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Title Page

Hypersensitivity of HIV-1 Infected Cells to Reactive Sulfonamide Metabolites Correlated to Expression of the HIV-1 Viral Protein Tat

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Non-standard abbreviations

ADRs	Adverse Drug Reactions
SMX	Sulfamethoxazole
SMX - HA	Sulfamethoxazole Hydroxylamine

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Abstract

Impairment of HIV-infected cells to deal with reactive drug metabolites may be a mechanism for the increased rate of adverse drug reactions seen in AIDS. Human Immunodeficiency Virus (HIV) Tat protein expression may be associated with increased oxidative stress within HIV-infected cells. To determine the relationship between expression of HIV Tat and sensitivity to reactive drug metabolites, we studied toxicity of sulfamethoxazole (SMX) and its reactive hydroxylamine intermediate (SMX-HA) in lymphocytes transfected with the HIV *tat* gene. Over a concentration range from 0 to 400 μM SMX-HA, there was a significant concentration-dependent increase in cell death in transfected cell lines expressing Tat compared to controls. Jurkat T cells transfected with a dose dependent inducible *tat* gene showed increased toxicity in response to SMX-HA as more Tat expression was induced. Enhanced sensitivity to SMX-HA was accompanied by significantly lower concentrations of total intracellular glutathione compared to controls ($P<0.05$). Sensitivity to reactive drug metabolites in HIV infected cells appears to be mediated by the viral protein Tat.

Introduction

Adverse drug reactions (ADRs) are important problems for people living with Human Immunodeficiency Virus (HIV) (Rieder and Dekaban 2000, Tantisiriwat and Powderly, 2000). The rate of ADRs to sulfonamide drugs among people with AIDS is as high as 50% compared to 5 % in the general population (Carr *et al.* 1993a, Carr *et al.* 1993b, Rieder *et al.* 1997). Sulfonamide-induced ADRs among HIV infected individuals are primarily hypersensitivity reactions, characterized by a severe rash and often associated with involvement of organs such as liver, kidney or bone marrow (Rieder *et al.* 1988, Rieder *et al.* 1995, Hess and Rieder 1997). People with AIDS are at an increased risk for hypersensitivity ADRs to many drugs, including other anti-infectives, anticonvulsants and antiretroviral drugs (Carr *et al.* 1993a, Carr *et al.* 1993b, Koopmans *et al.* 1995,).

The increased risk for ADRs appears to parallel progression of HIV infection to AIDS. During the asymptomatic stage, people positive for HIV are not at a higher risk for hypersensitivity ADRs than the general population. However, as the patient's $CD4^+$ cell count falls and viral load increases, risk for ADRs increases, according to most investigators (Carr *et al.* 1993a, Rieder *et al.* 1995, Rieder and Dekaban 2000, Rabaud *et al.* 2001, Elisazewicz *et al.* 2002, Sterling *et al.* 2003). Thus, factors associated with a decline in $CD4^+$ count, such as increasing viral load, may be associated with increase in ADR risk.

Both plasma and peripheral blood mononuclear cells (PBMCs) of AIDS patients exhibit significantly decreased levels of glutathione and concomitant marked increases in oxidized products (Buhl *et al.* 1989, Choi *et al.* 2000). Impairment of HIV-infected cells to deal with oxidative stress may explain the compromised ability of the cells to deal with reactive drug metabolites, generated during oxidative metabolism of the parent drug (Repetto *et al.* 1996, Rieder *et al.*1988, Rieder *et al.* 1989, Rieder *et al.* 1995). The HIV-associated decrease in glutathione (GSH) content can result from diminished GSH synthesis or an increased rate of loss. GSH consumption may increase in HIV infection as a result of increased oxidative stress (Favier *et al.* 1994, Pace and Leaf 1995, Walmsley *et al.* 1997). The systemic decrease in total GSH content and decreased metabolic labelling of GSH in HIV infected individuals suggest reduced GSH biosynthesis (Helbling *et al.* 1996, Jahoor *et al.* 1999). Thus, decreased GSH content may allow for development of oxidative stress rather than the converse.

We have previously demonstrated that HIV infection of T-cells is associated with increased cellular sensitivity to reactive drug metabolites (Rieder *et al.* 1995). Recent work has suggested expression of the HIV protein Tat is associated with increased oxidative stress (Flores *et al.* 1993, Westendorp *et al.* 1995a, Opalenik *et al.* 1998). Tat is a 14 kiloDalton protein encoded by HIV type 1 (HIV-!) that serves as a transcriptional/post-transcription transactivator essential for efficient viral replication (Dingwall *et al.* 1989, Kashanchi *et al.* 1994). In addition to regulating HIV gene expression and replication, Tat has been reported to influence cellular phenotype by affecting expression of cellular genes (Li-Weber *et al.* 2000). HIV-1 Tat has been found

to modulate the redox status of cells, lowering the total and reduced GSH concentration *in vitro* in various cell lines and decreasing the total sulfhydryl content *in vivo* in a Tat transgenic mouse model (Choi *et al.* 2000). Further investigation of this transgenic mouse model has revealed that Tat expression was associated with a linear decrease in hepatic and erythrocyte glutathione content accompanied by a decrease in the glutathione synthetase regulatory subunit mRNA and protein content, resulting in an increased sensitivity of the synthetase to glutathione feedback inhibition (Choi *et al.* 2000). This, together with the documented down-regulation of manganese superoxide dismutase by Tat, may explain the observed reduction in GSH content in HIV infected cells (Flores *et al.* 1993). Tat can cause both enhanced activation of NF-κB and increased susceptibility to apoptosis by increasing sensitivity to apoptotic signals through up-regulation of FLICE/Caspase-8 and CD95L expression in T cells (Campioni *et al.* 1995, Westendorp *et al.* 1995a, Westendorp *et al*. 1995b,Demarchi *et al.* 1996, Bartz and Emerman 1999, Kelly *et al.* 1999).

In this study, we sought to determine the relationship between expression of HIV viral protein Tat and cellular sensitivity to reactive drug metabolites using sulfamethoxazole as a model compound. This is the first report to investigate the relationship between viral protein expression and cellular sensitivity to reactive drug metabolites.

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Methods

Cell lines

The following cell lines used in this study were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The HIV infected lymphocytic $CD4^+$ cell line Jurkat J1.1 was originally from Dr. Thomas Folks, while the uninfected parent cell line Jurkat E6.1 was originally from Dr. Arthur Weiss. The Jurkat J1.1 and the parent Jurkat E6.1 cell lines were maintained in Complete RPMI 1640 medium [supplemented with 1% L-glutamine (Invitrogen), 20% FCS (Invitrogen) and 100 units/ml of penicillin G and streptomycin (Invitrogen)]. The HIV-1 Tat-expressing stable cell line Jurkat-tat, was originally from Drs. Antonella Caputo, William Haseltine, and Joseph Sodroski and was grown in Complete RPMI 1640 medium plus 800 μg/ml geneticin (G418; Gibco-BRL) to insure the maintenance of Tat expression.

Drug Incubations

All tissue culture cells were harvested during logarithmic growth (minimum 97% viability), washed repeatedly in Hank's Balanced Salt Solution (HBSS), resuspended in HBSS and pipetted into either 96-well plates at densities of 8×10^4 cells/well for the cell viability assessments or into 6-well plates at corresponding densities of 5×10^5 cell/well for the glutathione quantitation. Cells were incubated in the presence and/or absence of various concentrations of freshly prepared solutions of sulfamethoxazole-hydroxylamine

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(SMX-HA) or the parent drug sulfamethoxazole (SMX) for 2 hours, 5% CO_2 and 37^oC. The series of final SMX-HA concentrations (in 1% DMSO vol/vol [v/v]) were 0, 25, 50, 100, 200, 300, and 400 µM; that of SMX was 400µM; while the vehicle control final concentration was maintained throughout all the assays at 1% DMSO except where specified differently. All treatments were performed and measured in quadruplicate. Following drug exposure, the supernatants were removed and the cells were incubated in the Complete RPMI 1640 for 18 h, 5% CO_2 and 37^oC.

Drug Cytotoxicity Assessment

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye conversion to a dark blue formazan product. Eighteen hours following drug exposure, MTT was added to the wells at a final concentration of 1mg/ml and incubated at 37^oC for 4 hours. The formazan reaction product was solubilized overnight at room temperature using 50% dimethylformamide/ 20%SDS. The 96 well plates were read at 590 nm on a plate reader (Molecular Probes, Sunnyvale, CA). Viability was determined by comparison to a standard curve generated for each assay by concomitantly plating the corresponding cell type at 0, 25, 50, 75 and 100% of the initial cell density.

Cell viability was also determined by flow cytometry of Jurkat E6.1 cells stably transfected with a pBIG plasmid containing a *tat* gene fused to GFP or GFP alone that were induced in a concentration-dependent manner by doxycycline, 0-1000ng/ml.. The stable tat-transfected cells were incubated with vehicle alone or increasing

concentrations of doxycycline for 38 hours, harvested and analyzed for Tat expression using Western blots and 10µg of total protein for each differentially induced population. The blots were probed with both anti-tat antibodies and anti-β-actin antibody. These cells were also differentially induced with doxycycline for 38 hours, and treated with vehicle alone, 1000µM SMX, 50 or 200µM SMX-HA for 2 hours. They were subsequently incubated overnight in complete RPMI, harvested and stained with Annexin V-PE, a marker of apoptosis, and 7AAD, a viable cell marker (BD Pharmingen Apoptosis Detection Kit I).

Quantitation of Total Intracellular Glutathione

High performance liquid chromatography with fluorescence detection (HPLC-FD) was utilized to determine the total amount of intracellular glutathione in the transfected and parent Jurkat cell lines. Following a two hour exposure of 1.5×10^6 cells to either vehicle control or 200µM SMX (final concentration 1% DMSO v/v), the cultures were washed and allowed to recover in complete media for 18 h in 5% CO_2 and 37^{o}C. Triplicate aliquots of the treated cells were individually harvested and washed three times in Hepes Buffered Saline solution (Invitrogen). Cell suspensions were then prepared for total glutathione quantitation following a slightly modified method described by Jacobsen *et al*. involving the simultaneous sodium borohydride reduction of disulfide bonds and derivatization of sulfhydryl groups with monobromobimane followed by HPLC-FD (Jacobsen *et al.*1994). Using a Hewlett Packard 1090 Liquid Chromatograph, the reduced and derivatized samples were injected as 10 µL aliquots onto a C8 column (10 cm x 3.2 mm i.d., 5 µ) at a flow rate of 0.5 ml/min and column temperature of 40^{o}C.

The mobile phase consisted of a 4% acetonitrile/ 25mM ammonium formate buffer (pH 3.8 with formic acid). Following chromatographic separation, the thiolbimane adducts were detected fluorometrically with a Shimadzu fluorescence detector with the excitation wavelength set at 390 nm and the emission wavelength set at 480 nm.

Western blot detection of HIV-1 Tat

Jurkat-tat and Jurkat E6.1 cells at a density of 8 x 10^4 cells/well, were incubated in the presence of a series of SMX-HA concentrations ranging from 0 to 400μM or to the parent drug SMX at a final concentration of 400μM (all in a final v/v of 1% DMSO) for 2 hours, followed by the removal of the drug-containing supernatants and further incubation in complete RPMI 1640 for 18 hours at 5% CO_2 and 37^{o}C. Cells were harvested and lysed in cold extraction buffer (pH 7.6; 100 mM NaCl, 10 mM sodium phosphate, 1% v/v Triton X-100, 0.5% [wt/vol] sodium deoxycholate, 0.1% w/v sodium dodecyl sulphate, 1 mM EDTA) in the presence of a serine and cysteine protease inhibitor cocktail (Complete; Roche). Following the addition of an equal volume of 8M urea DTT-complete Laemmli loading buffer, the samples (pool of 10 wells) were sonicated briefly. HPLC purified HIV-1 Tat protein (0.45 μg; MW 14000 Daltons) was concomitantly run on each Tricine-6 M urea 15% SDS-PAGE gel to serve as a positive control. The HIV-1 Tat protein was originally from Dr. John Brady supplied through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Supernatant of cell lysates and purified Tat protein were electrophoresed for 7 hours at 50 volts in a tricine buffer system and transferred from the gel to an Immobilon P membrane (Millipore). Blots were blocked in a solution of 5% skim milk powder at room

temperature for 5 h. Blots were incubated in an anti-tat antibody cocktail diluted in blocking buffer, overnight at 4^{o}C with rocking. The anti-Tat antibody cocktail consisted of the mouse monoclonal NT3 2D1 1 antibody (IgG_{1a}; final concentration of 10 μg/ml) specific for the N-terminal 15-mer peptide of HIV-1 Tat [amino acid (a.a.) 73-86], and the HIV-1_{BH10} Tat mouse monoclonal antibody 15.1 (IgG_{1k}; final concentration of 42.5 pg/ml) specific for the epitope spanning a.a.1-16 of the HIV-1 Tat protein. Both Tat monoclonal antibodies were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Blots were then washed in blocking buffer and exposed to the secondary antibody solution consisting of goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs Inc.) at a final dilution of 1:8200 for 1 h at room temperature. The blots were then washed and exposed to the enhanced chemiluminescence substrate (Amersham) according to the manufacturer's instructions.

Statistics

GraphPad InStat® programs were used to calculate mean and standard error of the means for the toxicity data, and mean and standard deviations for the cytometry data. This program was also used to perform one way ANOVA and Dunnett post hoc multiple comparison tests.

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Results

The first objective of the current study was to determine whether the differential toxicity to sulfamethoxazole metabolites previously observed between HIV infected and uninfected cells was a tissue culture cell-type specific phenomenon or truly a consequence of HIV infection. HIV infected $Molt3_{IIIB}$ lymphoblastoid ($CD4^+$) cells had previously been shown to exhibit increased sensitivity to sulfonamide reactive metabolites compared to the uninfected parental cell line (Rieder *et al.* 1995). In the current study, we investigated the toxicity of sulfamethoxazole hydroxylamine on HIV infected T4-lymphoblastoid Jurkat J1.1 ($CD4^+$) cells. To determine the viability of the cell lines following exposure to the sulfamethoxazole and its hydroxylamine metabolite, uninfected Jurkat E6.1 and HIV infected Jurkat J1.1 cells were incubated with SMX-HA at varying concentrations for two hours, allowed to recover during an overnight incubation and then exposed to MTT. As expected, when cytotoxicity was assessed by the measurement of MTT dye conversion, Jurkat E6.1, Jurkat J1.1 and stably transfected Jurkat tat cells exposed to 400 μM parent compound sulfamethoxazole exhibited no significant differences in viability (92% versus 90% and 70% respectively; $P>0.05$ in both cases). In contrast, Jurkat E6.1, the HIV infected Jurkat J1.1 cell lines both exhibited concentration-dependent toxicity following exposure to the reactive metabolite SMX-HA (Figure 1). The Jurkat J1.1 cell line exhibited significantly greater levels of sensitivity and cell death to SMX-HA at all concentrations above 200 μM, with a calculated (Graph Pad InStat®) LC_{50} of 62.0 +/-39.5 μM SMX-HA, compared to the uninfected parent cell line E6.1 that demonstrated a LC_{50} of 280.8 +/- 59.4 μM. This

increased toxicity to SMX-HA exhibited by the HIV infected Jurkat J1.1 cells is consistent with our previous findings with HIV infected Molt3$_{IIIB}$cells and further suggested that the exhibited hypersensitivity to SMX-HA was a direct consequence of HIV infection. The Tat-expressing Jurkat-tat cells (LC_{50} of 55.4 +/- 16.7 μM) were studied concomitantly with Jurkat E6.1 and HIV_{IIIB} infected Jurkat J1.1 cells and exhibited a greater degree of dose-dependent toxicity to the drug metabolite SMX-HA (0-400 μM). (**Figure 1**).

In order to examine the level of Tat expressed during drug treatment Western blotting was performed and correlated to increasing drug concentrations. The samples analyzed on the Western blot shown in **Figure 2**, were collected from cell cultures initially plated at the same density prior to drug exposure for 2 hours followed by an 18 hour recuperation period. At the time of cell harvest for Western blot analysis, parallel samples were assessed for cell viability by the MTT dye conversion assay. To reflect the viability effects of the drugs, the collected cell lysate equivalents destined for Western blot analysis were not corrected for total protein content. Purified HIV-1 Tat protein was simultaneously immunoblotted to serve as a positive control. Upon incubation of the blot with an anti-Tat antibody cocktail, untreated Jurkat-tat cell cultures [0 μM HA] and those exposed to the parent drug sulfamethoxazole [400 μM SMX], both exhibiting greater than 92% viability, expressed the 14 000 Dalton Tat protein in comparable amounts (**Figure 2**). The level of the Tat expression detected decreased as the drug concentration increased and cell viability decreased.

To further demonstrate the increased cell death in stably transfected Jurkat E6.1 cells, we transfected Jurkat E6.1 cells with an inducible plasmid containing *tat* fused to GFP or with GFP alone. Increased induction with doxycycline showed a concentration dependent increase in Tat-GFP and GFP expression. The cells were induced with vehicle alone or increasing concentrations of doxycycline for 38 hours, harvested and analyzed by Western blot for the expression of Tat protein, and the expression of β-actin as a protein-loading control. The Western blot **(Figure 3)** clearly demonstrates that Tat-GFP expression from the pBIG vector platform is inducible in a dose dependent fashion, and not expressed in the absence of doxycycline. Flow cytometric analysis also demonstrated a dose response of induction of both Jurkat Tat-GFP and control GFP to be in the range of 31-500ng/ml doxycycline with a plateau beginning at 250 ng/ml. Furthermore, the more doxycycline-induced Tat-GFP there was in the Jurkat transfected cells, the more susceptible the cells were to SMX-HA-mediated toxicity, but not to SMX or DMSO. Increasing levels of control GFP expression induced in stably transfected cells by increasing doses of doxycycline did not affect the viability of these cells (>95% viable). However, over the same range of doxycycline, the *tat*-GFP transfected Jurkat cells showed a consistent and repeatable increase in cell death on exposure to increasing doses of SMX-HA, but not to SMX. As shown in **Table 1**, vehicle-treated cells transfected but not induced were viable (90-96.7%), but increasing both the expression of Tat-GFP and the concentration of drug in the incubation caused increased cell death, as evidenced by increased staining with both by Annexin V and 7AAD staining (**Table 1**). Flow cytometry results indicate the amount of *tat*-GFP fusion protein present in the cells makes the cells more susceptible to toxicity of sulfa

metabolites. No synergistic toxic effects of doxycycline and SMX-HA were found in Jurkat E6.1 or Jurkat-tat cells using the MTT assay. These effects on cell viability were not seen in control cells that expressed only GFP (data not shown).

High performance liquid chromatography with fluorescence detection (HPLC-FD) was utilized to determine the total intracellular glutathione concentration of both the transfected and parent Jurkat cell lines. Following a two hour exposure of cultures to either vehicle or 200μM SMX (final concentration 1% DMSO v/v), and 18-hour recovery in complete media, triplicate aliquots of the treated cells were prepared for total glutathione quantitation. Total cellular glutathione was determined by borohydride reduction of disulfide bonds and derivatization of sulfhydryl groups (thiols) with monobromobimane followed by HPLC-FD. **Figure 4** demonstrates that the intracellular concentrations of total GSH in the Tat-expressing Jurkat-tat cells, were significantly lower than those found in their corresponding parental cell lines ($P<0.03$), both in the presence and absence of the control parent drug SMX. Upon exposure to both sets of control conditions, the Tat-expressing Jurkat cells possessed the lowest concentrations of total GSH, exhibiting only 23% +/-1% of the total GSH concentration compared to its parent cell line Jurkat E6.1.

Discussion

ADRs are common and important problems for people living with HIV. (Hess and Rieder 1997). There is an increase in sulfonamide ADR risk associated with HIV infection, including a significantly increased risk for sulfonamide hypersensitivity ADRs (Carr *et al.* 1993a, Carr *et al.* 1993b). We have previously suggested that reactive drug metabolites appear to be the key elements in the pathogenesis of ADRs to drugs such as the sulfonamides. This appears to involve increased cellular sensitivity to reactive drug metabolites and this increased cellular sensitivity is more marked among HIV-infected cells. It appeared likely that clinical hypersensitivity to sulfonamides was related to HIV-specific alterations in cellular homeostasis, which would ultimately place the HIV infected cells at greater risk for cellular injury resulting from various reactive chemical species, including sulfonamide reactive intermediates (Koopmans *et al.* 1995, Rieder *et al.* 1995, Hess *et al.* 1999). It appears that this increased risk for cellular injury was related to factors unique to HIV infection, which could include the expression of proteins specific to HIV-infected cells. Thus, to test whether the altered ability of HIV infected cells to detoxify reactive sulfa drug metabolite intermediates was a direct consequence of a fundamental HIV viral function, we decided to focus on Tat protein due to its important role in viral gene expression and replication, the ability of Tat to alter host gene expression, and its known proapoptotic activity.

We have demonstrated that expression of Tat produced an increased cellular sensitivity to reactive drug metabolites equivalent to that seen among HIV-infected cells. This sensitivity occurred over drug concentrations that would be predicted to be found in the tissues of patients being treated clinically with the sulfonamides (i.e. 25 – 100 μM). The concentration of metabolite in tissues has not been directly measured, but is certainly highest at the site of metabolite generation. Cells expressing increasing susceptibility to drug metabolites with increasing levels of Tat suggest that Tat may be the most likely candidate for these effects. The dose-response curve for toxicity associated with incubation with reactive drug metabolites in the presence of Tat is not as smooth as would be anticipated if there was a simple linear relationship between toxicity and Tat expression. This suggests that toxicity of reactive metabolites in the context of HIV infection and Tat expression is likely to be a complex process. Thus, although Tat appears to be an important contributor to the toxicity of reactive metabolites, there may be additional molecular determinant(s) of this toxicity. More experiments are needed to determine if the amount of increased cell death in the transfected cells versus the HIV infected cells is due to changes in glutathione levels, an increased intracellular concentration of Tat or a combination of both factors in the transfected cells.

The marked toxicity experienced by the Tat-expressing transfected Jurkat-tat cells to SMX-HA was also observed upon Western blot analysis of the differentially drug-treated cells. This demonstrated that the Tat expression in Jurkat-tat cells was unaltered, both in the presence of the parent drug SMX and the solvent vehicle, 1% DMSO, that was common to all of the tested drug-exposure culture conditions.

Incubation of Jurkat-tat cells with varying concentrations of the reactive SMX-HA metabolite was associated with an increasing degree of cell death and was reflected in a concomitant decrease in the amount of Tat protein present. Preliminary results with another Tat-expressing cell line (CEM-tart cells received from Drs. Herbert Chen, Terence Boyle, Michael Malim, Bryan Cullen, and H. Kim Lyerly, and parental CEM-SS obtained directly from Dr. Peter Nara [Frederick Cancer Research and Development Center, Frederick, Maryland]) yielded similar results (data not shown). Thus this appears to be a consistent result in this third human T cell line.

Increased cell death on exposure to sulfa metabolites was also seen when transfected Jurkat cells were induced to express Tat in a dose dependent fashion. The increased expression of Tat was documented by Western blot and flow cytometry, and provided a correlation between expression of Tat protein and increased susceptibility to SMX-HA. The Annexin V data suggests the cells died by apoptosis; however, further experiments are needed to confirm this mechanism of cell death.

With respect to potential targets for Tat, our previous work and the work of others strongly suggests a possible link with the intracellular thiol glutathione. We have previously demonstrated that thiols modulate the effect of reactive sulfonamide metabolites (Rieder et al. 1988). Recent reports have suggested that Tat expression is associated with increased oxidative stress by lowering the total and reduced glutathione (GSH) concentration *in vitro* in various cell lines and also decreasing the total sulfhydryl content *in vivo* in a Tat transgenic mouse model (Choi *et al.* 2000). Thus, we were interested in determining if the total intracellular GSH concentrations of the transfected Jurkat-tat cell line used in this study also exhibited altered GSH levels, since these had

not yet been documented. We have demonstrated that expression of Tat significantly decreased total intracellular GSH concentrations. Under both control culture conditions tested, the cell viability of both cell lines was greater than 97%, thus the observed differences in total GSH were not merely reflections of differences in cell number or cytotoxicity.

It has been believed for some time that factors expressed during viral infection may play a role in the induction of adverse drug reactions, but there has been little evidence supporting this theory. It is unlikely that the HIV infection itself is contributing an increased immunostimulation to coadministered drugs because the increased risk for ADRs is so late in the infection process when immune suppression is clearly evident (Coopman *et al*. 1993), but this is a factor to consider. We have demonstrated that expression of the HIV Tat significantly increases cellular sensitivity to reactive drug metabolites and significantly decreases intracellular GSH concentration. This is the first demonstration of a potential mechanism for the previously described increased cellular sensitivity in the setting of HIV infection. The levels of other compounds affecting oxidative stress may also have a role in the incidence and severity of ADRs in HIV-infected patients. In particular, one study demonstrates that a deficiency in plasma ascorbate is associated with decreased detoxification of sulfa metabolites in HIV-infected individuals (Trepanier *et al*, 2004). This suggests that altered intracellular homeostasis may be key in the pathogenesis of the increased rate of ADRs seen in the setting of HIV infection.

Future work will need to better define the effect(s) and target(s) of Tat in the induction of enhanced cellular sensitivity. In future experiments, to further confirm the Tat-mediated increase in sensitivity to reactive drug metabolites, we hope to use Tat inhibitor/antagonists such as Ro24-7429 (7-chloro-5-1H-pyrrol-2-yl-3H-benzo[e][1,4]diazepin-2-yl-methylamine) to determine if this antagonist is capable of inhibiting the enhanced drug sensitivity demonstrated in the Tat expressing cells. Our findings detailing the association of Tat protein with increased susceptibility to adverse drug metabolite reactions does not preclude the possibility that other HIV proteins may also contribute to this phenomenon, or be involved in the modulation of the cellular oxidative status. Thus, future research will need to include the development of transfected cell lines expressing other HIV genes for similar sensitivity testing and glutathione quantitation. As well, the effects we have studied have been in relation to total cellular glutathione, which is well known to act by cycling between reduced and oxidized forms. Thus, it would be of great interest to dissect the effect(s) of Tat expression on the kinetics of GSH synthesis and cycling.

The recognition that Tat may be instrumental in viral modulation of host oxidative status underscores the potential importance of this HIV protein. This effect may be direct as has been demonstrated here in HIV infected cells. However, Tat can also be secreted from infected cells and taken up by uninfected neighbouring cells. Tat can then effect transcellular transactivation of gene expression (Marcuzzi *et al*. 1992, Frankel and Pabo 1998). Hence, host tissues not actually infected may nevertheless have their oxidant status modified and thereby escalate the progression of the disease.

Remembering that HIV infection is a dynamic process, with active traffic in viral particles, it can be appreciated that there would be marked changes in the expression of Tat, with viral protein expression increasing dramatically as the HIV infection progresses to AIDS. If this viral regulatory protein modulates, at least in part, the ability of HIV infected cells to deal with oxidative stress, such as that produced by reactive drug metabolites, this provides a mechanistic framework for the increased risk of hypersensitivity and adverse drug reactions as HIV infection progresses. This would also explain why the effective control of HIV viral load through the use of anti-retroviral therapy appears to lower the risk of developing the most severe adverse reactions associated with drug therapy (Ryan *et al.* 1998).

Understanding the molecular mechanism(s) responsible for these effects will be important in designing safer and more effective therapy for patients with HIV infection and other chronic viral infections.

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Footnotes

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Legends for Figures

Figure 1: Jurkat E6.1, HIV infected Jurkat J1.1 cells and Jurkat-tat cell lines exhibit concentration-dependent toxicity following exposure to SMX-HA, one of the reactive metabolites of sulfamethoxazole. The cells (at a density of 8 x 10^4cells/well) were exposed to a range of concentrations of SMX-HA (0-400μM) for 2 hours and then allowed to recover for 18 hours (see *Experimental Procedures*). The Jurkat J1.1 cell line and tat-expressing Jurkat-tat cells exhibited significantly increased levels of sensitivity to SMX-HA compared to the uninfected parent cell line E6.1. The parent cell line showed significant difference from vehicle at 300μM SMX-HA, the Jurkat 1.1 cells at 200μM SMX-HA, and the Jurkat-tat cells at 50μM SMX-HA. (* indicates pP0.05, ** P<0.01). The graphs were analyzed by one way ANOVA and Dunnett Multiple post hoc Comparison Test (GraphPad Instat®) showed Jurkat E6.1 and Jurkat 1.1 cells were significantly different (P<0.001), and the Jurkat E6.1 and Jurkat-tat were also significantly different (P<0.001). The viability results assessed by MTT dye conversion, are the means +/- standard error of the mean of three separate trials involving the three Jurkat cell lines.

Figure 2: Western blot analysis revealed that the HIV-1 Tat protein continues to be expressed in Jurkat-tat cells in detectable amounts, following a 2 hour exposure to either 400 μM SMX or low concentrations (0 to 100 μM) of the reactive metabolite SMX-HA. All cells were initially plated at the same density of 8 x 10^4/well prior to drug exposure for 2 hours and followed by an 18 hour recuperation period, at which time the cell viability was assessed. To reflect the toxicity effects of the drugs, the collected cell

lysate equivalents were not corrected for total protein content. The blot was incubated in a primary anti-Tat antibody cocktail consisting of the mouse monoclonal NT3 2D1 1 antibody (final concentration of 10 μg/ml) specific for the N-terminal 15-mer peptide of HIV-1 Tat (a.a. 73-86), and the HIV-1_{BH10} Tat mouse monoclonal antibody 15.1 (final concentration of 42.5 pg/ml) specific for the epitope spanning a.a. 1-16 of the HIV-1 Tat protein. Purified HIV-1 Tat protein was concomitantly immunoblotted to serve as a positive control.

Figure 3: A)Cell lysates of JurkatpBIG tatGFP cells treated with different doses of doxycycline were probed with anti-tat or anti-β-actin antibodies. Differential tatGFP expression depended on the dose of doxycycline. Cell populations were induced with 0 (lane 1), 31 (lane 2), 62 (lane 3), 125 (lane 4), 250 (lane5), 500 (lane 6) or 1000 (lane 7) ng per ml of doxycycline per ml of media. The experiment was repeated three times.

B) Densitometric reading of Western blots shows per cent of maximal (JurkatpBIG tatGFP induced with 1000 ng per ml doxycycline) tatGFP expression. The data are presented as mean +/- standard deviation.

Figure 4: High performance liquid chromatography with fluorescence detection (HPLC-FD) was utilized to determine the total intracellular glutathione amounts of both the transfected and parent Jurkat cell lines. Following a two hour exposure of cultures (1.5 x 10^6 cells/well) to either vehicle alone or 200μM SMX (final concentration 1% DMSO v/v), and 18-hour recovery in complete media, triplicate aliquots of the treated cells were then prepared for total glutathione quantitation (μM/L). Quantitation by HPLC-FD revealed that intracellular concentrations of total GSH in Jurkat-tat cells were significantly lower than those found in the parent cells (23% +/-2 upon comparison,

P<0.03), both in the presence and absence of the control parent drug SMX (200 μM SMX or vehicle alone, respectively).

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Table 1

Cells expressing increasing concentrations of Tat protein show an increasing susceptibility to sulfamethoxazole metabolite. Cells stably transfected with a doxycycline-inducible Tat plasmid were stained with Annexin V-PE, a stain for early apoptosis, and with 7AAD, a vital stain. The table shows the percentages of cells stained with neither (live cells), Annexin V alone (cells in early stages of apoptosis) or both stains (dead cells). The number of cells staining indicates an increase in dead and apoptotic cells as either Tat expression or SMX-HA concentration increases. All cells treated with the parent sulfamethoxazole showed high viability. Percent viability is expressed as the mean and standard deviation of three separate experiments.

SMX – HA concentration		0 µM	50 µM	200 µM	-----
SMX concentration		-----	-----	-----	1000 µM
Uninduced	Live	88.3 ± 10.7	81 ± 7	73.5 ± 4	-----
	AnnexinV$^+$	5.0 ± 1.5	5.7 ± 1.9	9.6 ± 1.7	-----
	7AAD$^+$, AnnexinV$^+$	5.9 ± 8.2	10.4 ± 4.0	15.4 ± 6.0	-----
31 µM doxycycline	Live	92.4 ± 1.1	51.3 ± 14.9	38.7 ± 16.6	-----
	AnnexinV$^+$	6.6 ± 0.7	15.3 ± 5.1	14.3 ± 5.6	-----
	7AAD$^+$, AnnexinV$^+$	1.0 ± 0.4	29.8 ± 13.5	43.0 ± 13.1	-----
500 µM doxycycline	Live	85.4 ± 5.8	55.6 ± 21.4	40.1 ± 27.7	97.6
	AnnexinV$^+$	9.0 ± 0.3	17.6 ± 7.1	18.2 ± 4.7	1.55
	7AAD$^+$, AnnexinV$^+$	5.0 ± 5.3	25.5 ± 13.3	40.9 ± 23.7	0.73

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Figure 1

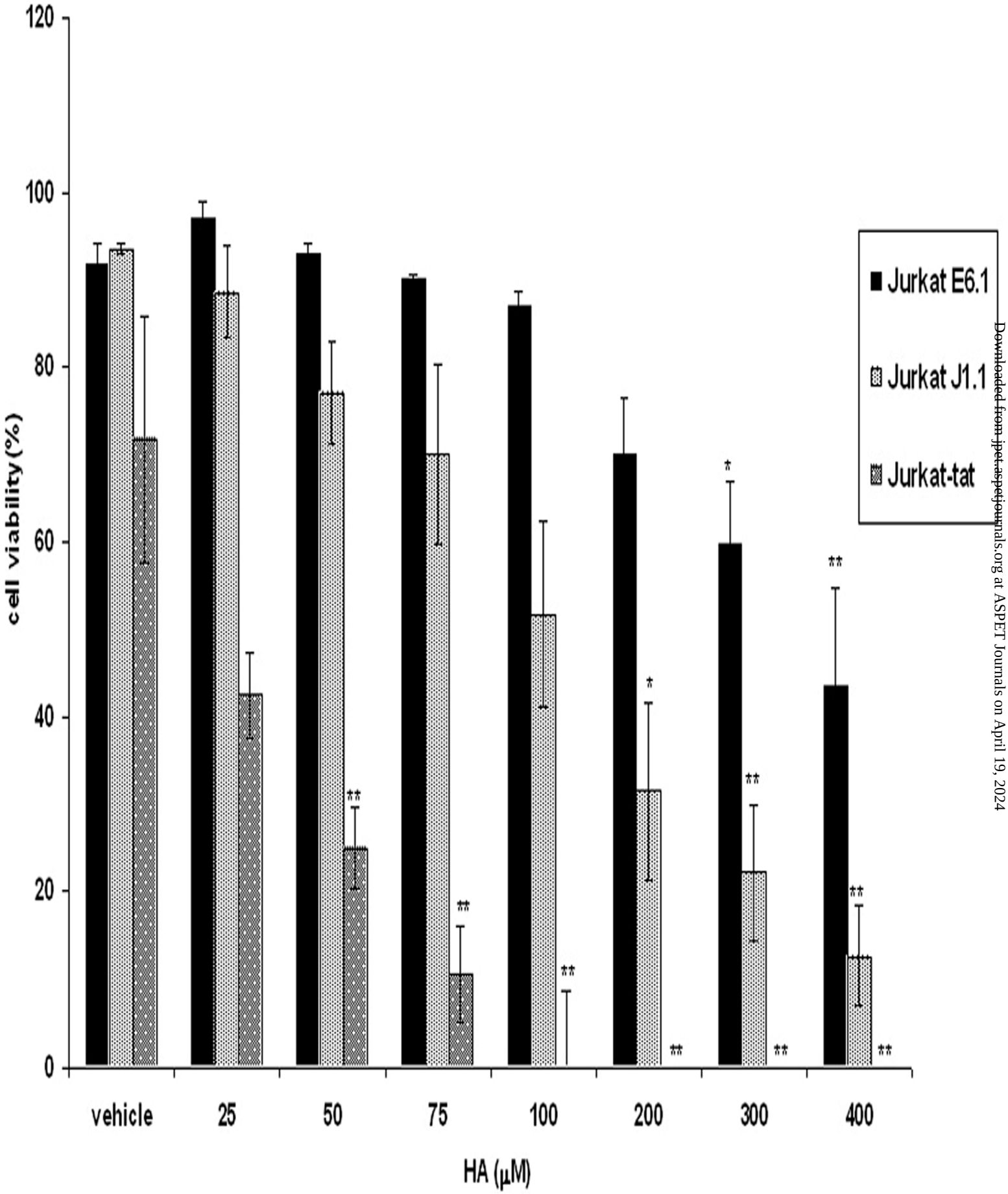
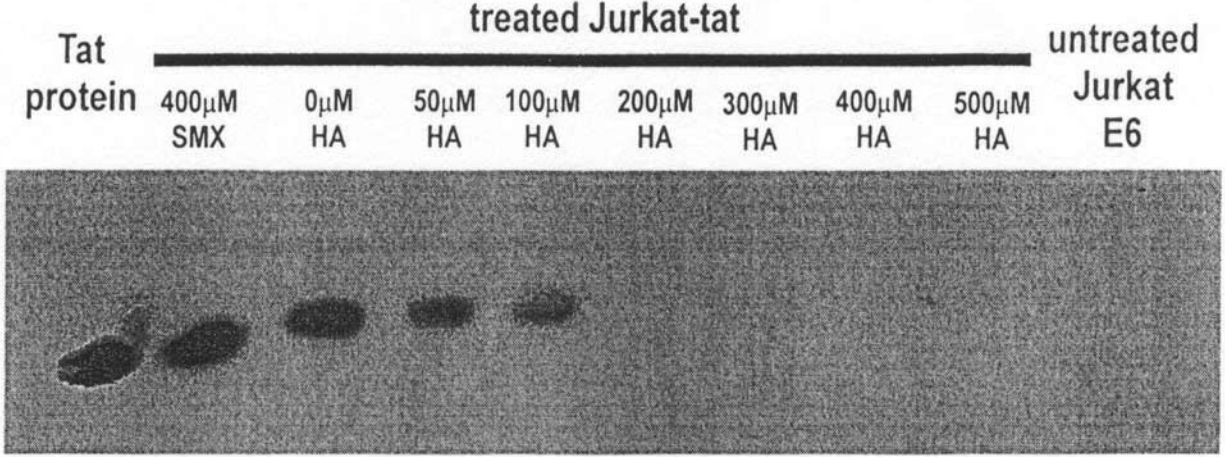
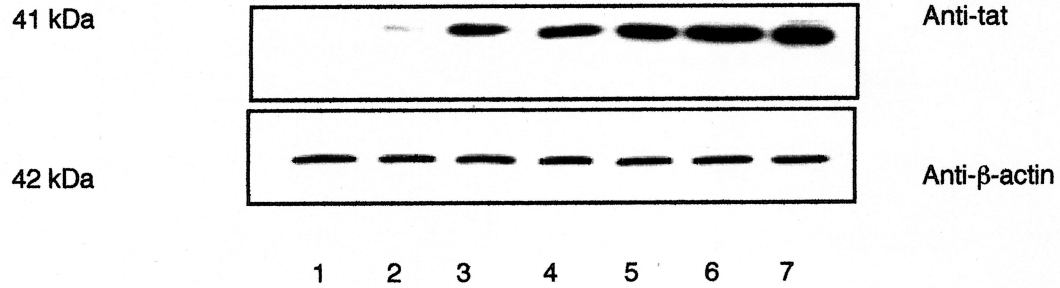


Figure 2


treated Jurkat-tat
Tat protein
400μM SMX
0μM HA
50μM HA
100μM HA
200μM HA
300μM HA
400μM HA
500μM HA
untreated Jurkat E6

A)


41 kDa
Anti-tat
42 kDa
Anti-β-actin
1
2
3
4
5
6
7

B)

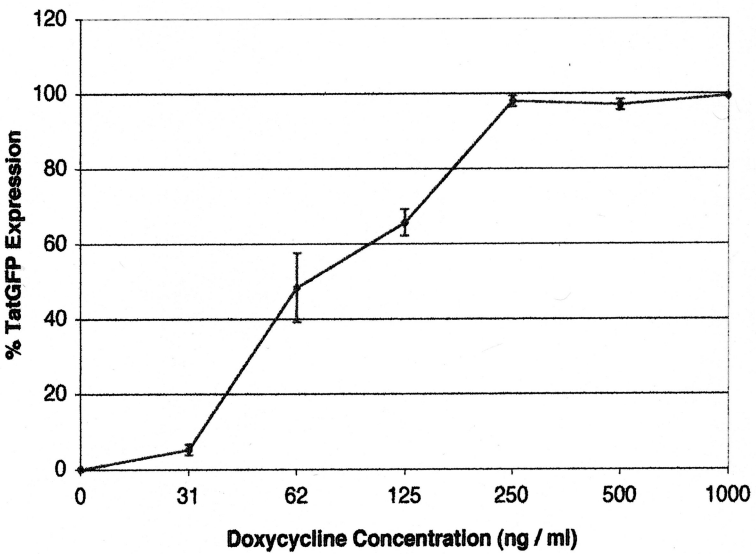

120
100
80
60
40
20
0
% TatGFP Expression
0
31
62
125
250
500
1000
Doxycycline Concentration (ng / ml)

Figure 3

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Figure 4