT and 1-Δ) were used in the luciferase reporter assays. The transcriptional activity of the two Tat proteins is regardless of any potential differences in the two Tat variants. The cell death appears to remain the same.

We have determined the stable transfectant profile of HIV mutant that retains the protein transduction domain (PTD) nuclear localization sequence (NLS) exclusively, to distribute protein in the nucleus. Indeed the results of our micro experiments suggest that these proteins (Tat101GFP, Tat8) ventured out of the nucleus to impact other organelles biochemically. To verify these results, we could carry out mitochondrial subfractionation by differential centrifugation followed and immunoblotting with an anti-Tat antibody.

The data in Chapters 2a and 3 show that transiently transfected T cell lines showed a decrease in cell viability when treated with HA. However, the difference in cell viability between Tat101GFP and Tat48GFP was not significant. It would be interesting to determine if differences in cell viability between Tat101GFP and Tat48GFP are due to differences in cell viability when the deletion constructs were applied to the T cell culture, both in the presence and absence of HA. The extracellular application of Tat and its deletion constructs also mean that the number of Tat molecules in a particular cell would then be multiplied many fold to determine what effect expression of Tat might have. The use of the deletion constructs in particular region of Tat had a synergistic effect on T cell viability.

Another potential area of research is to evaluate the cytokine profile produced by the incubation of peripheral blood mononuclear cells with HSA. This was first followed by Smith and Ares (1991) who showed that HSA reduced the production of the inflammatory cytokines IL-1 and IL-6, but had no effect on IFN- γ production. A significant decrease in the production of these cytokines by PBMCs exposed to HSA would lead to a higher immune response. This is thought to account for some of the characteristics of hypersensitivity ADRs such as delayed onset and fever. The recruitment of monocytes to peripheral sites, cytokines including IL-1 and TNF (Chertanin 1997; Nataraj 1999) such an experiment to establish if the addition of the HIV protein Tat to more rodent systems might account, at least in part, for the higher incidence of hypersensitivity ADRs.

5. Conclusions

Drug hypersensitivity does not contribute substantially to patient mortality, especially in the developed countries, a major public health problem in developing countries and increase the burden and cost of an overstretched healthcare system. The development of hypersensitivity ADRs by rodent studies have shown a contribution of the Tat protein in the HIV population. In this thesis I lay the groundwork for the role played by the HIV protein Tat in the sensitivity of T cells to the HSA and its metabolic pathway.

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APPENDIX

Curriculum Vitae

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Postsecondary Education and Degrees: Honours Bachelor of Science
Department of Biochemistry
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PhD, Microbiology and Immunology
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2002-2011

Honours and Awards: CIHRHIV/AIDS Research Initiative Doctoral Award
Biomedical/Clinical Str
Schulich Graduate Scholarship, 2006
Western Graduate Scholarship, 2004

Related Work Experience: Research assistant
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2002-2004

Research skills include

- Flow cytometry and analysis
- ELISA; Enzyme-linked Immunosorbent Assays
- Virus culture and plaque assays and titration
- Mammalian cell culture; transfections and colony i
- Western, Northern and Southern blots
- Recombinant DNA techniques; subcloning
- Polymerase Chain Reaction (PCR)
- Isolation of genomic and plasmid DNA
- Gel electrophoresis
- Restriction enzyme analysis
- Redox -dimensional SDS

Publications:

Paper

Adeyanju K., Krizova A., Gilbert P.A., Dekaban G.A. and potentiates cell toxicity model for sulphamethoxazole drug reactions versus G6PD(3):-827.2

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Adeyanju K., Krizova A., Rieder M. and Dekaban G. A. S cellular toxicity of HIV expressing cells in the presence of full protein. Canadian Therapeutics Congress, May 2007.

Adeyanju K., Rieder M. and Dekaban G. A. -Differential Expression by T cells Confers Differential Sensitivity to Sulphamethoxazole. IXth World Conference on Clinical Pharmacology and Therapeutics

Oral Presentations:

Adeyanju, K. The Contribution of the HIV Tat protein to adverse drug reactions induced by Sulphamethoxazole. MICROIMM 540y, Department of Microbiology and Immunology, The University of Western Ontario April 2005.

Adeyanju, K. The Contribution of the HIV Adverse Drug Reaction induced by Sulphonamides. MICROIMM 540y, Department of Microbiology and Immunology, The University of Western Ontario. London, Ontario. October, 2006.

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Adeyanju, K. The HIV Tat protein and Adverse Drug Reaction utilizing sulphamethoxazole. MICROIMM 9250y, Department of Microbiology and Immunology, The University of Western Ontario. London, Ontario. November 20, 2006.

Adeyanju K. Understanding the mechanism behind adverse drug reactions in AIDS Patients. MICROIMM 9250y, Department of Microbiology and Immunology, The University of Western Ontario. October, 2008.

Adeyanju K. Differential Expression of HIV T cells Co-receptor CXCR4 and Differential Sensitivity to HIV-1 by CXCR4 antagonists. Guest speaker in Pharmacology Grand Rounds, Department of Medicine, The University of Western Ontario. London, Ontario. April, 2009.

Adeyanju K. Understanding the mechanism behind adverse drug reactions in AIDS Patients. MICROIMM 9250y, Department of Microbiology and Immunology, The University of Western Ontario. November, 2009.

Activities and Contributions:

I was a member of the governing council of the Society of Microbiology and Immunology, representative of the department of Microbiology and Immunology, The University of Western Ontario. 2008-2005

I also sat on the Academic Committee of the Society of Microbiology and Immunology, charged with organizing the Western Research Forum. Established in 2005, the Research Forum is an annual, multidisciplinary conference. The Academic Committee and the Research Forum features both poster presentations and oral presentations from graduate students in the arts, biosciences and social sciences. 2008-2005

I was on the editorial board of the Western Graduate Review, a publication that profiles the outstanding research conducted by graduate students in a variety of disciplines at UWO. The Western Graduate Review is an online resource for current graduate students, as well as a useful resource for prospective graduate students. It can be viewed at <http://www.uwo.ca/sogs/WGR/index.html>. 2008.