The Pennsylvania State University
The Graduate School
Immunology and Infectious Diseases

TARGETING HIV TAT-MEDIATED TRANSCRIPTION BY COMPLEMENTARY THERAPEUTIC MODALITIES

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Abstract

Human Immunodeficiency Virus (HIV) infection and Acquired Immunodeficiency Syndrome (AIDS) still claim over 2 million lives annually, despite the emergence of Highly Active Anti-Retroviral Therapy (HAART) over 15 years ago. The main cause for this has been the advent of viruses resistant to the HAART therapy. Besides this, there is very irregular adherence to the recommended therapy for HIV/AIDS because of the highly unpleasant side-effects prevalent due to the drugs used in conventional therapy. Due to these reasons, there is a need to establish additional forms of therapy against this disease.

There is a considerable amount of epidemiological data suggesting a direct relation between selenium status and the severity of HIV infections. The mechanism underlying this effect has not been studied in detail, although it has been suggested that selenium supplementation alleviates oxidative stress by inhibiting the NF-κB pathway. Selenium exerts its antioxidant effects via incorporation into selenoproteins as selenocysteine. We show here that selenium, via incorporation into the antioxidant selenoenzyme, thioredoxin reductase-1 (TR1), inhibits HIV replication in macrophages by modulating the activity of a crucial HIV transcription factor, Transactivator of transcription (Tat). Our data suggests that TR1 reduces the disulfides in Tat which are important for its activity.

Another mechanism by which selenium may inhibit HIV replication, besides incorporation into macrophages, is to cause the inhibition of the lysine acetyltransferase (KAT) activity of the enzyme, p300 (KAT3B). We present data in this thesis suggesting

that selenium, via the upregulation of endogenous levels of the cyclopentenone prostaglandin (CyPG) Δ^{12}-PGJ_2, inhibits the activity of p300 by covalently modifying a key cysteine residue in the enzyme active site. Furthermore, another CyPG, 15d-PGJ_2, which is formed endogenously as a result of selenium supplementation, covalently binds to and inhibits the transactivation activity of Tat. Exogenous addition of 15d-PGJ_2 and other Michael acceptor electrophiles such as celastrol, inhibits Tat-mediated transcription and HIV replication in cell culture.

Thus, we show here that selenium can inhibit HIV replication by inhibiting the activity of Tat by affecting its modification, and that this inhibition may be enhanced by treatment with compounds of natural origin which covalently modify the Cys-thiols which are highly conserved in the Tat proteins across different HIV strains worldwide.

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List of abbreviations

HIV – human immunodeficiency virus
Tat – Transactivator of transcription
rTat – recombinant Tat
MDM – monocyte derived macrophage
TAR – transactivation-responsive region
HAART – Highly active antiretroviral therapy
CDK9 – cyclin dependent kinase 9
P-TEFb – positive transcription elongation factor b
Sec – selenocysteine
Cys - cysteine
SECIS – selenocysteine insertion sequence
GPx – glutathione peroxidase
GSH – glutathione
TR – thioredoxin reductase
SPS2 – selenophosphate synthetase 2
AA – arachidonic acid
COX – cyclooxygenase
PG – prostaglandin
CyPG – cyclopentenone prostaglandin
H-PGDS – hematopoietic prostaglandin D2 synthase
mPGES-1 – microsomal prostaglandin E synthase – 1
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
ELISA – enzyme-linked immunosorbent assay
Δ^{12}-PGJ_2 – Δ^{12}-prostaglandin J_2
15d-PGJ_2 – 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
iNOS – inducible nitric oxide synthase
TNF-α – tumor necrosis factor –α
LPS – lipopolysaccharide
NF-κB – nuclear factor - kappa B
IκB – inhibitor of kappa B
IKK – inhibitor of kappa B (IκB) kinase
GA –gambogic acid
DGA – 9,10-dihydro gambogic acid
Cur – Curcumin
tCur – Tetrahydrocurcumin
RA – Rosmarinic acid
Cel – Celastrol
bCel – biotinylated celastrol
HAT – histone acetyltransferase
KAT – lysine acetyltransferase
LTR – long terminal repeat

Acknowledgements

How time flies! I still remember my first day in the United States (8th August, 2007) like it was yesterday. Before that, I had never even stayed away from my family for two days! And now here I was, many thousand miles away from them. And the journey back would not be a couple of hours' train ride either (not to mention the cost!!). I was wondering what I had gotten myself into, and if this was worth the distance separating me from my family and my girlfriend (now wife). It was with these tumultuous thoughts that I started my graduate life in Penn State. And I say now with the utmost confidence that coming to Penn State for my PhD was one of the best decisions I have ever made. My journey (and a PhD can be described as nothing less), was made possible due of the support of many individuals throughout my life. It is to these people that I owe whatever little ability and knowledge that I have, and I wish to acknowledge the contributions of some of them.

I would like to start with my Advisor, Dr. K. Sandeep Prabhu. I have not known a person more involved in the scientific process than Sandeep. In my opinion, he is close to being the perfect PI. He is one of the smartest people I know. While being heavily involved in the projects of every lab member, he is not overbearing. He allows you space to think about your project and is open to considering new ideas. I have learnt how to develop and nurture ideas under his tutelage, and also to look at the positive aspect of things when nothing is going right. He is a fantastic mentor and I owe my growth as a researcher to him.

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Believe it or not, pursuing a PhD is an extremely stressful ordeal. While you are the only one who can learn how to deal with the stress, having a support system of peers and friends will only help you along. At this time I would like to acknowledge the huge part played by my lab-mates, past and present - Parisa Kalantari, Ujjawal Gandhi, Ryan Arner, Chris Chiaro, Naveen Kaushal, Ravindra Kodihalli, Shakira Nelson, Avinash Kudva, and Emily Finch. The long discussions I have had with them have helped me a lot these last six or so years. Without their suggestions and technical expertise, I wouldn't have been able to accomplish even half of the work that I have presented in this thesis.

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I would also like to mention the only undergraduate student I “mentored” during my PhD, Sara Lynn Nicole Farwell. I want to thank her for providing me with the opportunity to be her mentor, and for putting up with my crazy ideas and work hours. As I write this, she is now pursuing a PhD herself. I wish her all the luck in the world, and hope that I have had a positive impact on her research career.

And where would I be today if it weren’t for my family! My parents, Mr. L. R. Narayan and Mrs. Nalini Narayan, have always been a constant source of inspiration to me. I cannot thank my parents enough for allowing me to pursue my passion, and for supporting my decision to travel to a foreign country to achieve it. I would never have been able to finish my PhD without their constant support and encouragement. I know their blessings are always with me in whatever I do. My younger brother, Vishal, is the one I look to when I feel a lack of dedication and motivation. Talking to him is always like a breath of fresh air.

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Chapter 1: Literature Review

The Human Immunodeficiency Virus

The human immunodeficiency virus (HIV-1) is a retrovirus which belongs to the genus *Lentivirus*; a part of the subfamily Orthoretrovirinae, of the family Retroviridae, and is the causative agent of a disease called acquired immunodeficiency syndrome (AIDS). It is spherical in structure, and has a diameter of 120 nm (Gelderblom *et al.* 1987). The virus has 2 copies of positive-sense RNA (~ 9 kb) strands as its genome. The viral genome has nine open reading frames and encodes for three polypeptides, Gag, Pol and Env. These polypeptides are proteolyzed to form 15 proteins that are needed by the virus at various stages of its life cycle. Thus, the virus has been described as an entity consisting of "15 proteins and one RNA" (Frankel *et al.* 1998). The Gag polypeptide is cleaved into four proteins namely, matrix (MA), nucleocapsid (NC, also p7), capsid (CA, also p24) and p6. Env is cleaved into two proteins, gp120 (surface glycoprotein) and gp41 (transmembrane). These six proteins are the structural components of the virus, making up the core and the outer envelope of the virion. Pol is cleaved to PR (protease), IN (integrase) and RT (reverse transcriptase), which provide essential enzymatic functions during the viral life cycle. Reverse transcriptase is essential for reverse transcribing the viral RNA genome into DNA, which can then be integrated into the host genome by IN (Fig. 2). Protease is important during the maturation phase of the viral life cycle (Fig. 2). In addition to these essential proteins, the HIV genome also encodes for other "accessory" proteins such as Vif, Vpr, Vpu, Tat, Rev and Nef. The mRNAs coding for these proteins are formed as a result of alternative splicing of the HIV transcripts.

These proteins play important roles at different points during the viral life cycle. Early in the infection, only the completely spliced mRNAs encoding the regulatory proteins Tat, Rev and Nef, are transported into the cytoplasm and subsequently translated. Rev acts as the transport protein and transports the viral mRNAs into the cytoplasm from the nucleus to be translated. As the infection progresses and sufficient levels of Rev are achieved, the incompletely spliced and unspliced viral RNAs, which encode the polyproteins, are transported out of the nucleus. Such alternative splicing is an important step during the life cycle of HIV as the different efficiencies with which the splice sites are used control the relative abundance of the proteins required for the formation of the virion. The process of alternative splicing during HIV replication has been reviewed by Dr. C. Martin Stoltzfus (Stoltzfus 2009).

The viral RNAs in the mature virus are tightly associated with the nucleocapsid proteins, and are encased by a conical capsid made up of the capsid protein (Fig. 1). The capsid also encases the enzymes and proteins needed by the virus (not available in the host) including reverse transcriptase, integrase, protease, Vif, Nef, p6 and Vpr. The capsid is surrounded by the matrix protein, which is in turn surrounded by the viral envelope formed of phospholipids from the host cell membrane, taken up during budding. The viral transmembrane protein, gp41, is embedded into the lipid membrane and non-covalently associates with gp120.

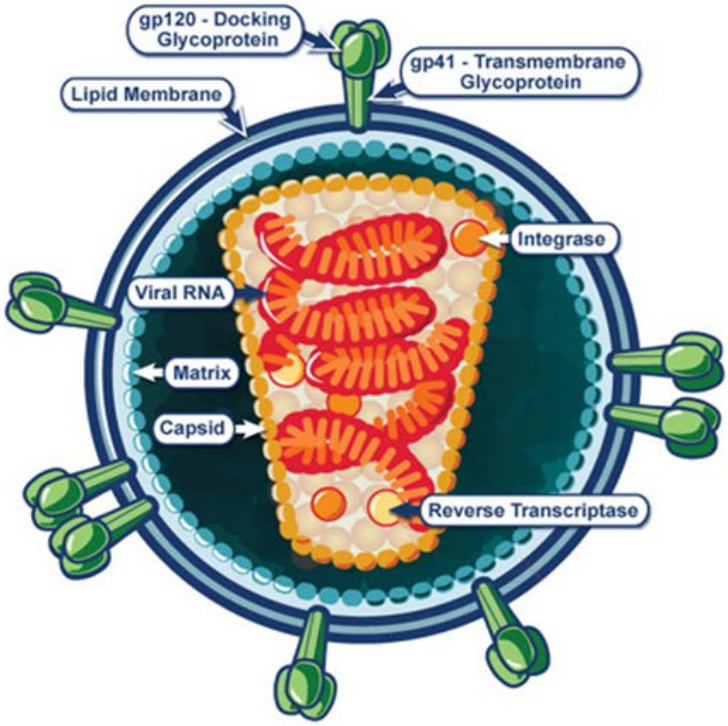

FIGURE 1. **The HIV virus.** A representation of the HIV virus showing some of the major proteins found in the virion. (www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/Pages/structure.aspx)

Life Cycle of HIV

The life cycle of HIV can be divided into six major steps – viral entry (into the cell via viral attachment to receptors on the host cell), reverse transcription (of the viral RNA inside the host cell by viral RT), integration (of the newly formed viral DNA into the host genome), transcription and translation (of the integrated viral genome by the host machinery), assembly (of the virus at the host membrane) and budding (of the immature virion), and maturation (of the immature virion into the infectious viral particle) (Fig. 2). Table 1 identifies the viral proteins involved in each step of the viral life cycle, and drugs which have been approved for clinical use against the respective proteins.

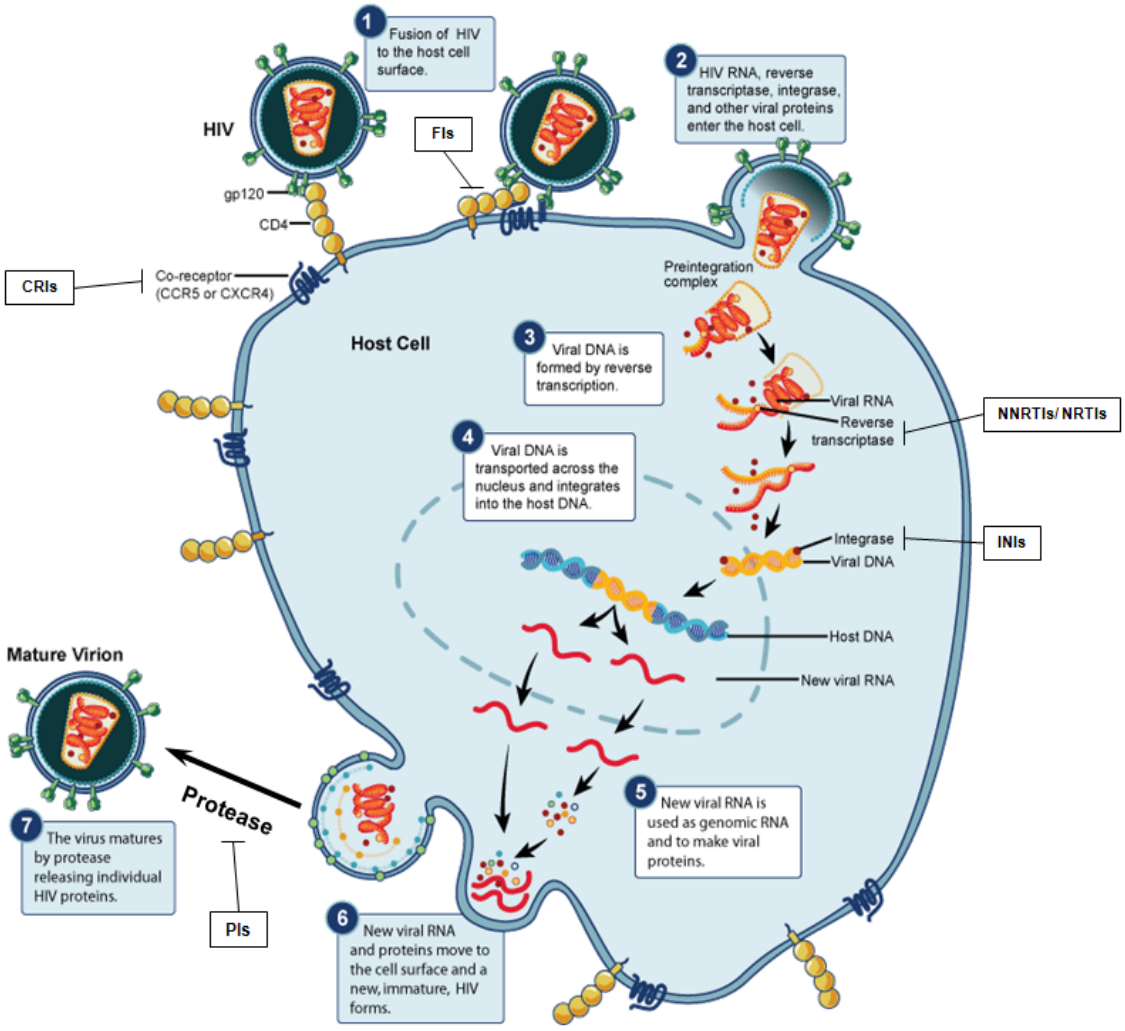

FIGURE 2. **HIV Life Cycle.** The various steps in the life cycle of the HIV virus. Different classes of inhibitors targeting the viral life cycle have been depicted. NNRTIs – non-nucleoside reverse transcriptase inhibitors (RTIs), NRTIs – nucleotide/nucleoside RTIs, INIs – integrase inhibitors, PIs – protease inhibitors, CRIs – co-receptor inhibitors, FIs – fusion inhibitors.
(http://www.niaid.nih.gov/topics/hivaids/understanding/biology/Pages/hivReplicationCycle.aspx)

Stage of the HIV life cycle	Proteins involved	FDA approved drug therapy	References
Viral entry	Viral – gp41, gp120 Host – CD4, CCR5, CXCR4	Entry inhibitors, e.g. Maraviroc	Tilton *et al.* 2010
Reverse transcription	Reverse transcriptase (RT)	Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), e.g. Zidovudine Non-nucleoside reverse transcriptase inhibitors (NNRTIs), e.g. Nevirapine	El Safadi *et al.* 2007, Cihlar *et al.* 2010
Integration	Integrase (IN)	Integrase inhibitors (INIs), e.g. Raltegravir	McColl *et al.* 2010
Transcription and translation	See text		
Assembly and budding	Viral – Gag polyprotein Host – Proteins of the ESCRT family, etc.	-	Freed 1998, Ganser-Pornillos *et al.* 2008, Briggs *et al.* 2011, Ganser-Pornillos *et al.* 2012
Maturation	Protease (PR)	Protease inhibitors (PIs), e.g. Saquinavir	Wensing *et al.* 2010, Briggs *et al.* 2011

TABLE 1. **Table listing the different stages of the HIV Life Cycle, the major proteins involved, and the inhibitors thereof.** (See references for more information)

Transcription of the HIV genome

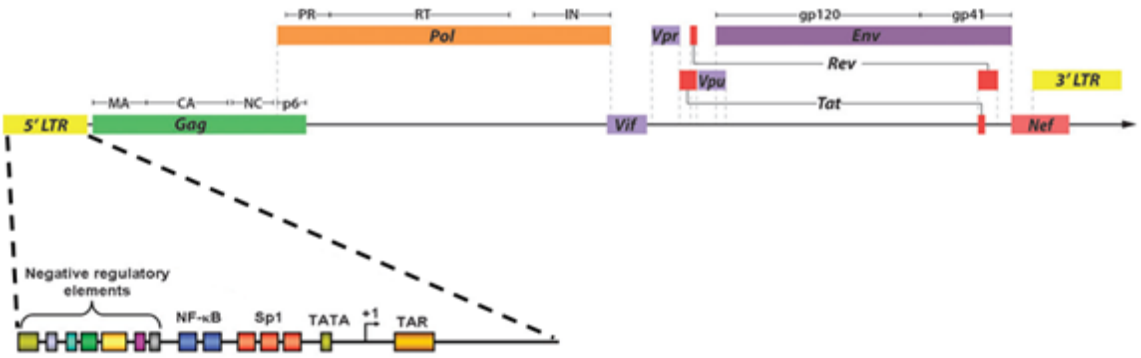

FIGURE 3. **The organization of the HIV genome.** Schematic representation of the HIV genome depicting the different genes (and gene products), and the organization of the HIV LTR. (Adapted from Romani *et al.* 2010, Tavassoli 2011)

Transcription of the viral DNA to form RNA, which will further be translated by the host machinery to form viral proteins, is a very critical step in the life cycle of HIV. HIV co-opts the cellular transcriptional machinery to facilitate the transcription of its genome, and regulates the process using its own viral proteins. Unlike murine and avian retroviruses, the HIV long terminal repeat (LTR), which regulates proviral expression at the transcriptional level, is an inducible promoter. The novel transactivating factor which induces transcription from the HIV LTR is named Tat (Transactivator of transcription) (Sodroski *et al.* 1985).

The HIV LTR (Fig. 3) is generally divided into four elements (Karn 1999), three of which recruit various host transcription factors like NF-κB, SP-1, C/EBPβ (Pierson *et al.* 2000, Rohr *et al.* 2003), histone acetyltransferases (HATs), and chromatin remodeling machinery (Agbottah *et al.* 2006, Mahmoudi *et al.* 2006). One of the domains, called the transactivation-responsive region (TAR), forms an RNA stem-loop upon transcription,

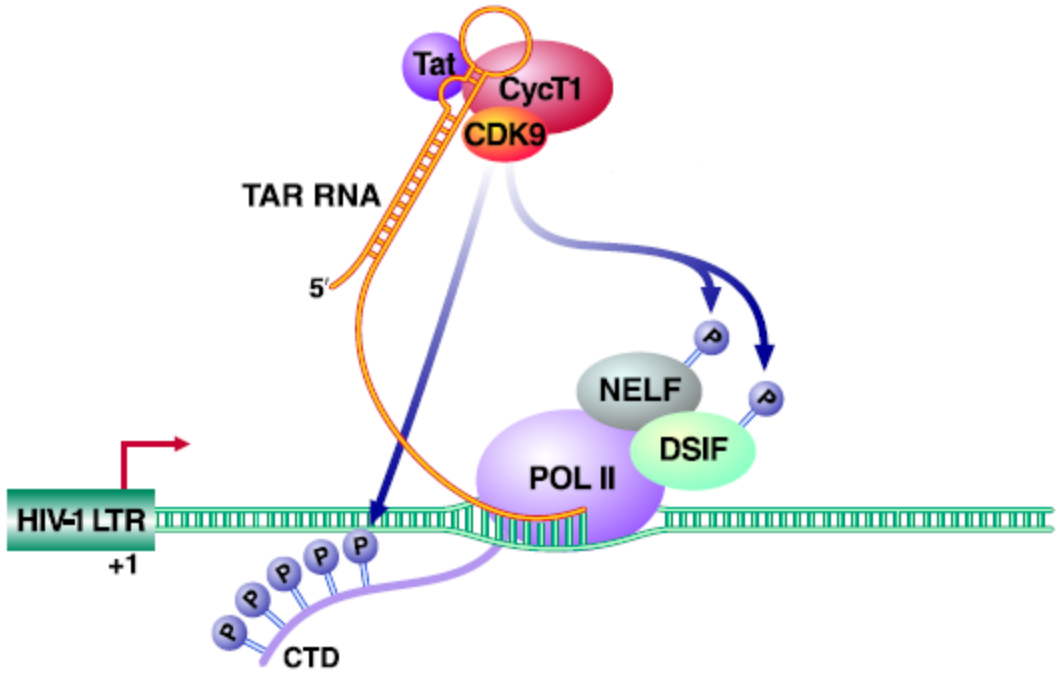

FIGURE 4. **Tat recruits the P-TEFb complex to facilitate viral transcriptional elongation.** Tat forms a ternary complex with cyclin T1 at the TAR loop of the transcribing viral RNA, thus recruiting the P-TEFb complex to the HIV LTR. This results in the phosphorylation of the C-terminal domain of RNAPII, thereby enhancing its processivity; resulting in the formation of elongated viral transcripts. CDK9 also phosphorylates the negative elongation factors, NELF and DSIF, thereby inactivating them (Ott *et al.* 2011).

and is involved in recruiting Tat to the LTR (Karn 1999). Tat is known to be a factor involved in enhancing viral transcription elongation (Feinberg *et al.* 1991). It is involved in the recruitment of the transcription factor P-TEFb to the LTR (Garber *et al.* 1998). Cyclin T1, a constituent of P-TEFb along with CDK9, binds to the loop region of the TAR RNA and forms a ternary complex with Tat (Garber *et al.* 1998, Imai *et al.* 2009) (Fig. 4). Tat and cyclin T1 interact with each other via zinc bridges, which involve the cysteine-rich domain of Tat and Cys^{261} of cyclin T1 (Garber *et al.* 1998). The interaction between Tat and cyclin T1 leads to a conformational change in CDK9, resulting in its autophosphorylation and activation. CDK9, upon activation, hyperphosphorylates the

carboxy-terminal domain of RNA polymerase II (Garber *et al.* 1998, Karn 1999, Imai *et al.* 2009), which increases the processivity of the polymerase, enhancing the transcription of the viral genome. Thus, in the absence of Tat, viral transcription is impaired, which is evident by the accumulation of short viral transcripts (Kao *et al.* 1987, Marciniak *et al.* 1991). The presence of Tat, on the other hand, promotes the formation of elongated viral transcripts (Laspia *et al.* 1989, Marciniak *et al.* 1990, Marciniak *et al.* 1991). It can be hypothesized that the initial lack of processivity during viral transcription enables the creation of key check-points for HIV transcription, which contribute to the establishment of proviral latency. As noted earlier, Tat is one of the earliest proteins to be produced upon infection, encoded by spliced HIV mRNAs. It can be speculated that inefficient RNA processing in the infected cell or a translational block would result in insufficient levels of Tat expression (e.g. in resting or quiescent cells), which could serve as a signal to the provirus indicating suboptimal conditions for replication In such a situation, it would be beneficial for the integrated provirus to “pause” transcription and replication till the infected cell again gets activated to actively transcribe and process RNA so that viral transcription and translation may proceed efficiently.

Depending of the HIV strain, *tat* encodes for Tat protein of 86 to 104 amino acids (Kuppuswamy *et al.* 1989, Romani *et al.* 2010). Tat is encoded by two exons (Fig. 1), the first of which codes for the first 72 amino acids (Sodroski *et al.* 1985, Schwarze *et al.* 1999). Due to this, the molecular weight of the Tat protein can vary between 14 to 16 kDa (Romani *et al.* 2010). Based on mutation-based mapping studies, Tat has been divided into six domains (Fig. 5). Of the six, the first four domains, N-terminal domain (a. a. 1-19), cysteine-rich domain (a. a. 20-39), core domain (a. a. 40-47), and basic

domain (a. a. 48-56, also called the arginine-rich motif; ARM) are considered essential for the transactivation property of Tat, while the auxiliary domain of Tat (a. a. 57-67) enhances the activity of Tat (Kuppuswamy *et al.* 1989). Of these domains, the cysteine domain, which has seven cysteine residues, has been found to be crucial in the transactivation activity of Tat (Kuppuswamy *et al.* 1989). Studies have shown that deleting the cysteine domain of Tat severely impairs its transactivation ability (Kuppuswamy *et al.* 1989). It has also been shown that reducing agents inhibit the activity of Tat (Koken *et al.* 1994), suggesting that at least some of the cysteine residues form intramolecular disulfide bonds (Kirsch *et al.* 1996).

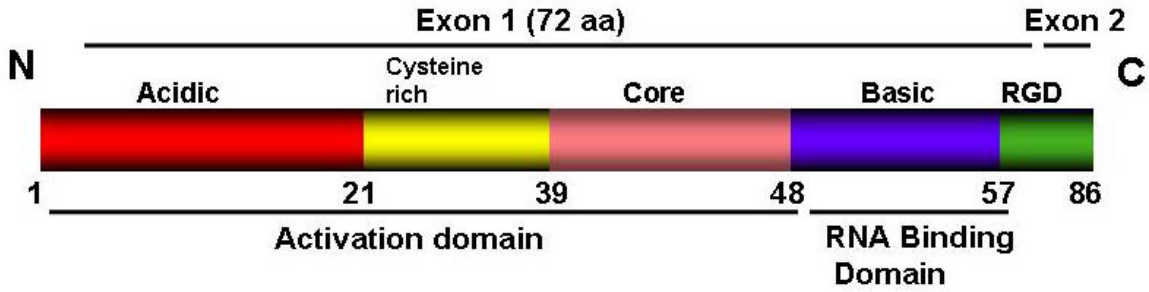

FIGURE 5. **The domains of HIV-1 Tat.** HIV-1 Tat protein is divided into the following domains: acidic, cysteine-rich, core, basic, and C-terminal glutamine-rich domain. The first three domains make up the "Activation Domain" which is absolutely required for the activity of Tat. The basic domain is involved in binding to the TAR element of the HIV RNA, and in binding to certain transcription factors like p300.

The cysteine domain has been a major obstacle in obtaining a three-dimensional crystal structure of HIV Tat. Recently, the crystal structure for HIV Tat complexed with human P-TEFb was resolved (2.1 Å resolution) by Tahirov *et al* (Tahirov *et al.* 2010). The crystal structure shows that the first 49 residues of Tat, collectively called the activation domain (AD), adopt a structure complementary to the surface of P-TEFb, interacting

extensively with both cyclin T1 (88% of the interacting Tat surface) and CDK9 (12% of the interacting Tat surface). Thus, Tat is known to be an intrinsically unfolded protein, which adapts to the structure of its binding partners to adopt a conformation to enable its interaction with host proteins (Shojania *et al.* 2006, Tahirov *et al.* 2010).

Tat undergoes several post-translational modifications in order to function efficiently. Some modifications like methylation of certain arginine or lysine residues interfere with the formation of the Tat/TAR/P-TEFb complex, thus turning Tat "off". On the other hand, modifications like phosphorylation, polyubiquitination, and acetylation are known to be critical in activating Tat or enhancing its function.

HIV-1 Tat is acetylated by enzymes called histone acetyltransferases (HATs, also called lysine acetyltransferases; KATs), which also modify histones. Tat is acetylated at Lys^{28}, located in the cysteine-rich motif; by PCAF (also called KAT2B) (Kiernan *et al.* 1999, Bres *et al.* 2002). This modification has been found to be critical for the binding of Tat to TAR and P-TEFb. Tat is also acetylated at Lys^{50} and Lys^{51} (in the ARM domain) by the enzymes p300/CBP (KAT3B) and GCN5 (KAT2A) (Kiernan *et al.* 1999, Ott *et al.* 1999, Col *et al.* 2001). Acetylation of Lys^{50} perturbs the electrostatic interactions between Tat and TAR, dissociating Tat from the TAR RNA and P-TEFb (Kaehlcke *et al.* 2003). This modification also unveils a new binding interface in Tat for transcriptional coactivators, such as PCAF and members of the chromatin remodeling SWI-SNF complex, leading to the next stages of transcription (Bres *et al.* 2002, Dorr *et al.* 2002, Mahmoudi *et al.* 2006).

In addition to facilitating its own acetylation, Tat also recruits histone-modifying enzymes, like p300/CBP, to the HIV promoter, which acetylates the nucleosomes on the promoter (Hottiger *et al.* 1998, Lusic *et al.* 2003, Marcello *et al.* 2003). Such a Tat-induced acetylation of the nucleosomes relieves the repression of the HIV LTR, enabling the RNA polymerase II to efficiently transcribe the HIV genome (Benkirane *et al.* 1998, Hottiger *et al.* 1998, Kiernan *et al.* 1999), forming elongated HIV RNA transcripts. Some studies have also shown that conformation of p300/CBP changes upon binding to Tat, increasing its HAT activity (Deng *et al.* 2000, Deng *et al.* 2001). The recruitment of p300/CBP to the HIV promoter also assists in chromatin remodeling which allows the binding of basal transcription factors like the TATA-binding protein and TFIIB to the LTR (Easley *et al.* 2010).

HIV Infection

An HIV infection can be divided into three main stages:

Acute infection – This is the period following initial infection with the virus. Many patients display flu-like or mononucleosis-like symptoms in this period of infection. This period can last upto 12 weeks, and is associated with high levels of viremia (Clark *et al.* 1991, Daar *et al.* 1991). An immune response to HIV is mounted during this time leading to a decline in the viremia (Clark *et al.* 1991, Daar *et al.* 1991). But this response is rendered inadequate as the virus persists in the lymph nodes while HIV mRNA is almost undetectable in the peripheral blood immune cells (Michael *et al.* 1992).

Clinical latency – Following the initial response to HIV infection and apparent decline of viral load, most patients have a period of “clinical latency” which can last many years.

This term is misleading because there is no period where a true state of latency (Harper *et al.* 1986, Schnittman *et al.* 1989), i.e. no expression of viral mRNA and proteins, is achieved; although there may be no detectable viral mRNA in the infected individual. This period is marked by a gradual deterioration of the patients' immune system, quantifiable due the depletion of the $CD4^+$ T lymphocytes (Fauci 1988, Fauci *et al.* 1991). Thus, even during the so-called latent phase of the infection, the disease is steadily progressing.

Clinically apparent disease/ AIDS – AIDS is the outcome of the progressive deterioration of the immune system. AIDS is diagnosed when the $CD4^+$ T-cell titer decreases to 200 cells per µl and below, and the appearance of opportunistic infections (e.g. pneumonia) or viral induced cancers (e.g. Kaposi's sarcoma) (Fauci *et al.* 1991). In the absence of treatment, most patients develop AIDS within 10 years of infection. According to statistics from UNAIDS, as of 2010, approximately 34 million people (adults and children) worldwide were living with HIV, with over 2.7 million new infections in 2010. There were approximately 1.8 million deaths due to AIDS in 2010 alone (available at http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2011/jc2216_worldaidsday_report_2011_en.pdf).

Highly Active Antiretroviral Therapy (HAART)

A lot of research has been devoted to the discovery of drugs which could control HIV replication. Since the discovery of the first antiretroviral drug, AZT (zidovudine; 3'-azido-2',3'-dideoxythymidine), twenty-five anti-HIV drugs have been licensed by the FDA (Fig. 2, Table 1). These can be classified into seven different groups such as,

nucleoside reverse transcriptase inhibitors, nucleotide reverse transcriptase inhibitors (collectively called NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), fusion inhibitors (FIs), co-receptor inhibitors (CRIs), integrase inhibitors (INIs), and protease inhibitors (PIs). The common hindrance to anti-HIV monotherapy is the emergence of resistant strains of the virus, rendering the drug ineffective. Due to the low fidelity of HIV RT, the absence of a proofreading function, and the high rate of HIV replication in the host, the viral proteins targeted would acquire mutations, making them resistant to the respective anti-HIV drug. To counter this, a strategy called highly active antiretroviral therapy (HAART) was introduced about 17 years ago, in which drugs from different classes were used, in concert, to try and inhibit HIV at different stages of its life cycle. Typically, drugs from the NRTI, NNRTI and PI classes of anti-HIV drugs were used in the HAART regimen, with members of the other classes of anti-HIV drugs being added recently. Incorporation of HAART changed AIDS from being a fatal disease that caused certain death, to one which could now be reduced to a chronically manageable state. This means that patients have to continue adhering to the HAART regimen for their entire lives. Even with the incorporation of HAART, drug-resistant HIV virus particles are still isolated from patients, due to the high infidelity of the RT, coupled with the high replication rate of the virus. Furthermore, discontinuing the HAART regiment results in a relapse of HIV infection, even though virus levels would reduce to below detection limits (with standard tests) while on medication. One reason for discontinuation of the regimen (very frequently, a decision taken by patients without the physician’s approval) is the related toxicities associated with prolonged use of the antiretroviral drugs in the HAART

regimen. Increasingly, patients are looking towards alternative forms of therapy to fight off the virus while potentially reducing the adverse effects of the existing therapies.

Targeting HIV transcription as a form of anti-HIV therapy

Transcription elongation has been demonstrated to be a limiting step for HIV expression (Feinberg *et al.* 1991, Karn 1999). HIV Tat binds the RNA stem loop structure formed by TAR and by recruiting PTEFb to the LTR, enhances processive transcription. pTEFb, which is composed of CycT1 and CDK9, modifies RNA polymerase II (Pol II) activity by hyperphosphorylating the carboxy-terminal domain (CTD) of Pol II as well as negative elongation factors NELF and DSIF (Ivanov *et al.* 2000, Ping *et al.* 2001, Bourgeois *et al.* 2002, Ping *et al.* 2004). In the absence of Tat, transcription elongation by RNA Pol II from the HIV promoter is very inefficient leading to an accumulation of short transcripts (Kao *et al.* 1987, Marciniak *et al.* 1991). The accumulation of short transcripts and the identification of a site in this region, where purified Pol II strongly pauses elongation, have led to the speculation that transcription of the integrated provirus is repressed by premature termination (Feinberg *et al.* 1991, Kessler *et al.* 1991, Zhang *et al.* 2007). The activity of Tat substantially increases the production of long transcripts (Kao *et al.* 1987, Laspia *et al.* 1989, Feinberg *et al.* 1991). Furthermore, Tat recruits chromatin remodeling machinery to assure efficient transcription (Emiliani *et al.* 1996, Agbottah *et al.* 2006, Mahmoudi *et al.* 2006). Since Tat is necessary for HIV-1 transcription and replication, regulation of its activity would be novel and complementary to current anti-retroviral treatments targeting reverse transcriptase, integrase, and protease.

Complementary and Alternative Medicine (CAM) in HIV-AIDS

There is evidence from epidemiological studies, which have suggested a direct correlation between the severity of AIDS and micronutrient deficiencies. In particular, deficiencies in antioxidants during HIV infection facilitate the development of oxidative stress and, thus, may contribute to immune dysregulation and exacerbated HIV replication. Many studies have shown that optimal nutrition can help boost immune function, maximize effectiveness of antiretroviral therapy, and contribute to a better life. HIV-infected individuals are prone to malnutrition due to increased energy requirements, enteropathy, and increased catabolism (Khalili *et al.* 2008). Although the introduction of highly active antiretroviral (HAART) therapy has increased the quality of life and longevity, current studies indicate that many CAM interventions, which have gained popularity, could improve the quality of life of people living with HIV-AIDS (Power *et al.* 2002). CAM therapies such as acupuncture, massage, meditation/yoga, herbal medicine, and dietary supplements are commonly used with mixed success. Of these, the use of herbal medicine and antioxidant micronutrients has increased in the last few years among the HIV-infected population. Antioxidant micronutrients, such as selenium, have largely been effective as adjuvants in majority of the studies (reviewed in (Stone *et al.* 2010)). However, the mechanism underlying the antiretroviral properties of selenium is still unclear; while the antiretroviral effects of botanicals are relatively better understood.

Selenium in health and disease

Selenium is a nutritionally essential trace element that is important for optimal immune system function. Selenium deficiency has been associated with several pathological

conditions, including cardiovascular abnormalities as in the case of Keshan disease in China, rheumatoid arthritis, HIV/AIDS, and most notably cancer (Rayman 2000). In particular, cigarette smokers (Preston 1991) and HIV/AIDS positive individuals (Kassu *et al.* 2008), have significantly reduced plasma selenium levels. In fact, an investigation of the causal relationship between selenium in forage-crops and county levels of cancer mortality in the USA and cancer mortality rates for the major cancer sites were found to be higher in low selenium counties (Clark *et al.* 1991)**.** Selenium intakes may be suboptimal with respect to disease risk, notably in population of adults in the UK, Europe, China, and New Zealand. Based on several studies, the Institute of Medicine of the National Academy of Sciences (USA), has recommended a daily intake of 55 μg/day, with a tolerable upper daily intake of 400 μg/day (Antioxidants *et al.* 2000). There is a growing body of evidence suggesting that intakes of selenium above the normal nutritional range may be beneficial (Rayman 2000). However, at high concentrations (>600-800 μg/day), symptoms of selenium toxicity (selenosis) in the form of discoloration of skin, blotchy nails, nausea, vomiting, and diarrhea has been reported (Fan *et al.* 1990).

HIV and selenium deficiency

While general micronutrient deficiencies may contribute to HIV/AIDS, selenium deficiency has been strongly and independently associated with disease progression and mortality in HIV infected individuals (Baum *et al.* 1997, Kupka *et al.* 2004, Kupka *et al.* 2005). Kupka *et al* demonstrated that selenium-deficiency was associated with increased infectivity of HIV (Kupka *et al.* 2005). This observation is corroborated by two large trials in Kenya and Tanzania showing that selenium deficiency correlated with increased

genital shedding of HIV DNA and increased risks of fetal and child mortality as well as intrapartum mother-to-child transmission (Kupka *et al.* 2004, Kupka *et al.* 2005, Kupka *et al.* 2005). In a longitudinal study which employed multivariate statistical models that included $CD4^+$ T-cell counts, protein status, deficiency of vitamins A and B12, and zinc, selenium-deficiency was independently related to mortality and disease progression (Baum *et al.* 2006). A much debated report from Hurwitz *et al* suggested that daily selenium supplementation suppresses the progression of HIV-1 viral burden and provides improvement of $CD4^+$ T-count supporting the use of selenium as a simple, inexpensive, and safe adjunct therapy in HIV spectrum disease (Hurwitz *et al.* 2007). The beneficial therapeutic effects for maintaining optimal selenium status in HIV-1 infected populations are also supported by a 30-month double-blind trial with 186 HIV positive drug users in Florida, which demonstrated that supplementation with 200 μg of selenium yeast slowed disease progression, increased $CD4^+$ counts, increased vigor (Shor-Posner *et al.* 2003), decreased anxiety, reduced the need for hospitalization, and lowered the cost of health care when compared to those receiving a placebo (Burbano *et al.* 2002). Similar increases in $CD4^+$ and $CD8^+$ T-cell counts were observed with short-term supplementation with 200 μg selenium for 6 weeks in a cohort of 400 HIV-infected women in Kenya (McClelland *et al.* 2004). In addition, selenium treatment decreased oxidative stress in HIV-infected people positively impacting patient immune function and health (McDermid *et al.* 2002). Ogunro *et al* also demonstrated a significant decrease in plasma levels of selenium accompanied by a decrease in erythrocyte selenoenzyme glutathione peroxidase-1 (GPX1) activity in HIV-infected population (Ogunro *et al.* 2006). Taken together, the findings from selenium supplementation trials suggest that administration of

selenium to HIV-1 seropositive individuals at nutritional levels (50-200 μg/day) is an effective method to retard progression of AIDS, reduce morbidity, and enhance survival by stimulating the immune system.

Although there are epidemiological data suggesting the beneficial role of selenium in HIV infection, the mechanism of action of selenium is still not understood. While the general thought is that selenium supplementation negatively impacts HIV infection by elevating the antioxidant protection by GPX and other selenoenzymes, it is possible that selenium directly impacts HIV replication by influencing different steps in the virus life cycle including virus entry, establishment of HIV provirus, transcription, packaging and release of the virus. Dr. Taylor's group has suggested that HIV-1 encodes a sequence overlapping env gp41 with high similarity to selenium-GPXs to deprive HIV-infected individuals of selenium (Zhao *et al.* 2000). On the other hand, several viruses, including HIV and influenza, induce an imbalance of intracellular redox state toward pro-oxidant conditions. Such imbalances contribute both to virus replication and to the pathogenesis of virus-induced disease. It is believed that small changes in intracellular redox state can regulate cellular signaling pathways that favor virus replication, and that this can be inhibited via the use of antioxidants (Garaci *et al.* 1997, Nencioni *et al.* 2007, Nencioni *et al.* 2011).

Selenoprotein biosynthesis

Selenium is co-translationally inserted into proteins as selenocysteine (Sec), now widely accepted as the 21st amino acid. Sec is synthesized on its own tRNA, tRNA$^{[Ser]Sec}$ (Lee *et al.* 1989, Leinfelder *et al.* 1989), and is encoded by the stop codon, UGA, which

generally functions in translation termination (Hatfield *et al.* 1970, Diamond *et al.* 1981, Hatfield *et al.* 1982). Thus, decoding the UGA codon to facilitate the insertion of Sec requires unique machinery (Fig. 6). The main components in eukaryotic Sec biosynthesis, besides $tRNA^{Ser[Sec]}$, are selenophosphate synthetase 2 (SPS2), phosphoseryl-$tRNA^{Ser[Sec]}$ kinase, and selenocysteine synthase (SecS). Briefly, $tRNA^{Ser[Sec]}$ is initially aminoacylated with serine to form seryl-$tRNA^{Ser[Sec]}$ (Lee *et al.* 1989, Leinfelder *et al.* 1989). This is then phosphorylated by phosphoseryl-$tRNA^{Ser[Sec]}$ kinase, in the presence of ATP and Mg^{2+}, to form *O*-phosphoseryl-$tRNA^{Ser[Sec]}$ (Carlson *et al.* 2004). Simultaneously, selenide (reduced from dietary selenium) is converted to monoselenophosphate by the action of SPS2 (Kim *et al.* 1995, Low *et al.* 1995, Guimaraes *et al.* 1996, Kim *et al.* 1997, Kim *et al.* 1999). *O*-phosphoseryl-$tRNA^{Ser[Sec]}$ is dephosphorylated by SecS, followed by the transfer of the monoselenophosphate onto the tRNA (carried out by the same enzyme) to now form selenocysteyl-$tRNA^{Ser[Sec]}$, completing Sec biosynthesis (Lee *et al.* 1989, Leinfelder *et al.* 1989).

The next step is the incorporation of Sec into the nascent polypeptide during translation. In addition to selenocysteyl-$tRNA^{[Ser]Sec}$ and the in-frame UGA codon in the selenoprotein mRNA transcript, other factors are required for insertion of Sec to protein, such as the *cis*-acting Sec insertion sequence (SECIS) element, the SECIS-binding protein 2 (SBP2), the Sec-specific elongation factor (EFsec), and the ribosomal protein L30 (Low *et al.* 1996, Copeland *et al.* 2000, Fagegaltier *et al.* 2000, Tujebajeva *et al.* 2000, Hatfield *et al.* 2002, Chavatte *et al.* 2005). SECIS elements are highly conserved stem-loop structures present in the 3’ untranslated regions (3’-UTRs) of all eukaryotic selenoprotein genes. SBP2 binds to the SECIS element to form a stable SBP2-SECIS

complex, thus directing the decoding of the UGA codon to insert Sec into the growing polypeptide. SBP2 also binds EFsec, which functions to specifically recruit selenocysteyl-tRNA$^{[Ser]Sec}$ and insert Sec into nascent polypeptides. Finally, L30 is known to trigger the conformational transition of the SECIS element from an open to kinked form by competing with SBP2, suggesting that L30 and SBP2 act sequentially during UGA recoding to recruit EFsec and deliver selenocysteyl-tRNA$^{[Ser]Sec}$ to the ribosomal 'A' site during translation of selenoproteins (Chavatte *et al.* 2005), thus facilitating the release of SBP2. Recently, two additional proteins, SECp43 and SLA (soluble liver antigen), have been shown to play a role in selenoprotein biosynthesis by interacting with the selenocysteyl-tRNA$^{[Ser]Sec}$ complex and help in its shuttling across the nuclear membrane (Xu *et al.* 2005).

Due to the presence of Sec, a superior nucleophile with a better leaving group ability relative to Cys because of a significantly lower pKa (5.2 vs. 8.3) (Hondal *et al.* 2011), in selenoproteins, they act as excellent antioxidants and function to relieve oxidative stress in biological systems. Of particular interest to our group is the ability of selenoproteins to influence the production of certain fatty acid molecules, which are known to have anti-inflammatory and anti-proliferative effects as described in the next section.

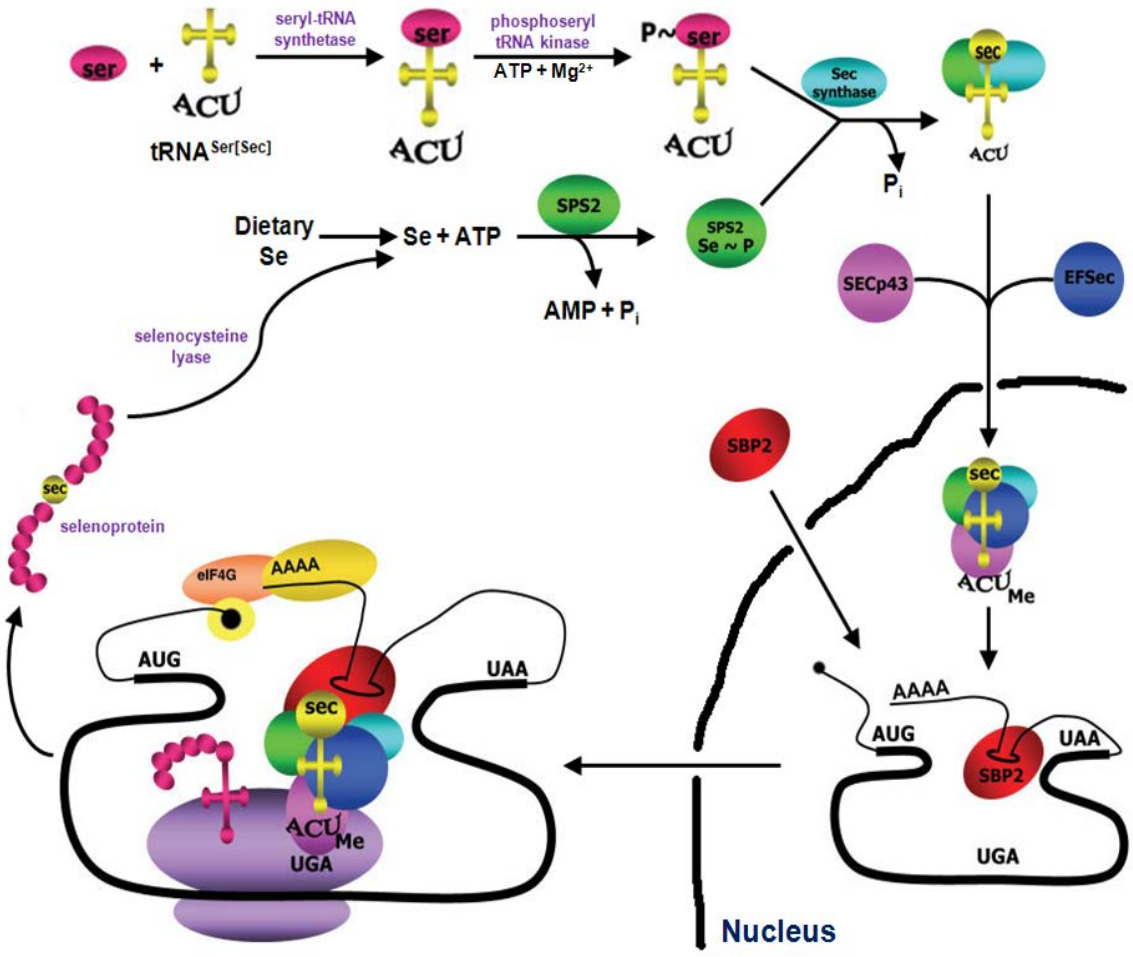

FIGURE 6. **Selenoprotein biosynthesis.** A schematic representation of the mechanism of selenocysteine biosynthesis and incorporation into selenoproteins. Dietary selenium is converted to monoselenophosphate by the action of SPS2 and is added onto the phosphoseryl-tRNA$^{Ser[Sec]}$ by SecS to form selenocysteyl-tRNA$^{Ser[Sec]}$. Recruitment of the selenocysteyl-tRNA$^{Ser[sec]}$ to the UGA codon in the mRNA occurs with the help of factors such as SBP2, SECp43 and EFSec, leading to the subsequent incorporation of selenocysteine into the growing polypeptide chain. (Adapted from Small-Howard *et al.* 2006, Squires *et al.* 2008)

Cyclopentenone prostaglandins, selenium, and HIV replication

Cyclopentenone prostaglandins (CyPGs) are derived from membrane fatty acids, mostly arachidonic acid (AA, Fig. 10). AA released by phospholipases undergoes a two-step reaction involving cyclooxygenation and peroxidation, which are catalyzed by the cyclooxygenase (COX) enzymes; thus called the COX pathway. The product, PGH_2 can

then be transformed by various prostaglandin synthases, generating PGE_2 [by the action of prostaglandin E synthases, microsomal PGE_2 synthases-1 and -2 (mPGES-1/2)] and PGD_2 (by the action of prostaglandin D synthases (PGDS), lipocalin-type (L-PGDS) and hematopoietic-type (H-PGDS)], in addition to other prostanoids. CyPGs are formed through dehydration reactions within the cyclopentane ring of the PGs derived from PGH_2. Dehydration of PGD_2 yields the CyPG PGJ_2 (Δ^{13}-PGJ_2) (Narumiya *et al.* 1985, Shibata *et al.* 2002). PGJ_2 can further spontaneously dehydrate to yield the CyPG 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), or can transform into Δ^{12}-PGJ_2 in an albumin-dependent manner (Shibata *et al.* 2002).

Recently, Gandhi *et al* have reported a positive correlation between selenium supplementation, and the expression of a critical enzyme in the prostaglandin (PG) biosynthesis pathway (Fig. 7), H-PGDS, *in vitro* and *ex vivo* in murine macrophages, and also in a murine leukemia model (Gandhi *et al.* 2011). Supplementation with selenium has been shown to shunt macrophage maturation from the classical pathway (leading to a M1, inflammatory phenotype) to the alternative pathway (leading to a M2, anti-inflammatory phenotype) (Nelson *et al.* 2011). This results in a shift in prostaglandin production from the pro-inflammatory PGE_2 to the anti-inflammatory CyPGs, Δ^{12}-PGJ_2 and 15d-PGJ_2 (Fig. 7) (Gandhi *et al.* 2011).

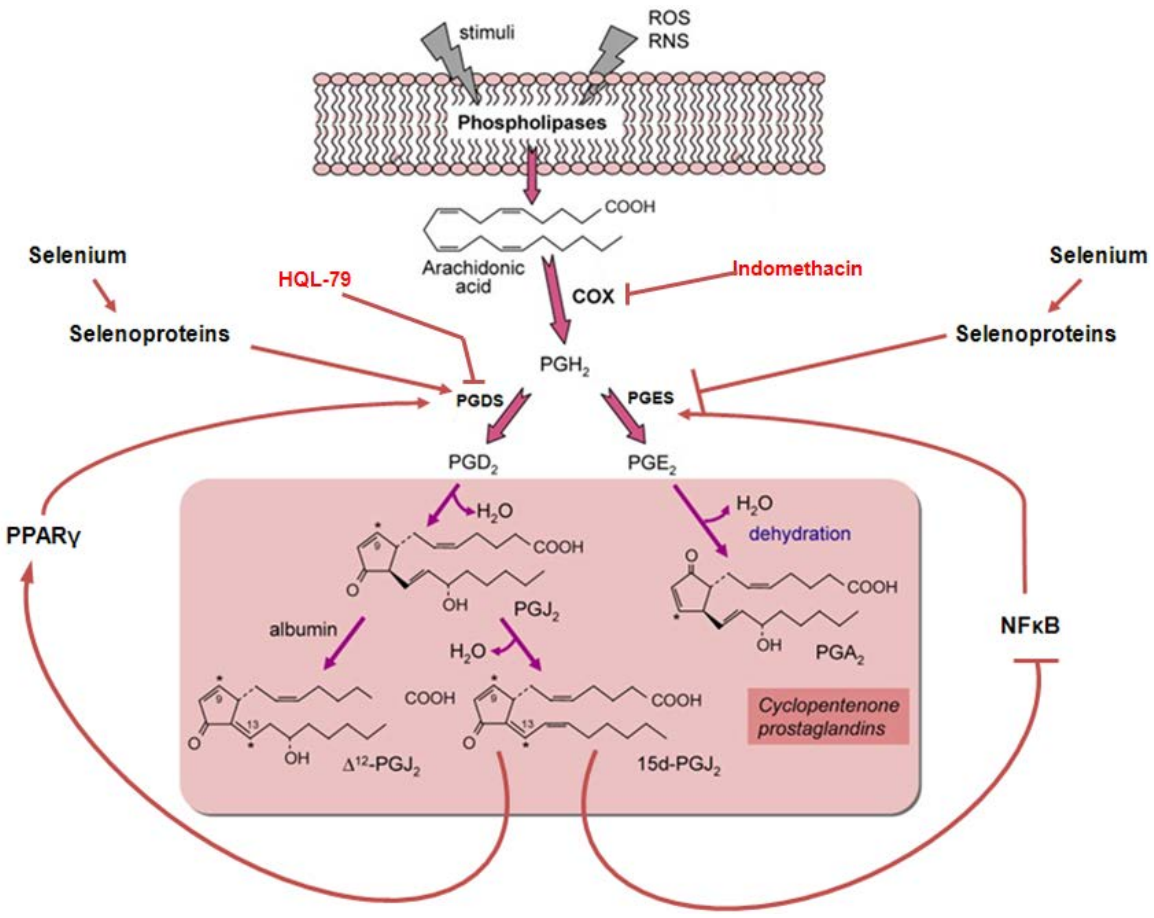

FIGURE 7. **The Arachidonic acid-Cyclooxygenase pathway.** A representation of the AA-COX pathway leading to the production of different prostaglandins. Following an inflammatory stimulus, AA from the cell membrane is acted upon by COX enzymes to form PGH_2, which is in turn converted into PGD_2 and PGE_2 due to the action of PGDS or PGES respectively. PGD_2 can get spontaneously dehydrated to CyPGs. Selenium, via selenoproteins, can shunt the pathway towards the preferential production of the anti-inflammatory CyPGs, by inhibiting the PGES arm of the pathway, while enhancing the PGDS arm of the pathway at the same time. Use of inhibitors against the COX or PGDS enzymes negates the effect of selenium. (Adapted from Gandhi *et al.* 2011, Garzon *et al.* 2011)

Cyclopentenone PGs contain an electrophilic carbon in the cyclopentenone ring, which allows these molecules to form Michael adducts with cellular nucleophiles such as glutathione (GSH) or nucleophilic residues in cellular proteins, like cysteine (Cernuda-Morollon *et al.* 2001, Shibata *et al.* 2003, Palempalli *et al.* 2009) or histidine (Yamaguchi *et al.* 2010). The reactions of CyPG with thiols have been studied in detail for GSH and

some cysteine derivatives (Suzuki *et al.* 1997, Bickley *et al.* 2004). In the case of GSH it has been observed that though adduct formation is reversible, there are important differences in kinetics and reversibility of adduct formation with CyPGs of different structures. As for immobilized thiol groups or those present in proteins, adducts with CyPG are generally considered irreversible under physiological conditions.

Depending on the degree of unsaturation and the constituents of their lateral chains, CyPGs display varied structural features. Unlike acrolein or 4-hydroxynonenal (HNE), and other electrophilic lipids of lower molecular mass, the complex structure of CyPGs either favors or hinders their interaction with proteins. Also, the presence of one or more electrophilic carbons in the PG structure may be an important determinant for selective CyPG-protein interaction. Thus, the inter- and intra-molecular selectivity of protein modification by these electrophilic lipids may be determined by the structural features of the target protein and of the CyPG (Diez-Dacal *et al.* 2010), and also the redox status of the cell type in question.

Cyclopentenone PGs are known to have anti-proliferative and pro-apoptotic effects in several tumor cell lines and *in vivo* (Tsubouchi *et al.* 2000, Kondo *et al.* 2002, Shimada *et al.* 2002, Nencioni *et al.* 2003, Pignatelli *et al.* 2005, Ciucci *et al.* 2006, Sanchez-Gomez *et al.* 2010), and thus have generated interest as potential antineoplasic agents. Their anti-proliferative effects can be mediated by several different mechanisms like the blockade of the cell cycle progression (Kim *et al.* 1993, Kamagata *et al.* 2007), activation of the mitochondrial apoptotic pathway (Nencioni *et al.* 2003), induction of oxidative stress (Kondo *et al.* 2001), cytoskeletal disruption (Gayarre *et al.* 2006, Stamatakis *et al.* 2006, Aldini *et al.* 2007), and activation of the mitogen activated protein kinase (MAPK)

cascades (Shan *et al.* 2004, Moriai *et al.* 2009), or by downregulation of the human telomerase reverse transcriptase (hTERT) (Moriai *et al.* 2009). Recently, our group has characterized a novel CyPG, Δ^{12}-PGJ_3, formed by the activity of the COX and H-PGDS enzymes on the ω-3 fatty acid, eicosapentanoic acid (EPA). This compound has been shown to cause the apoptosis of leukemic stem cells (LSCs) in mouse models of acute and chronic leukemia (Hegde *et al.* 2011).

The antiviral effects of CyPGs have also been established in literature. Dr. Gabi Santoro's group has shown that the antiviral effects of CyPGs correlated with the induction of stress response, the expression of cytoprotective heat shock proteins like Hsp70, and with the inhibition of factors important for viral replication, like NF-κB (Santoro 1997). Moreover, the interference with virus protein glycosylation and the direct binding to viral proteins (Rossi *et al.* 1997, Kalantari *et al.* 2009) may contribute to the inhibition of the replication of a wide variety of RNA and DNA viruses (Rossi *et al.* 1997, Santoro 1997, Kalantari *et al.* 2009). Rozera *et al* have clearly demonstrated the anti-viral effect of the cyclopentenone prostaglandins (CyPGs), 15d-PGJ_2 and PGA_1 (Fig. 23A), which dramatically suppressed HIV-1 replication in a T-lymphocyte cell line during acute infection, likely through a transcriptional block (Rozera *et al.* 1996). Our work, studying the modulation of HIV replication by 15d-PGJ_2, corroborates their observations and also delineates a mechanism by which a transcriptional block in the HIV life cycle may be established.

There exist many small molecules of natural origin which have a similar α,β-unsaturation in their molecular structure, and are known to have anti-inflammatory and, in some cases, anti-HIV activity. Indeed, we have previously shown that naturally occurring MAEs may

modify certain Cys-thiols in proteins to regulate their function (Palempalli *et al.* 2009). Given the abundance of such natural compounds, it seems prudent to explore their activity against HIV further.

Anti-retroviral activities of botanicals

Since HIV was first discovered, several drugs have entered the market as anti-HIV agents. Although successful, most of the drugs have associated toxicities and serious side effects such as rash, hypertension, fatigue, anemia, granulocytopenia, pancreatitis, and peripheral neuropathy (Cuzin *et al.* 2008). Thus, there is an urgent need to discover new ways to manage the disease using highly effective CAM approaches. The diversity of the plant kingdom has proven to be an excellent source of novel entities with anti-retroviral properties (Singh 2005). The number of compounds, such as alkaloids, coumarins, flavonoids, lignans, phenolics, quinones, and terpenes, isolated from plant sources exhibiting anti-HIV activity is rising steadily (Asres *et al.* 2005, Singh 2005). Many of these compounds have anti-HIV replication (Fujioka *et al.* 1994), integrase, and protease activities in addition to inhibiting viral entry (Robinson *et al.* 1996, Xu 1996, Kim *et al.* 1999, Tewtrakul *et al.* 2003, Cos *et al.* 2004, Notka *et al.* 2004, Asres *et al.* 2005, Pommier *et al.* 2005). The compounds from the above mentioned classes, being antioxidants, also target the NF-κB pathway. Although NF-κB inhibitors may prevent the early transcriptional initiation of the provirus, it is important to note that the antiretroviral effects of these compounds stem from their ability to target other HIV-1 proteins. A classic example is that of ascorbate and curcumin. Drs. Jariwalla and Linus Pauling have demonstrated the anti-HIV properties of ascorbate to be independent of the NF-κB pathway (Harakeh *et al.* 1990). Curcumin has also been shown to exert antiretroviral

effects via integrase-mediated effects, but with an IC_{50} of ~ 40 μM (Barthelemy *et al.* 1998). Thus, it may be beneficial to have an antioxidant botanical with multiple viral targets, as long as it is inhibitory without any effect on the host cell viability. Botanicals have been investigated for their anti-viral activities by targeting many viral proteins and processes, in addition to the NF-κB pathway. Of these, a subset of compounds with a common structural entity in the form of an α,β-unsaturated carbonyl "enone" function (Fig. 23B), display the property to target NF-κB signaling pathway. Examples such as curcumin (*Curcuma longa*), gambogic acid (*Garcinia hanburyi*), celastrol (*Tripterygium wilfordii*), and rosmarinic acid *(Coleus parvifolius*) are known to inhibit NF-κB, upregulate a battery of antioxidant genes, and most importantly, act on various HIV targets such as integrase, reverse transcriptase, and protease (Barthelemy *et al.* 1998, Asres *et al.* 2005). Surprisingly, there is no report on the ability of these compounds to modulate Tat-function, particularly given their ability to modify protein thiols such as those present in Tat.

Research hypotheses

The use of complementary and alternative medicine (CAM), in the form of antioxidant botanicals and micronutrients, is prevalent among HIV-positive individuals worldwide. Narrow specificity, drug resistance, and adverse side effects of currently approved anti-HIV drugs has turned many towards natural sources as a potential CAM approach to combat HIV/AIDS. Epidemiological studies have suggested a direct correlation between the severity of AIDS and micronutrient deficiencies. In particular, deficiencies in antioxidants during HIV infection facilitate the development of oxidative stress and, thus, may contribute to immune dysregulation and exacerbated HIV replication. Selenium, which exerts antioxidant activity through insertion into selenoproteins, is significantly lowered in the plasma of HIV-infected population, particularly in sub-Saharan Africa. selenium supplementation trials have demonstrated benefits including reduced risk of mortality, increased $CD4^+$ T-lymphocyte counts, and decreased hospitalization suggesting an intriguing CAM strategy for HIV/AIDS. It is believed that in virus-infected cells, selenium abrogates increased activation of the redox-sensitive transcription factor, nuclear factor-κB (NF-κB), which is pivotal for the increased transcription of HIV. Previous studies have also demonstrated that during HIV infection, the cellular levels of thioredoxin reductases (TRs), a selenium-containing pyridine nucleotide-disulfide oxidoreductase, which reduces protein disulfides to free thiols, is dramatically decreased. Based on the preliminary results, we believe that cytosolic TR (TR1) plays an important role in modulating the redox status of the key HIV-1 transactivator protein Tat. Tat is an essential viral protein with seven of the four cysteine (Cys) amino acids in the form of two intramolecular disulfide bonds that are absolutely required for its transactivating

function. We hypothesize that **selenium, by activating TR1, reduces the disulfide bonds required for Tat activity, thus repressing HIV-1 transcription and replication.** Given the ability of such reduced Cys thiols in Tat to oxidize and form disulfide bonds, several electrophilic compounds of natural origin with a conserved α,β-unsaturated enone moiety could prevent the redox cycling by interacting with the free thiols in Tat. Thus, an additional hypothesis is that **interaction of Tat with α,β-unsaturated enone electrophiles of natural (or endogenous) origin leads to functional inactivation of Tat by covalent modification of thiol residues and that the modification is further augmented by TR1-dependent reduction of Tat such that it prevents redox cycling of Tat.**

Another critical checkpoint during HIV viral transcription is the remodeling of the chromatin structure at the promoter sites of the integrated provirus. Data from our laboratory suggest that CyPGs can modulate the activity of the histone acetyltransferase KAT3B (p300), the dysregulation of which has been implicated in AIDS, cancer and cardiovascular diseases, among others. KAT3B is also essential for the activity of Tat. The active site of KAT3B has an essential thiol residue (Cys^{1438}), which could be a target for modification by CyPGs. Thus, we hypothesize that **selenium supplementation, via the upregulation of the CyPG Δ^{12}-PGJ_2, inhibits the activity of KAT3B by affecting the covalent modification of its critical Cys^{1438} residue.** We have explored the role of selenium supplementation in the modulation of KAT3B activity in a chronic HIV infection model.

Chapter 2: Effect of Selenium supplementation on HIV transcription and replication in human macrophages - targeting of thiol disulfides in Tat by thioredoxin reductase-1

The data presented in this chapter has been published in –

Parisa Kalantari, Vivek Narayan, Sathish K. Natarajan, Kambadur Muralidhar, Ujjawal H. Gandhi, Hema Vunta, Andrew J. Henderson, and K. Sandeep Prabhu. **Thioredoxin Reductase-1 Negatively Regulates HIV-1 Transactivating Protein Tat-dependent Transcription in Human Macrophages.** *The Journal of Biological Chemistry* 283(48):33183–33190, November 28, 2008 © the American Society for Biochemistry and Molecular Biology.

(Contributions: The work with the HIV virus in this study was performed by Dr. Parisa Kalantari. Vivek Narayan and Dr. Parisa Kalantari performed the luciferase assays. Vivek Narayan performed the redox western blot. Dr. Sathish Natarajan performed the mechanism-based affinity assay. Dr. Kambadur Muralidhar determined the free thiols in native and reduced rTat.)

Abstract

Epidemiological studies suggest a correlation between severity of acquired immunodeficiency syndrome (AIDS) and selenium deficiency, indicating a protective role for this anti-oxidant during HIV infection. Here we demonstrate that thioredoxin reductase-1 (TR1), a selenium-containing pyridine nucleotide-disulfide oxidoreductase that reduces protein disulfides to free thiols, negatively regulates the activity of the HIV-1

encoded transcriptional activator, Tat, in human macrophages. We used a small interfering RNA approach as well as a high affinity substrate of TR1, ebselen, to demonstrate that Tat-dependent transcription and HIV-1 replication were significantly increased in human macrophages when TR1 activity was reduced. The increase in HIV-1 replication in TR1 small interfering RNA-treated cells was independent of the redox-sensitive transcription factor, NF-κB. These studies indicate that TR-1 acts as a negative regulator of Tat-dependent transcription. Furthermore, *in vitro* biochemical assays with recombinant Tat protein confirmed that TR1 targets two disulfide bonds within the Cys-rich motif required for efficient HIV-1 transactivation. Increasing TR1 expression along with other selenoproteins by supplementing with selenium suggests a potential inexpensive adjuvant therapy for HIV/AIDS patients.

Introduction

The physiological functions of the micronutrient selenium are mediated through a co-translational insertion of selenocysteine (Sec), into specific proteins (Lee *et al.* 1989, Leinfelder *et al.* 1989, Stadtman 1990, Mustacich *et al.* 2000). Commonly studied selenoproteins such as the glutathione peroxidases, thioredoxin reductases (TR), deiodinases, and other selenoproteins share a redox mechanism that is driven by Sec present in the active site making selenium a required vital nutrient. As a specific example, selenium deficiency has been strongly and independently associated with disease progression and mortality in HIV-infected individuals, and increased risks of fetal and child mortality as well as intrapartum mother-to-child transmission (Baum *et al.* 1997, Kupka *et al.* 2004, Kupka *et al.* 2005, Kupka *et al.* 2005, Kaiser *et al.* 2006, Durosinmi *et al.* 2008, Kupka *et al.* 2008, Kupka *et al.* 2009). Studies have demonstrated that daily

selenium supplementation of HIV-1 positive individuals suppresses the progression of HIV-1 viral burden, provides improvement of CD4 counts (Hurwitz *et al.* 2007), increases vigor (Shor-Posner *et al.* 2003), decreases anxiety, and reduces the need for hospitalization when compared with those receiving a placebo (Burbano *et al.* 2002, McDermid *et al.* 2002).

HIV-1 infection alters the expression of selenoproteins, including TR (Gladyshev *et al.* 1999). TRs are homodimeric enzymes belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases that includes lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase (Arner *et al.* 2000, Becker *et al.* 2000, Mustacich *et al.* 2000). Each monomer of TR includes a FAD prosthetic group, and a NADPH binding site. Mammalian TR contains an active site motif, namely Cys^{59}-Val-Asn-Val-Gly-Cys^{64}, similar to glutathione reductase (Karplus *et al.* 1987, Gasdaska *et al.* 1995, Zhong *et al.* 1998). The C-terminal end of mammalian TRs contains a penultimate Sec residue within the sequence Gly^{496}-Cys-Sec-Gly^{499}, and forms the selenenylsulfide active site which is conserved in mammalian TRs (Gladyshev *et al.* 1996, Tamura *et al.* 1996, Zhong *et al.* 1998, Gasdaska *et al.* 1999, Lee *et al.* 1999, Miranda-Vizuete *et al.* 1999, Zhong *et al.* 2000). Removal of the Sec residue results in total loss of the activity of TR (Zhong *et al.* 1998). The mechanism of the reaction of TR with its natural substrate thioredoxin (Trx) is illustrated in Fig. 8. The two subunits of the homodimeric TR are arranged in a head to tail fashion, positioning Sec^{498} and Cys^{497} of one molecule adjacent to Cys^{59} and Cys^{64} of the other molecule (Fig. 9) (Zhong *et al.* 2000). The oxidized enzyme (Fig. 8, A) is first reduced by a moiety of NADPH to give rise to a reduced enzyme (EH_2) species with a $FADH_2$ (Fig. 8, B). This $FADH_2$ forms a charge-transfer complex with Cys^{64} in the same

subunit (Fig. 8, C). Next, this motif is believed to transfer electrons to the C-terminal selenenylsulfide of the second subunit in the dimer, which is now reduced to a selenolthiol moiety (Fig. 8, D). The reduction of this species by another equivalent of NADPH gives rise to the reduced form of the enzyme (Fig. 8, F) which is the actual state of TR (EH_4) where it reduces its substrate Trx. This form of TR has a thiolate-FAD

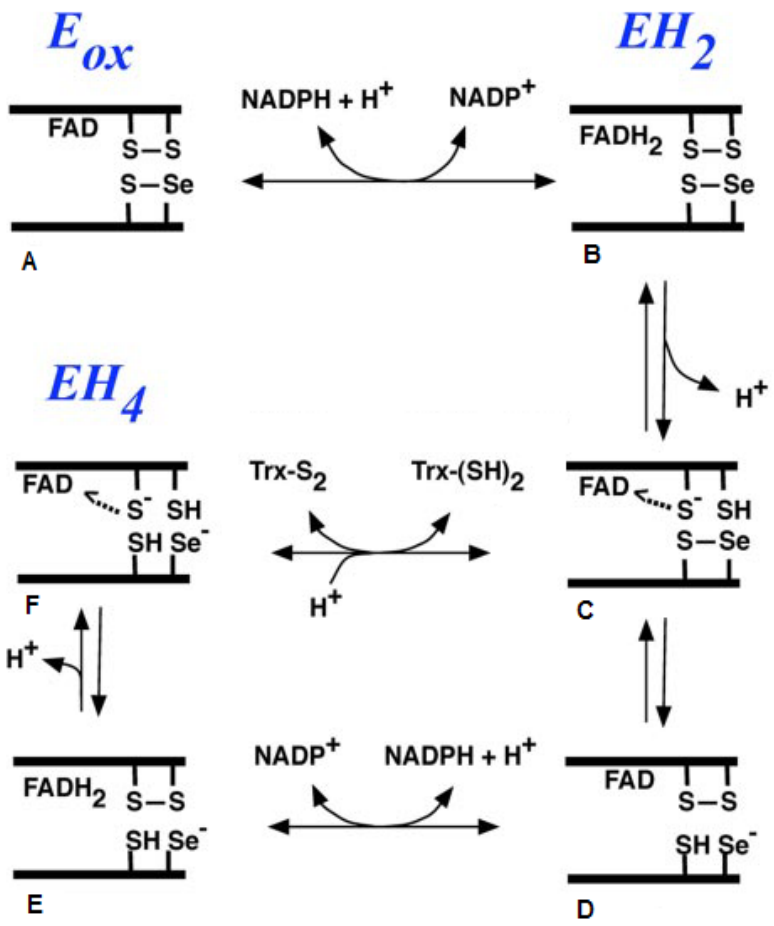

FIGURE 8. **Mechanism of the reduction of Trx by TR.** This scheme depicts one of the two active sites of the TR1 homodimer. The oxidized enzyme (E_{ox}) is reduced by NADPH to the two-electron reduced enzyme (EH_2). The charge-transfer complex is denoted by a dashed arrow in (C)**.** A second equivalent of NADPH reduces EH_2 to the four-electron reduced EH_4 form. The EH_4 form reduces $Trx\text{-}S_2$ to $Trx\text{-}(SH)_2$, and returns to the EH_2 form. (Adapted from Cheng *et al.* 2009)

charge-transfer complex concomitant with the selenolthiol at the C-terminus. The enzyme returns to the EH_2 form after reducing Trx. Reduced Trx in turn catalyzes thiol-disulfide exchange to reduce key Cys residues in transcription factors (Arner *et al.* 2000). Extracellular Trx inhibits the production of HIV-1, whereas its degradation product, eosinophil cytotoxicity-enhancing factor, promotes HIV-1 production (Newman *et al.* 1994). Apart from Trx, cytosolic TR (TR1) exhibits broad substrate specificity reducing cytotoxic protein NK-lysin, tumor suppressor protein p53, and non-protein substrates such as lipoic acid, lipid hydroperoxides, vitamin K3, dehydroascorbic acid, and the ascorbyl free radical (Arner *et al.* 1996, Mustacich *et al.* 2000).

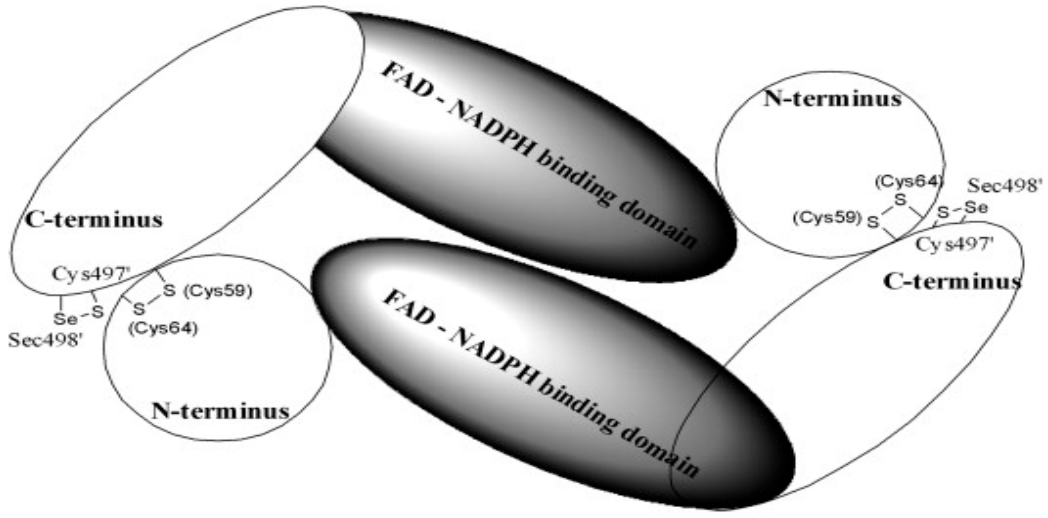

FIGURE 9. **The Thioredoxin Reductase homodimer.** The subunits of the TR1 homodimer are arranged in a head-to-tail fashion with Sec^{498} and Cys^{497} of one molecule adjacent to Cys^{59} and Cys^{64} of the other molecule. (Cai *et al.* 2012)

Tat is an HIV-encoded transactivating protein that is required for virus replication. Tat binds to a RNA stem loop structure (TAR) and recruits the positive transcription

elongation factor, P-TEFb, to the HIV long terminal repeat (LTR), increasing RNA polymerase II processivity (Herrmann *et al.* 1995, Bourgeois *et al.* 2002). In the absence of Tat, transcription is impaired; leading to the accumulation of prematurely terminated short transcripts (Kao *et al.* 1987, Marciniak *et al.* 1991). The primary structure of Tat is made up of 101 amino acids. Based on mutagenesis studies, the Cys-rich region (amino acids 20–39) has been demonstrated to be necessary for Tat activity (Frankel *et al.* 1988, Kuppuswamy *et al.* 1989). Furthermore, Koken *et al.* (Koken *et al.* 1994) have shown that the transcriptionally active form of Tat is a monomer, and reducing agents strongly inhibit Tat activity, suggesting that Cys residues in Tat form intramolecular disulfide bonds. Similarly, increased transactivation activity was seen in bacterially expressed Tat that was subjected to slow-protein refolding, which permits the formation of disulfide bonds (Kirsch *et al.* 1996). These data suggest that the activity of Tat is governed in part by Cys groups via the formation of disulfide bonds and that Tat is sensitive to the redox state of the cell. Here, we report that TR1 targets the disulfide bonds in Tat and functions as a negative regulator of Tat-dependent proviral transcription.

Materials and Methods

Cell Culture – The human U937 promonocytic cell line (ATCC, Manassas, VA) was cultured in RPMI 1640 medium supplemented with 5% defined fetal calf serum (Hyclone), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.2 M L-glutamine. Peripheral blood macrophages were isolated from whole blood obtained from healthy HIV-1-seronegative donors. Mononuclear cells were obtained by differential centrifugation using a Ficol/Hystopaque (Sigma-Aldrich) gradient and adherence to plastic culture flasks as previously described (Henderson *et al.* 1997). Macrophages were

separated from other cells by an initial adherence to plastic culture flasks overnight. After removing nonadherent cells, monocytes were cultured for 5–10 days to mature into monocyte-derived macrophages (MDM). All protocols were preapproved by the IRB at Penn State University. Fetal calf serum lots containing low selenium were selected for the study. Selenium was measured using atomic absorption spectrometry. The basal media (containing 7 nM selenium) was supplemented with selenium in the form of sodium selenite.

Expression and Activity of TR – U937 cells were cultured in basal media with or without exogenously added selenium for 1 week with three media changes in between. The washed cell pellets were lysed with mammalian protein extraction reagent (M-PER; Pierce) and clarified by centrifugation at 16,000*g* at 4 °C for 15 min. Cell lysates were used in immunoblot and enzymatic assays for TR. Assays for cytosolic TR activity in these lysates were performed as described by Holmgren and Bjornstedt (Holmgren *et al.* 1995), where recombinant Tat (rTat) was substituted for Trx in the assay. 0.1 μM purified rat liver TR1 from Sigma was used. Change in the absorbance at 314 nm was used to calculate the activity.

Transient Transfections, siRNA, and Luciferase Assay – Replication competent HXB.2, bacterial and mammalian HIV-1 Tat expression vectors, pTat86R His (Patki *et al.* 1996), and pCI-Tat1 (Frankel *et al.* 1988) were obtained from the AIDS Research Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. pCI vector (Promega, Madison, WI) was used as an empty vector control. LTR reporter constructs, LTR-Luc (wild-type -205 LTR), ΔTARLTR-Luc (-205 LTR lacking the TAR region), and ΔκBLTR-Luc (-205 LTR lacking the NF-κB binding sites) were prepared as

previously described (Henderson *et al.* 1995). DNA for transfections was prepared using plasmid purification systems from Marligen Biosciences (Ijamsville, MD) following protocols provided by the manufacturer. Transfection of U937 cells was performed using *Trans*IT-Jurkat transfection reagent from Mirus (Madison, WI). Transfection efficiency was assessed by co-transfecting pEGFP-N3 (Clontech, Palo Alto, CA) and monitoring enhanced green fluorescence protein expression by fluorescence microscopy. Cell viability was confirmed by trypan blue staining. siRNA for TR1 as well as siRNA control were reconstituted based on the manufacturer's guidelines (Dharmacon). Luciferase assays were performed using a commercial luciferase assay kit (Promega) and a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA). U937 cells were infected with HIV-Luc virus (1 X 10^6 infectious particles/1 X 10^6 cells). The next day cells were transfected with TR1 or control siRNA using the Amaxa nucleofector system, and luciferase activity was measured 72 h post-infection.

Generation of HIV Infectious Titers, Infections, and Transduction of Macrophages – Infectious HXB.2 virus was generated by cotransfecting 293T cells with 15 μg of HXB.2 cDNA, 3 μg of vesicular stomatitis virus-glycoprotein, and 3 μg of RSVRev by calcium phosphate transfection (Pear *et al.* 1993). Transfection efficiency was assessed by measuring p24 levels (p24 ELISA, PerkinElmer Life Sciences). We consistently generated titers of 1.0 X 10^6 infectious particles/ml. Supernatants were collected and filtered through 0.45 μm syringe filter (Whatman, Clifton, NJ). One ml of undiluted virus stock was added to 1.0 X 10^6 U937 cells or human MDMs (differentiated 10 days) for 24 h and then replaced with fresh media. Cells were then transfected with TR1 siRNA using the Amaxa nucleofector system and were subjected to luciferase assays 3–5 days post-

infection. For some experiments transfected cells were cultured in the presence or absence of ebselen (5 μM; Sigma) or DMSO. Supernatants were collected at 4 days post-infection and assayed for viral replication by p24 ELISA. To assess the effect of selenium supplementation on p24 levels, 1.0 X 10^6 U937 cells were cultured in a selenium-deficient media and infected with HXB.2 for 24 h followed by media changes containing selenium from 0.05 μM to 1 μM. After 3 days, the culture media supernatants were used to measure p24 levels.

Immunoblots – MDMs and U937 cells were washed twice with phosphate-buffered saline, and protein extracts were prepared by treating cells with M-PER reagent (Pierce) at 4 °C for 30 min. Lysates were mixed with 2X SDS loading buffer containing dithiothreitol and heated at 100 °C for 5 min before resolving by SDS-PAGE (%T = 4–20) followed by immunoblotting onto a nitrocellulose membrane. Rabbit polyclonal antibodies against TR1 and GPX1 (Abcam) were used to analyze expression of the two selenoproteins. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) was used as the secondary antibody. To normalize loading, membranes were treated with Western blot stripping buffer (Pierce) and reprobed with anti-β-actin or anti-glyceraldehyde-3-phosphate dehydrogenase followed by an appropriate horseradish peroxidase-conjugated secondary antibody.

Expression, Purification, and Refolding of rTat – rTat was expressed in *Escherichia coli* transformed with pTat86RHis as a His-tagged fusion protein using the autoinduction system (Novagen). The purification of rTat was performed using the method of Kirsch et al. (Kirsch *et al.* 1996) with some modifications. Briefly, the *E. coli* pellet from a 24-h culture was lysed in 6 M guanidine-HCl for 12 h. All steps were performed at 4 °C unless

otherwise mentioned. The cleared cell lysate was applied to a nickel-nitrilotriacetic acid column (Novagen) and developed as described by Kirsch *et al.* (Kirsch *et al.* 1996). The eluate was dialyzed against 0.1 N HCl for 2 days at 4 °C followed by an overnight lyophilization. The lyophilized powder was reconstituted in 6 M urea followed by reduction of the thiols with tris[2-carboxyethyl] phosphine (25 mol/mol of Tat) in the dark for 8 h at room temperature as per the instructions of the supplier (Pierce). The mixture was subjected to sequential dialysis in 0.1 M phosphate buffer (pH 6.3) containing 4 M urea, 2 M urea, and finally in 0.1 M phosphate buffer (pH 4.3) containing 200 mM NaCl. The dialysate was filtered through 0.45-μm filter and stored in small aliquots at -20 °C for further use. Typical yields from a liter of bacterial culture were ~10 mg of pure Tat, based on SDS-PAGE (>95% purity).

Analysis of rTat Activity – U937 cells were transfected with 2 μg of LTR-Luc or an irrelevant reporter plasmid (pSV2-Luc) as a control by transient transfection. Cells were then treated with 2 μg of rTat or buffer alone, and luciferase activity was measured 48 h post-transfection as an indication of proviral transcription. After transfections, trypan blue staining was performed to control for cell viability. Transfections were performed in triplicate, and the data are presented as the % of luciferase with luciferase activity in the cells lacking Tat set at 100%.

Quantifying Free Cys Thiols in rTat – Purified and refolded rTat, as described above, was used in the standard Ellman's reagent [DTNB; 5,5'-dithiobis(2-nitrobenzoic acid)] assay as described by the supplier (Pierce). The concentration of rTat was 2 μM, whereas the concentration of rat liver TR1 (Sigma) was 100 nM in the reaction mixture containing 0.008% bovine serum albumin, 4.3 mM EDTA, 0.43 mM NADPH in a total of 1 ml of

phosphate buffer (pH 7.0) at 37 °C. Additional controls included heat-denatured TR1 and TR1 without NADPH. Free thiol (SH) groups in rTat were estimated before and after reduction by TR, reaction with heat-denatured TR, and reaction on ice (2 °C) using the standard Ellman's reagent with freshly prepared L-cysteine solution as a calibration standard (0–80 μM) (Ellman 1959).

Redox Western Blot Analysis – rTat protein (20 μM) was reacted with TR1 (100 nM, from Sigma) in the presence of 0.5 mM NADPH, 5mM EDTA, 0.1 mg/ml bovine serum albumin in 100mM phosphate buffer (pH 7.0) for 1 h at room temperature. The resultant mixture was then incubated with 25 mM AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; 250 mM stock in DMSO) for 30 min at 4 °C followed by 10 min at 37 °C. The mixture was subjected to a non-denaturing and nonreducing PAGE (%T = 18) followed by Western blotting with anti-Tat polyclonal antibodies. rTat (50 nM) reduced with 2.5 mM DTT and subsequently treated with AMS was used a positive control, whereas rTat (50 nM) oxidized with 2.5 mM *N*,*N*,*N'*,*N'*-tetramethylazodicarboxamide (diamide; Sigma) was used as a negative control.

E. coli Trx (100 μg) was treated with 200 μl of immobilized TCEP disulfide reducing gel (Pierce) and incubated with mixing at room temperature for 1 h in 100 mM phosphate buffer, 2 mM EDTA, pH 7.0. The mixture was centrifuged at 1000g at room temperature and the supernatant containing the reduced Trx was collected. The concentration was estimated by spectrophotometry using ε = 13,700 M^{-1} cm^{-1} at 280 nm (Holmgren *et al.* 1967). 80 nM of rTat was incubated with the reduced Trx at molar ratios of 1:1, 1:3, and 1:5 (rTat : reduced Trx) at room temperature for 1 h in the aforementioned buffer. Half of this mixture was incubated with 10 mM AMS for 30 min at room temperature. This

mixture was subjected to a native PAGE (%T = 18), followed by immunoblotting with polyclonal antibodies to rTat. rTat alone, rTat reduced with DTT, and rTat incubated with native Trx (not treated with the TCEP column), subsequently treated with AMS were used as controls.

Mechanism-based Affinity Chromatography – A Sepharose affinity matrix was prepared with two mutant forms of His-tagged human TR1 where the resolving Cys and Sec in the C-terminus were replaced with Ser in one (denoted as SU) and both Cys and SeCys to Ser in the other (denoted as SS) as described earlier (Turanov *et al.* 2006). The immobilized enzymes were initially incubated with 2 ml of 0.5 mM NADPH in phosphate-buffered saline for 20 min. 15 μg of rTat was applied to each column. After 1 h of incubation at room temperature on an end-over shaker, the resins were washed 5 times with phosphate-buffered saline (PBS) to remove nonspecifically bound rTat and then eluted with 500 μl of PBS containing 10 mM DTT. All the flow-through (wash) and eluate fractions were concentrated and analyzed for the presence of Tat using Western blots.

Action of reduced Trx on native rTat – Reduced Trx [Trx-$(SH)_2$] was prepared by the chemical reduction of oxidized Trx (Trx-S_2) as described earlier with some modifications (Holmgren 1979). Briefly, Trx-S_2 was incubated with TCEP-agarose beads (20 μl) in 500 μl of oxygen-free 0.1 M potassium phosphate buffer, 2 mM EDTA, pH 7.0 at room temperature for 30 min. The beads were centrifuged at 1500g to sediment them, and the supernatant with Trx-$(SH)_2$ was incubated with native rTat at molar ratios of 1:1, 1:2, and 1:5 [rTat:Trx-$(SH)_2$] at 37°C for 2 h. The resulting mixture was analyzed by redox western blot.

Statistics – Where necessary, data are expressed as mean ± sd of triplicate experiments. The data was analyzed using the Student's *t* test for comparison, and $p < 0.05$ was used as the criterion for statistical significance.

Results

Selenium Inhibits HIV-1 Transcription – An inverse correlation between selenium levels and HIV progression has been reported. To gain insight into mechanisms by which selenium inhibits HIV replication, we tested whether Tat-dependent HIV transcription was sensitive to changes in cellular selenium status. The human monocytic cell line U937, cultured under selenium-deficient conditions (containing 7 nM selenium), were supplemented with selenium at 0.05–1.0 μM. Cells were co-transfected with pCI-Tat and LTR-Luc reporter that contained the TAR element. Selenium at concentrations of 100 nM significantly reduced Tat-dependent transcription compared with the cells where no exogenous selenium was added (Fig. 10). Tat was required for selenium-dependent repression of LTR-Luc activity as selenium had negligible effect on basal LTR activity in the absence of Tat. Furthermore, selenium had no effect on the ΔTARLTR-Luc (data not shown). Similar repression of Tat-dependent LTR activity was observed in human MDMs, although the amount of selenium added was 500 nM (Fig. 10A, *inset*). Selenium also inhibited HIV-1 replication as determined by p24 ELISA (Fig. 10A). HIV-infected U937 cells cultured in the absence of selenium produced ~6-fold more virus than infected cells cultured in the presence of selenium. Maximum suppression of HIV-1 replication was observed at ~0.1 μM selenium, which was the concentration that repressed Tat-dependent LTR activity. To ensure that we were effectively depleting selenium, the expression of two selenoproteins, TR1 and GPX1, was monitored. We observed a

decrease in TR1 and GPX1 expression in U937 cells (Fig. 10B) and primary MDM cells (data not shown) that were cultured under selenium-deficient conditions. Selenium supplementation rescued TR1 activity (Fig. 10A) and expression (Fig. 10B) in U937 cells. The enzymatic activity of TR1 and expression of TR1 and GPX1 were saturated at ~100 nM selenium, which corresponded to the concentration that fully inhibited Tat-dependent LTR activity, suggesting an inverse relationship between HIV-1 transcription and selenoprotein activity.

HIV-1 Infection of Macrophages Down-regulates TR1 Expression – Selenium is incorporated as Sec into enzymes such as the glutathione peroxidases and TRs that control cellular redox homeostasis. Based on cellular localization and function, we focused on the cytosolic TR1 form (also called TXNRD1 or TrxR1) rather than the thioredoxin/glutathione reductase (TR3 or TrxR3) or the mitochondrial TR2 (TXNRD2 or TrxR2) (Turanov *et al.* 2006). To determine whether HIV-1 infection led to changes in TR expression, as reported earlier in T-cells (Gladyshev *et al.* 1999), primary MDM were infected with HIV-1 and TR1 levels were monitored by immunoblotting. There was ~85% decrease in TR1 expression within 48 h of HIV-1 infection (Fig. 11), suggesting that HIV-1 could potentially target selenoproteins, including TR1, to ensure generation of oxidative stress for efficient replication.

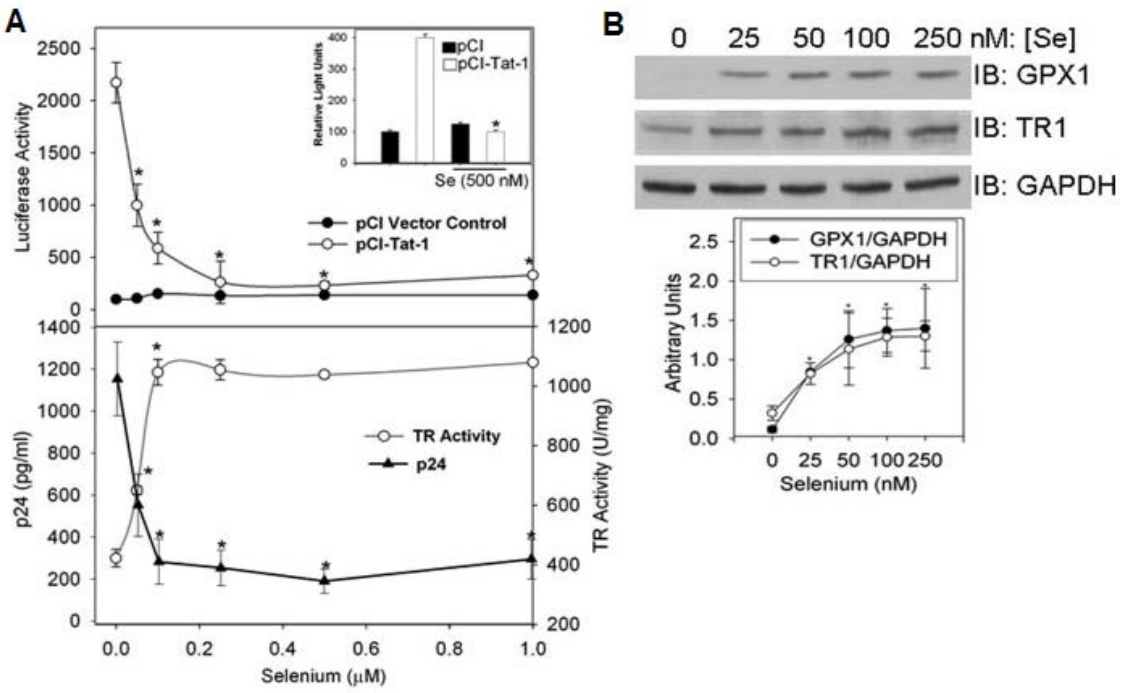

FIGURE 10. **Selenium inhibits Tat-dependent LTR activity in human macrophages.** A, U937 cells were transfected with 2 μg of LTR-Luc and then incubated with different concentrations of selenium (0.05–1.0 μM) for 72 h. Cells were lysed, and luciferase activity was measured as an indication of HIV-LTR activity. Inset, the same experiment performed with primary human MDMs. These cells were transfected with LTR-Luc, pCI-Tat, or empty vector using the Amaxa nucleofector system. TR activity was measured in the U937 cell lysates. For replication experiments, 1.0 X 10^6 U937 cells were cultured in a selenium-deficient media and infected with HXB.2 for 24 h followed by media changes containing selenium from 0.05 μM to 1 μM. After 3 days the culture media supernatants were used to measure p24 levels. B, Modulation of expression of selenoproteins (TR1 and GPX1) by exogenous selenium in macrophages. U937 cells cultured in low-selenium serum-containing media (7 nM selenium) were passaged for 3–5 days in media containing 0.025– 0.25 μM selenium (as sodium selenite). The cell lysates were analyzed for GPX1 and TR1 expression. Membranes were stripped and reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to normalize for protein loading. Densitometric evaluation of the data is shown. *; $p < 0.005$. IB, immunoblot.

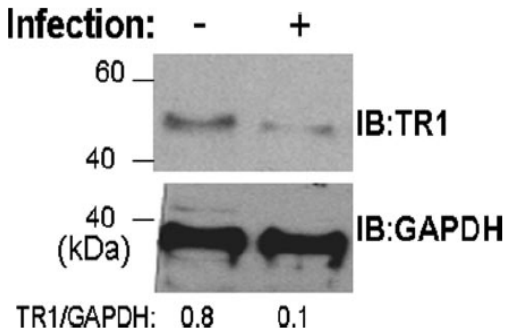

FIGURE 11. **Infection of MDMs by HIV-1 decreases the expression of TR1.** Human MDMs were infected with HXB.2 for 48 h. Cells lysates were probed with antibodies to TR1 and GAPDH..IB, immunoblot.

TR1 Negatively Regulates HIV-1 Transcription and Replication – The inverse correlation between TR1 activity and HIV-1 transcription and replication suggested that TR1 is a negative regulator of HIV-1 transcription. To investigate the role of TR1 in regulating Tat-mediated HIV-1 transcription, ebselen, a highly active substrate of TR (Zhao *et al.* 2002, Zhao *et al.* 2002), was used to "hijack" TR activity. As shown in Fig. 12A, ebselen increased Tat-dependent transactivation of the HIV-LTR by ~4-fold. The ability of ebselen to enhance transcription required Tat and the TAR element (Fig. 12A). In addition, we used siRNA to specifically diminish TR1 expression in U937 cells and MDM (Fig. 12B). TR1 siRNA depleted ~80% of TR1 in MDMs and U937 cells as determined by immunoblots (Fig. 12C). In U937 cells and MDMs treated with TR1 siRNA, Tat-dependent LTRLuc activity was increased by 8- and 4-fold, respectively (Fig. 12B). Furthermore, the decrease in TR1 did not ectopically activate NF-κB or enhance NF-κB-dependent transcription, as reducing TR1 expression and activity with siRNA or ebselen did not significantly alter activity of LTRs lacking NF-κB sites (Fig. 12D) or TAR (data not shown). To determine whether decreasing TR1 had an impact on

HIV-1 provirus transcription and replication, we infected cells with HIV-Luc, a replication incompetent HIV-1 clone in which *Env* was replaced with a luciferase reporter gene (Connor *et al.* 1995). Provirus transcription was monitored by measuring luciferase activity. Consistent with the transient transfection data, we observed a 3.5-fold increase in HIV-1 provirus transcription in U937 cells when TR1 was diminished by siRNA compared with the si-control (Fig. 13A). In addition, MDMs were infected with replication competent HIV-1, and virus replication was monitored by p24 ELISA (Fig. 13B). A 3.5-fold increase in HIV replication was observed in MDMs that were transfected with TR1 siRNA. A similar increase was also observed in HIV-1 replication in U937 cells that were treated with 5 μM ebselen (Fig. 13C). Taken together, these results indicate that TR1 impacts HIV-1 transcription and replication by repressing the activity of Tat.

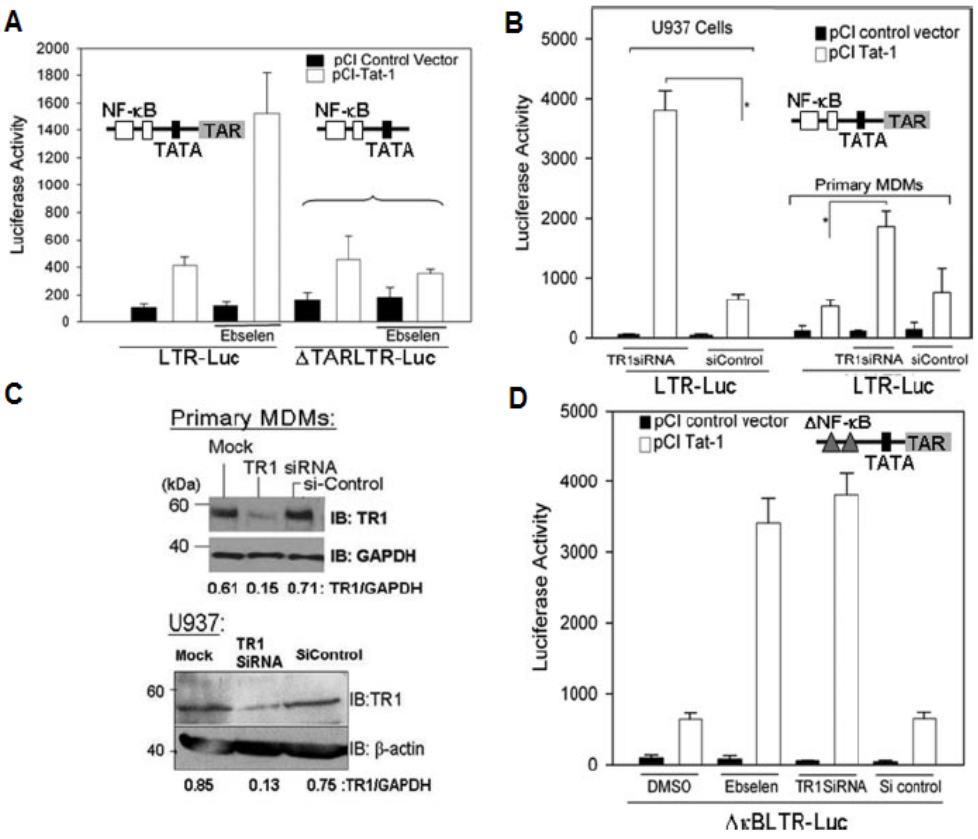

FIGURE 12. **Down-regulation of TR1 activity and expression increases Tat-dependent transcription.** A, Ebselen treatment of macrophages increases Tat-dependent transcription. U937 cells were transfected with LTR-Luc or TARLTR-Luc and pCI-Tat or empty pCI vectors. The transfected cells were cultured in the presence or absence of ebselen (5 μM) or DMSO (0.1% v/v) for 24 h. Luciferase activities in the lysates were quantitated after 48 h post-transfection and normalized to total protein. B, U937 cells were transfected with LTR-Luc and pCI-Tat or empty pCI vector and TR1 siRNA or si-control. Luciferase activities were measured as mentioned earlier. MDMs were transfected with LTR-Luc and pCI-Tat or empty pCI vector and TR1 siRNA using the Amaxa nucleofector system and processed as described earlier. C, TR1 expression in TR1 siRNA or si-control-treated U937 and MDM cells as determined by immunoblotting (IB). The blots were stripped and reprobed with either β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Densitometric values are shown below each panel. D, TR1 regulates Tat-dependent transcription independent of NF-κB. U937 cells were transfected with ΔκBLTR-Luc and pCI-Tat or empty pCI vector. Cells were then either treated with ebselen (5 μM) or TR1 siRNA or si-control. Cell lysates were used in luciferase activities 48 h post-transfection. The luciferase activity was normalized to total cell protein. *; $p < 0.05$.

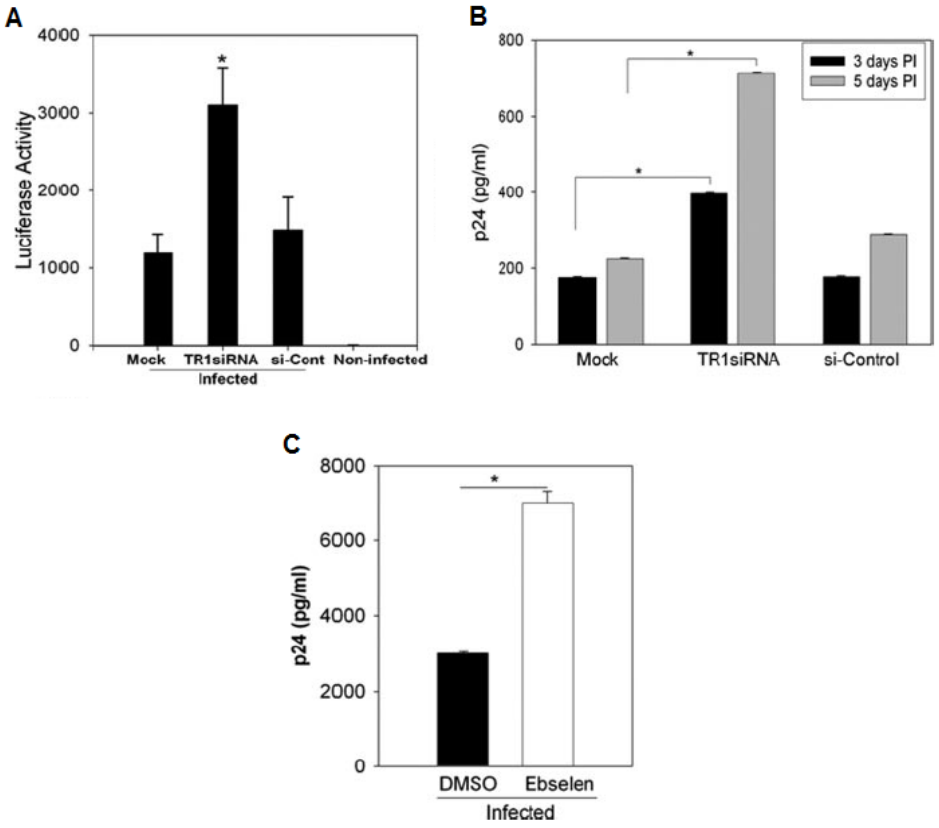

FIGURE 13. **TR1 negatively regulates HIV-1 replication in primary monocyte-derived-macrophages.** A, TR1 negatively regulates HIV-1 provirus transcription. U937 cells were infected with HIV-Luc virus. Cells (12–24 h post-infection) were transfected with TR1 siRNA. Luciferase activity was measured 72 h post-infection. B, MDMs were infected with HXB.2. 12–24 h post-infection cells were transfected with TR1 siRNA or si-control using the Amaxa nucleofector system and subjected to luciferase assay 3–5 days post-infection. C, ebselen treatment increases HIV-1 replication. U937 cells were infected with HXB.2. The transfected cells were cultured in the presence or absence of ebselen (5 μM) or DMSO (0.1% v/v). Supernatants were collected at 4 days postinfection and assayed for viral replication by p24 ELISA. Each data point represents three independent infections, and error bars show the S.D. of these replicates. *; $p < 0.05$.

In Vitro Reduction of rTat by TR1 – Tat has a Cys-rich region with three Cys-X-X-Cys motifs in the transactivation domain (Kuppuswamy *et al.* 1989). Previously, increased transactivation of a LTRLuc reporter was demonstrated by bacterially expressed rTat that was allowed to slowly refold in the presence of a reducing agent (tris[2-carboxyethyl]phosphine), indicating that the formation of intramolecular disulfide bonds was necessary for Tat function (Nordberg *et al.* 2001). Using an identical protocol,

our studies also demonstrated that refolded rTat, which is efficiently taken up by cells (Frankel *et al.* 1988, Herce *et al.* 2007), was able to activate TAR-dependent transcription in U937 cells, whereas rTat treatment did not activate the pSV2-Luc reporter, which lacks the LTR (Fig. 14). Furthermore, to demonstrate that rTat is a substrate for TR1, we used an *in vitro* assay with rat liver TR1 to assess the formation of free thiols from disulfide bonds using Ellman reagent [5,5'-dithiobis(2-nitrobenzoic acid)]. Coincubation of rTat with rat liver TR1 and NADPH increased the number of free thiols from three to seven, indicating that four out of the seven Cys in Tat form two disulfide bonds (Fig. 15A). Redox Western blot analysis was used to examine the electrophoretic mobility of rTat before and after reduction by TR1. AMS-modified proteins increased protein mass by 500 Da, which can be detected as a modest shift on a non-reducing and non-denaturing PAGE. The results indicate an upward shift in the mobility of rTat as a result of reduction by TR1, similar to the DTT-reduced rTat control (Fig. 15B). We used a mechanism-based affinity chromatographic approach to demonstrate that human TR1, where the resolving Cys was mutated to Ser (denoted as SU), was able to bind to rTat. Although there was some protein seen in the flow-through fraction, the eluate fraction had a relatively higher amount of rTat (Fig. 15C), which could be due to the saturation of TR1. Using human TR1 where both Cys and SeCys were mutated to Ser (denoted as SS), we noticed relatively low levels of rTat in the eluate fractions, whereas the flow-through fraction contained most of rTat (Fig. 15D). Taken together, these studies suggest that TR1 reduces disulfides in Tat by a direct interaction.

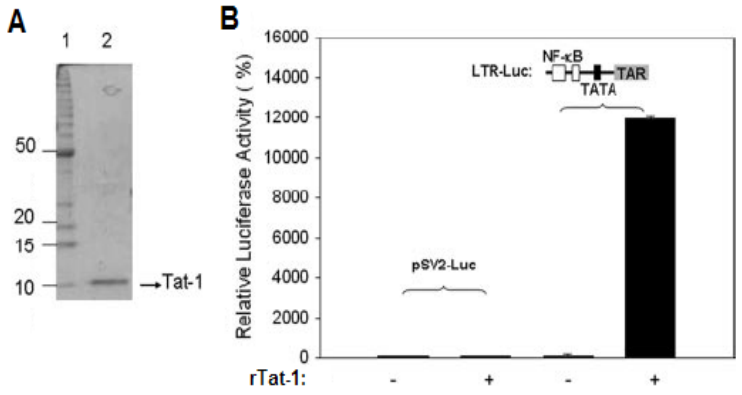

FIGURE 14. **Expression, purification, and biological activity rTat.** A, electrophoretic purity of rTat expressed in E. coli using SDS-PAGE. B, U937 cells were transfected with 2g of LTR-Luc or pSV2-Luc reporter constructs. Transfected cells were cultured in the presence and absence of 2 μg rTat protein. Luciferase activity was measured 48 h post-transfection as an indication of proviral transcription. After transfections, trypan blue staining was performed to control for cell viability. *; $p < 0.05$.

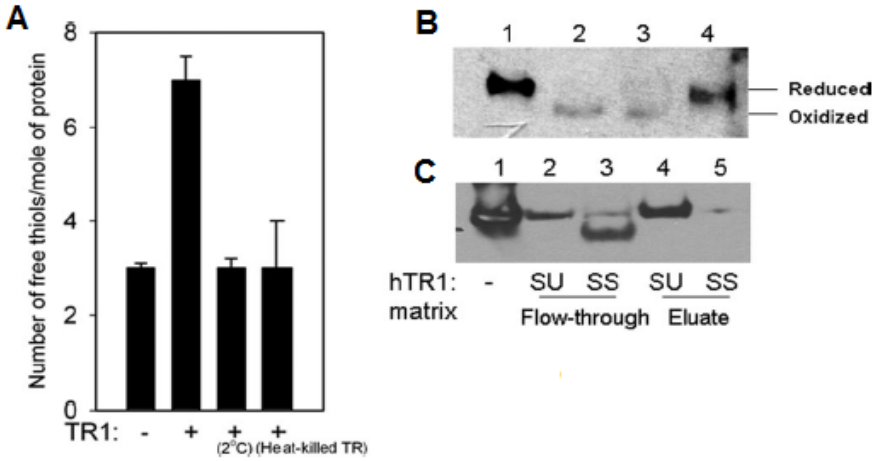

FIGURE 15. **Direct interaction of rTat with TR1 in vitro.** A, 2 μM rTat was mixed with 100 nM rat liver TR1 (Sigma) and 0.4 mM NADPH as described under Materials and Methods." Free thiols were determined using Ellman's reagent (5,5-dithiobis(2-nitrobenzoic acid). Data shown are the mean of triplicate experiments S.D. B, redox Western blot analysis of the reduction of rTat by TR1. The above reaction mixture was reacted with AMS followed by Western blot analysis with anti-Tat monoclonal antibodies. Lanes 1–4 represent DTT-reduced rTat, diamide (N,N,N,N-tetramethylazodicarboxamide)-oxidized rTat, native rTat, and TR1-reduced rTat, respectively. Although the protein load was identical in all the lanes, we consistently observed variations in the immunoreactivity of oxidized rTat. C, mechanism-based affinity chromatography showing the binding of rTat to hTR1SU and hTR1SS mutants. Protein in the flow-through and DTT eluate fractions were concentrated and analyzed by Western blot for the presence of Tat. Lanes 1–5 represent rTat positive control, flow-through from SU column, flow-through from SS column, eluate from SU column, and eluate from SS column, respectively.

In vitro reduction of rTat by reduced Trx – While TR1 can itself reduce disulfides in proteins, its natural substrate is Trx. TR1 reduces Trx, which can then reduce disulfides in a range of proteins. To determine the role of Trx in the TR1 mediated reduction of HIV-1 Tat-1, we incubated rTat with TCEP-reduced Trx, and analyzed the mixture by a redox western blot. As shown in Fig. 16, reduced Trx, when incubated with Tat, caused an upward shift of the Tat band in a redox western blot, indicating that the Cys residues in Tat were reduced.

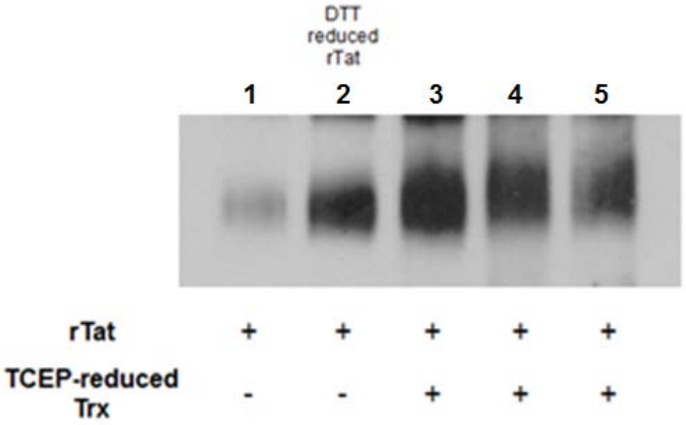

FIGURE 16. ***In vitro*** **reduction of rTat by reduced Trx.** 50 pmol of Tat was incubated with increasing concentrations of reduced Trx (50, 150, 250 pmol, lanes 3, 4, and 5 respectively) for 1 h at RT. The reaction mixtures were treated with 10 mM AMS for 30 min, following which they were separated by native PAGE and analyzed by immunoblotting with an anti-Tat antibody. Native Tat (lane 1) and DTT-reduced Tat (lane 2) were used as controls. Representative of n = 2 shown.

Discussion

Epidemiological studies have shown a correlation between decreased selenium status and increased mortality and morbidity of HIV patients (Baum *et al.* 1998, Kupka *et al.* 2004, Ogunro *et al.* 2006). However, there is sparse information regarding mechanisms by which selenium influences HIV-1 replication. Selenium decreases oxidative stress in

HIV-infected people by neutralizing highly reactive pro-oxidant species via selenoenzymes, which is thought to positively impact patient immune function and health (McDermid *et al.* 2002) and, thus, indirectly influence HIV-1 replication in infected cells Selenium has also been implicated in causing the inactivation of NF-κB, which is required for efficient provirus transcription. Based on our data, selenium appears to have a more direct effect on HIV-1 transcription, leading to decreased virus replication. In this report, we present data indicating that selenium, through the selenoprotein TR1, negatively regulates HIV-1 transcription by targeting Tat-dependent transcription, independent of NF-κB.

Tat is essential for HIV-1 replication and is required for efficient transcription elongation (Karn 1999). HIV-1 Tat binds the RNA stem loop structure formed by TAR and recruits the positive transcription elongation factor, P-TEFb, to the LTR, which enhances processive transcription. Thus, host mechanisms that specifically target its activity would provide attractive therapeutic options that would be complementary to current anti-retroviral treatments targeting reverse transcriptase, integrase, and protease enzymes. The conclusion that the intramolecular disulfide bonds are critical for Tat function is consistent with previous observations that reducing agents that disrupt intramolecular disulfide bonds inhibit Tat activity (Koken *et al.* 1994). Our results not only support earlier data but also demonstrate that TR1 negatively regulates Tat activity by reducing two disulfide bonds in Tat. Although the Cys-rich domain is not structurally well defined, recent NMR studies using recombinant Tat protein confirm that the disulfide bonds are essential for stabilizing its structure to possibly allow the cyclin T1 subunit of P-TEFb to bind Tat (Shojania *et al.* 2006). Therefore, TR-dependent reduction of the

disulfides may affect the interaction of Tat with P-TEFb, leading to a decrease in the transcription processivity. Although the mechanism based affinity chromatography experiments suggest a direct interaction between Tat and TR1, it is plausible that the redox modulation could also occur through TR modulation of Trx, as is the case for NF-κB p65 subunit (Hirota *et al.* 1999) and glucocorticoid receptor (Makino *et al.* 1999). Indeed, upon incubating the rTat protein with reduced *E. coli* Trx, we observed an increase in the free thiols in Tat, as indicated by the upward shift of such treated rTat on a redox western blot. Reduced Trx, formed due to the action of the enzyme TR1, has a high reductive potential and is able to reduce disulfides in many classes of proteins, thus indirectly increasing the reduction potential of TR1. It is possible that Tat is also targeted in this manner in our *in vitro* model.

Specifically inhibiting TR1 activity in macrophages with either a high affinity substrate or expression using siRNA resulted in more efficient HIV-1 replication. If oxidative stress is required for efficient HIV-1 replication, then the virus would be expected to circumvent the reducing activities of TR. Selenoproteins, including TR1, are down-regulated upon HIV-1 infection and during the progression of AIDS and associated diseases. Whether HIV-1 directly alters the redox status of the cell or indirectly promotes inflammation and oxidative stress is not clear; however, we show that infection of macrophages decreases TR1 protein, which is consistent with earlier reports (Gladyshev *et al.* 1999). Moreover, our data demonstrate that the expression of selenoproteins TR1 and GPX1 in U937 cells and MDMs can be modulated with selenium (Fig. 12D), providing a strategy to rescue selenoprotein expression as well as decreasing HIV-1 transcription and replication in HIV-1 infected cells.

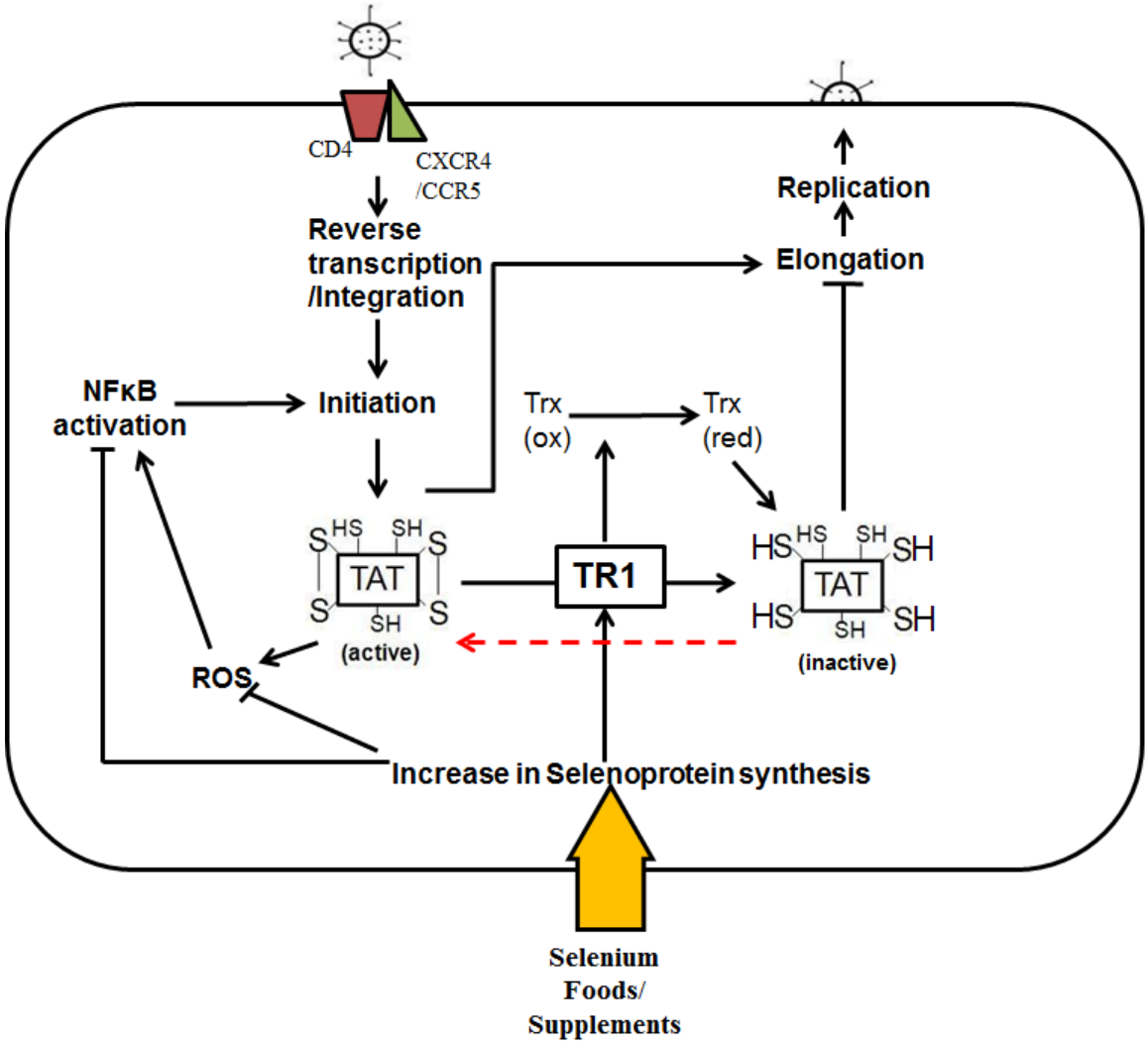

FIGURE 17. **Proposed model of regulation of HIV-1 transcription by TR1.** Selenium supplementation of macrophages leads to the decrease in reactive oxygen species (ROS) and NF-B-dependent pathways of pro-inflammatory gene expression possibly by the increased cellular peroxide scavenging ability of selenoenzymes. Although such effects may have an impact on the HIV-1 transcription, a more direct mechanism of action of the selenoprotein, TR1, is possible. Increased expression and activity of TR1 in selenium-supplemented cells reduces proviral transcription by targeting the disulfide bonds in Tat. Thus, TR1 acts as a negative regulatory molecule capable of suppressing proviral transcription of HIV-1 in *in vitro* culture models.

In conclusion, we have identified TR1 as a critical host selenoprotein that plays a pivotal role in repressing HIV-1 transcription by modulating the redox-status of the key viral protein, Tat (Fig. 17). It remains to be seen if the redox status of Tat correlates with the

plasma selenium in HIV-1 seropositive individuals. Such studies are necessary to understand the therapeutic benefits of selenium as an adjuvant in the management of HIV/AIDS. These studies partially elucidate one mechanism by which selenium inhibits HIV-1 replication. Furthermore, these findings suggest that administration of selenium to HIV-1 seropositive individuals at a daily-recommended intake of 55 μg/day, as per the National Institute of Medicine, may provide an effective method to slow the progression of AIDS, reduce morbidity, and enhance survival partly through the redox-dependent regulation of Tat-dependent transcription. Such supplementation studies need to be evaluated with caution given the toxicity associated with selenium, seen in the form of nausea, blotchy nails, vomiting, and diarrhea at doses higher than the tolerable upper intake levels of 400 μg/day. However, understanding the mechanism of action of selenium will allow more direct targets to be examined and set optimal doses to mitigate toxicity and achieve the desired effect.

Chapter 3: Alkylation of free thiols in Tat by Michael Acceptor Electrophiles (MAEs) affects HIV-1 transcription and replication in human macrophages

The data in this chapter have been published in two papers –

1. Parisa Kalantari, Vivek Narayan, Andrew J. Henderson, and K. Sandeep Prabhu. **15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits HIV-1 transactivating protein, Tat, through covalent modification.** *The FASEB Journal* 23(8):2366–2373, August, 2009

   (Contributions: Dr. Parisa Kalantari performed the work with the HIV virus. Vivek Narayan and Dr. Parisa Kalantari performed the luciferase assays and western blot analyses.)

2. Vivek Narayan, Kodihalli C. Ravindra, Chris Chiaro, Daniele Cary, Bharat B. Aggarwal, Andrew J. Henderson, and K. Sandeep Prabhu. **Celastrol inhibits Tat-mediated Human Immunodeficiency Virus (HIV) Transcription and Replication** *The Journal of Molecular Biology* 410(5):973-983, July 29, 2011

   (Contributions: Vivek Narayan performed the experiments in this study. Dr. Ravindra Kodihalli and Dr. Chris Chiaro helped in the synthesis and purification of biotinylated celastrol.)

Abstract

Controlling the HIV/AIDS epidemic remains a major challenge, with approximately 2 million new HIV infections annually. Current drugs used for anti-retroviral therapy against HIV have a narrow spectrum of activity, and more often have associated toxicities and severe side effects in addition to developing resistance. Thus, there is a need to develop new therapeutic strategies against HIV/AIDS to complement the already existing ones. Surprisingly, the viral early protein, Tat, an early virus encoded protein required for the efficient transcription of the HIV genome, has not been developed as a target for small molecular therapeutics. Cyclopentenone prostaglandins (CyPG), such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and certain plant-derived compounds such as celastrol, curcumin, rosmarinic acid and gambogic acid, belong to a class of compounds called Michael acceptor electrophiles (MAEs), and possess anti-HIV activity by an unknown mechanism. Given that the reactive α,β-unsaturated ketone in MAEs covalently modifies key Cys-thiols in select proteins, we hypothesized that certain MAEs could inhibit HIV transcription and replication by targeting Cys-thiols in HIV-1 Tat, which is a potent transactivator of viral gene expression. Our studies indicate that treatment of cells with MAEs like 15d-PGJ_2 and celastrol inhibits Tat-dependent transcription and replication of HIV-1 by modifying free Cys-thiols in Tat, while 9,10-dihydro-15d-PGJ_2, PGE_2, $PGF_{2\alpha}$, PGD_2, tetrahydrocurcumin or dihydrogambogic acid that lack the reactive α,β-unsaturated ketone were ineffective. Using circular dichroism (CD) spectroscopy, we show that alkylation of Tat changes its secondary structure, which likely leads to the inhibition of transcriptional elongation of the HIV proviral genome. Taken together, our data demonstrate that Tat acts as a molecular target of certain MAEs leading to the

inhibition of transcription and also suggest a novel therapeutic approach to complement current antiretroviral strategies for HIV/AIDS.

Introduction

HIV provirus expression is regulated at the transcriptional level, which is controlled by the upstream long terminal repeat (LTR). The HIV-1 LTR is often divided into four functional elements: the Tat activating region (TAR), the promoter, the enhancer, and the negative/modulatory regulatory element (Karn 1999). The promoter, enhancer, and modulatory elements recruit host-transcription factors, such as Sp-1, NF-κB, and C/EBPβ, which are necessary to initiate transcription (Pierson *et al.* 2000, Rohr *et al.* 2003), whereas the TAR element forms an RNA stem loop structure that recruits the HIV-encoded transcriptional activator Tat. Tat recruits pTEFb to the LTR, enhancing processive transcription. In the absence of Tat, transcription elongation by RNA polymerase II from the HIV promoter is very inefficient, leading to an accumulation of short initiated transcripts (Kao *et al.* 1987, Marciniak *et al.* 1991). The activity of Tat substantially increases the production of elongated transcripts (Kao *et al.* 1987, Laspia *et al.* 1989, Feinberg *et al.* 1991, Marciniak *et al.* 1991). Furthermore, Tat recruits chromatin-remodeling machinery to ensure efficient transcription (Emiliani *et al.* 1996, Agbottah *et al.* 2006, Mahmoudi *et al.* 2006). Because Tat is necessary for HIV-1 replication, compounds that specifically target its activity are likely to have the therapeutic potential as a complementary medicine to existing antiretroviral treatments targeting reverse transcriptase, integrase, and protease.

The primary structure of Tat is made up of 101 amino acids. On the basis of mutagenesis studies, six distinct domains have been mapped, which include N-terminal domain (residues 1–19), cysteine-rich domain (residues 20–39), core domain (residues 40–47), basic domain (residues 48–56), auxiliary domain (residues 57–67), and a C-terminal glutamine-rich (RGD) domain (residues 68–101) (Kuppuswamy *et al.* 1989). The first four domains make up the essential domain, which is necessary for Tat function, whereas the auxiliary domain enhances the activity of the essential domain (Kuppuswamy *et al.* 1989). Of particular interest is the Cys-rich domain, which contains seven Cys residues that are poorly defined with regard to intramolecular disulfide bonds. Functional analysis by deletion and site-specific mutations of the Cys-rich domain revealed that Cys residues are necessary for Tat activity. Although required for Tat transactivation function, the structural basis for this function is still not well understood. The Cys-rich domain could form a metal-linked dimer with a tetrahedral geometry in which each metal is liganded to twelve Cys residues; while the other two Cys act as terminal ligands (Frankel *et al.* 1988). However, the transcriptionally active form of Tat has been shown to be a monomer, and reducing agents dramatically inhibit Tat activity (Koken *et al.* 1994), suggesting that Cys residues in Tat form two intramolecular disulfide bonds that are essential for transactivation function (Kirsch *et al.* 1996). Given the formation of two disulfide bonds per molecule of Tat, the role of free Cys residues in the transcription elongation is unknown.

Arachidonic acid is enzymatically metabolized by cyclooxygenases (COX) to PGH_2 that is further metabolized by specific PG isomerases to PGE_2, PGD_2, $PGF_{2\alpha}$, thromboxane A_2 (TXA_2), and prostacyclin I_2 (Wada *et al.* 2007). Members of the PGJ2 class, particularly

15d-PGJ_2 (also called cyclopentenone PGs, CyPG), are derived from PGD_2 by two dehydration reactions that lead to the formation of a reactive α,β-unsaturated ketone functionality (Santoro *et al.* 1980, Fitzpatrick *et al.* 1983). CyPGs exhibit a unique spectrum of biological effects, including inhibition of IκB-kinase-β (Rossi *et al.* 2000), induction of synoviocyte and endothelial cell apoptosis (Koken *et al.* 1994), induction of glutathione *S*-transferase gene expression (Kirsch *et al.* 1996), and potentiation of apoptosis in neuronal cells (Kondo *et al.* 2002). In the case of IKKβ, 15d-PGJ_2 directly interacted with Cys^{179} of IKKβ to inhibit NF-κB activation (Rossi *et al.* 2000). In addition, 15d-PGJ_2 serves as an endogenous ligand for the nuclear hormone receptor PPAR-γ (Forman *et al.* 1995). The anti-inflammatory property of 15d-PGJ_2 also stems from its ability to initiate sumoylation of the liganded receptor that maintains a corepressor (NcoR) complex on NF-κB response elements (Pascual *et al.* 2005).

Rajakariar *et al.* (Rajakariar *et al.* 2007) have provided definitive proof in support of the *in vivo* production of 15d-PGJ_2 and its role as a crucial checkpoint controller of cytokine/chemokine synthesis, as well as leukocyte influx and efflux. In addition, natural and synthetic CyPGs also display antiviral activity (Santoro *et al.* 1980, Hughes-Fulford *et al.* 1992) by targeting different steps of DNA and RNA virus replication, including synthesis, glycosylation, intracellular translocation of viral proteins, and maturation of the virus particle (Santoro *et al.* 1983, Pica *et al.* 1993). Relevant to HIV, it has been demonstrated that 15d-PGJ_2 and PGA_1 suppress HIV-1 replication by inhibiting transcription (Rozera *et al.* 1996). In this study, we demonstrate that the mechanism by which 15d-PGJ_2 inhibits HIV transcription is independent of NF-κB and is due to covalent modification of the Cys residues in Tat.

Several drugs have entered the market as anti-HIV agents since HIV was first discovered. Most FDA approved drugs are synthetic and have associated toxicities and serious side effects such as rash, hypertension, fatigue, anemia, granulocytopenia, pancreatitis, and peripheral neuropathy (Raulin 2002, Cuzin *et al.* 2008, Mandas *et al.* 2009). These adverse effects, coupled with the narrow specificity of the available drugs and drug resistance, have turned many towards natural sources to combat HIV/AIDS. Thus, the use of herbal medicine as a complementary and alternative medicine (CAM), in the form of antioxidant botanicals, has become prevalent among HIV-positive individuals worldwide (Bedoya *et al.* 2001, Bedoya *et al.* 2002, Asres *et al.* 2005). Furthermore, due to the fact that the plant kingdom has served as a source for many of our modern day therapeutic drugs, we selected five compounds, ferulic acid (FA), curcumin (Cur), rosmarinic acid (RA), gambogic acid (GA) and celastrol (Cel) (see Fig. 18) extracted from medicinal plants with prior history of anti-retroviral and anti-inflammatory properties (Barthelemy *et al.* 1998, De Clercq 2000, Tewtrakul *et al.* 2003, Pommier *et al.* 2005, Vajragupta *et al.* 2005, Dubois *et al.* 2008), to determine their anti-HIV potential. The compounds, termed "Michael acceptor electrophiles" (MAEs; which 15d-PGJ_2 is also a part of) by virtue of the presence of α,β-unsaturated carbonyl functionality (enone), have the potential to form covalent Michael adducts by interacting with the cysteine sulfhydryls of certain proteins. Here we report the ability of some of the compounds of natural origin to inhibit the transactivation function of Tat and begin to delineate the underlying mechanism. Our data suggest that such MAEs could be developed as anti-retroviral therapeutics for HIV/AIDS, which may serve to complement the already existing therapies.

Materials and Methods

Cell Culture – The human promonocytic U937 cell line (ATCC, Manassas, VA, USA) was cultured in RPMI-1640 (Cellgro, Manassas, VA, USA) supplemented with 10 % defined fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine (Cellgro, Manassas, VA, USA), 100 U/ml Penicillin and 100 μg/ml streptomycin (Cellgro, Manassas, VA, USA). HEK 293T cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 4 mM L-glutamine, and 100 U/ml Penicillin and 100 μg/ml streptomycin.

Chemicals – Free and biotinylated 15d-PGJ_2, 9,10-dihydro-15d-PGJ_2, PGD_2, PGE_2, and $PGF_{2\alpha}$ were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 9,10-Dihydro-15d-PGJ_2-biotinamide was synthesized in our laboratory using 9,10-dihydro-15d-PGJ_2, as described previously (Shibata *et al.* 2003). Briefly, biotinpentylamine (Thermo-Pierce, Rockford, IL, USA) was used to conjugate the carbodiimide-activated carboxylic group of 9,10-dihydro-15d-PGJ_2 to form an amide bond. Such a biotin derivative of 9,10-dihydro-15d-PGJ_2 with a 5-carbon spacer arm was purified using RP-HPLC and quantitated by spectrophotometry. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was purchased from Thermo-Pierce (Thermo-Pierce). RA and Cel were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Cur and tCur were a kind gift from Dr. Bharat B. Aggarwal. GA and dihydro gambogic acid DGA was purchased from Gaia Chemical Corporation (CT, USA). FA was a kind gift from Dr. Devin Peterson, University of Minnesota. Celastrol-biotinamide (bCel) was synthesized in the laboratory by reacting Cel with 5-(biotinamido)pentylamine (Pierce) (1 : 2.5 :: Cel : pentylamine), in the presence of 1-ethyl-3-(3-diaminomethylaminopropyl)carbodiimide hydrochloride

(EDC) in MES buffer (100 mM, pH 5.5). The reaction was allowed to proceed for 4 hours, and extracted using ethyl acetate. bCel was purified by column chromatography on a silica gel column. bCel was subjected to LC-MS in our laboratory to confirm the modification. All compounds were tested for the presence of endotoxin and were found to be below the limits of detection using the QCL-1000 chromogenic LAL endpoint assay from Lonza (Basel, Switzerland). All other chemicals used were of analytical grade.

Expression, purification and refolding of rTat – Recombinant Tat (rTat) was expressed in *Escherichia coli* as a His-tagged protein using the methods previously described by Patki and Lederman (Patki *et al.* 1996) and Kirsch *et al.* (Kirsch *et al.* 1996) with some modifications. Briefly, pTatC6H-1 construct from the NIH AIDS Research and Reference Reagent Program was used to transform BL21 (DE3) bacterial cells. The bacterial clone was grown in terrific broth (TB) and induced with IPTG (1 mM) overnight at 37 ^{o}C. The bacterial cell pellet was subjected to lysis in 6 M guanidine hydrochloride for 12 h. All steps were performed at 4 °C unless otherwise mentioned. The clarified cell lysates were subjected to metal affinity chromatography using Ni-NTA matrix (Novagen). The eluate was dialyzed against 0.1 N HCl for 2 d followed by an overnight lyophilization. The lyophilized powder was reconstituted in 6 M urea followed by reduction by tris(2-carboxyethyl)phosphine (TCEP, 25 mol/mole of rTat) in the dark for 8 h at room temperature. The mixture was subjected to sequential dialysis in phosphate buffer (0.1 M, pH 6.3) containing 4 M urea, 2 M urea, 1 M urea, and finally in buffer containing 200 mM NaCl. The dialysate was filter sterilized, purged with nitrogen, and stored in small aliquots at -80°C for further use. The rTat solution tested negative for the presence of endotoxin (LPS) using the QCL-1000 chromogenic LAL

endpoint assay from Lonza (Basel, Switzerland). Using 5,5'-dithiobis-(2-nitrobenzoic acid; Ellman's reagent), the number of free Cys residues per mole of protein was deduced to be three in the rTat protein (Kalantari *et al.* 2008).

Plasmids, transient transfections, and luciferase assays – Replication competent HXB.2 virus, bacterial and mammalian HIV-1 Tat expression vectors, pTat86R His, pCI vector control, and pCI-Tat (Frankel *et al.* 1988), respectively, were obtained from the AIDS Research Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NAIAD), NIH. pCI vector (Promega, Madison WI, USA) was used as an empty vector control. LTR reporter constructs, LTR-Luc, and ΔκBLTR-Luc, which represent the wild-type -205LTR and ΔκB mutated -205LTR promoters, respectively, were prepared as described previously (Henderson *et al.* 1995). DNA for transfections was prepared using plasmid purification systems from Marligen Biosciences (Ijamsville, MD, USA) following protocols provided by the manufacturer. Transfection of U937 cells was performed using *Trans*IT-Jurkat transfection reagent from Mirus Corporation (Madison, WI, USA). Such cells were treated with DMSO or indicated concentrations of 15d-PGJ_2, 9,10-dihydro-PGJ_2, FA, Cur, tCur, GA, DGA, RA, and Cel. The final concentration of DMSO was kept constant at 0.1 %. Transfection efficiency was assessed by cotransfecting pEGFP-N3 (Clontech, Palo Alto, CA, USA) and monitoring EGFP expression by fluorescence microscopy. Cell viability was confirmed by Trypan blue staining. In addition, cell viability was assessed in PG-treated cells using the WST-8 cell counting kit from Dojindo (Gaithersburg, MD, USA). Luciferase assay was performed using the luciferase assay kit (Promega) and a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA, USA). The human inducible nitric

oxide synthase promoter luciferase reporter, pHiNOS-Luc (iNOS-Luc), was obtained from Dr. Kalipada Pahan (Rush University Medical Center, Chicago, IL, USA).

Generation of HIV infectious titer and infections – Infectious viral stocks of the HXB.2 clone of HIV-1 were prepared by cotransfecting 293T cells with 3.6 μg of pHXBnPLAP-IRES-N+ plasmid (NIH AIDS Research and Reference Reagent Program), 0.7 μg of vesicular stomatitis virus-glycoprotein (VSV-G) and 0.7 μg of RSV-Rev using the *Trans*IT-293 transfection reagent (MirusBio, Madison, WI, USA). Supernatants were collected for 5 d post-transfection, filtered through 0.45 μm filters and combined. Transfection efficiency was measured by p24 ELISA (Bio Academia, Japan). The infectious titer was found to be 1.0 X 10^6 infectious units/ml. One milliliter of undiluted virus stock was added to 1.0 X 10^6 U937 cells for 24 h and then replaced with fresh medium. Supernatants were collected at d 3 or 4 postinfection and assayed for viral replication by p24 ELISA.

For experiments with Cel, 10.0 X 10^6 U937 cells were incubated with 10 ml of undiluted viral stock for 24 h, after which the cells were cultured for 4 d in fresh media. As the HIV-1 clone being used is replication-competent, a progressive infection was established in the U937 cells. These cells were then treated with vehicle (DMSO, final concentration 0.1%) or Cel at 0.15 μM for 5 d. A fresh treatment of Cel was provided along with fresh media every day for the 5 d. Supernatants were collected by centrifugation every day and assayed for p24 protein by ELISA according to the manufacturer's instructions.

Immunoblotting – Cells were washed twice with phosphate-buffered saline, and protein extracts were prepared by treating cells with M-PER reagent (Thermo-Pierce) at 4°C for

30 min. Lysates were mixed with 6X SDS loading buffer containing DTT and boiled at 100°C for 5 min before resolving by SDS-PAGE, followed by immunoblotting onto a nitrocellulose membrane. Mouse anti-Tat monoclonal antibody from the NIH AIDS Research and Reference Reagent Program was used to analyze expression, and horseradish peroxidase-conjugated goat-anti-mouse (Sigma-Aldrich, St. Louis, MO, USA) was used as the secondary antibody. In the case of analysis of biotinylated Tat, streptavidin-HRP (Thermo-Pierce) was used.

Binding Studies –

A) Interaction between 15d-PGJ$_2$ and Tat – Studies to determine the covalent interaction of 15d-PGJ$_2$ with rTat were carried out as follows. First, the interaction of 15d-PGJ$_2$-biotinamide with rTat was examined *in vitro*. rTat was incubated with 15d-PGJ$_2$ at a molar ratio of 1:7 (protein:PG) for 3 h at 37°C in phosphate-buffered saline with or without DTT pretreatment. The reaction mixture was applied onto a Biogel P column (Bio-Rad, Hercules, CA, USA) to separate the unreacted 15d-PGJ$_2$-biotinamide from the protein-PG complex. The flow-through fractions, which tested positive for the presence of rTat, were analyzed by Western immunoblotting techniques with streptavidin-HRP and anti-Tat. Second, the interaction between 15d-PGJ$_2$ and rTat in the context of the cells was determined by using 15d-PGJ$_2$-biotinamide to pull down Tat from cell lysates. Cells were cultured for 12 h with rTat (2 μg/10^6 cells). After a medium change, cells were lysed and treated with 15d-PGJ$_2$-biotinamide (Cayman Chemicals) or 9,10-dihydro-15d-PGJ$_2$-biotinamide at a molar ratio of 1:7 (rTat:PG) for 3 h at 4°C. The cell lysates were subjected to biotin-pulldown assays using 20 μl immobilized neutravidin (Pierce), followed by SDS-

PAGE under reducing conditions and Western blot analysis. Biotinylated rTat was detected using streptavidin-HRP (Pierce). The blot was stripped and probed with anti-Tat antibodies.

B) Interaction between celastrol and Tat – Recombinant Tat (0.9 μM) was incubated with bCel (9.0 μM) for 3 h at 37 °C in a reaction volume of 100 μl. The buffer used for the reaction was 50 mM Tris-Cl, pH 7.5. rTat was incubated with bCel in the presence of 9.0 μM of Cel, as a "cold" competitor. As negative controls, bCel treated with 90 μM reduced Glutathione (GSH, Calbiochem), at 37 °C and rTat treated with 9.0 μM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, disodium salt (AMS, Invitrogen) were used. AMS is a thiol-reactive agent which forms charged adducts with proteins causing them to migrate slower when subjected to SDS PAGE. The samples were analyzed by SDS PAGE followed by Western blotting, and were probed with a polyclonal antibody to Tat (ab43014; abcam, Cambridge, MA, USA) and streptavidin-HRP (Pierce).

C) Spectrophotometric analysis of the interaction between celastrol and Tat – Recombinant Tat (0.9 μM) was incubated with Cel (9.0 μM) for 3 h at 37 °C. The reaction mixture was then extracted with ethyl acetate five times to remove unreacted celastrol. The aqueous phase containing the protein and bound Cel was then subjected to UV/Vis spectroscopy using a Beckman DU 7500 spectrophotometer. The spectrum was observed between 320 nm and 470 nm. Reaction mixtures containing buffer only and Cel only were subjected to the same extraction conditions as described above, and were used as controls.

D) **Interaction between celastrol and Tat in a cellular protein milieu** – Human U937 cell lysates was prepared using the mammalian protein extraction reagent (MPER, Pierce) supplemented with protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin. 100 μg of U937 cell protein was incubated with or without 250 ng (0.023 nmol) of rTat in the presence or absence of 173 ng (0.23 nmol) bCel (1:10 molar ratio) in a reaction volume of 100 μl. The mixtures were incubated on ice for 3 hours. These were then incubated with neutravidin-agarose beads overnight at 4 °C. Biotinylated proteins were separated by SDS-PAGE. Following Western transfer, the membrane was probed with anti-Tat antibodies.

Interaction of alkylated Tat with the HIV TAR element – A 29 bp ssRNA oligonucleotide representing the HIV-TAR element was obtained from Dharmacon to determine its interaction with Cel-modified rTat. The sequence of the oligo was 5' GGCAGAUCUGAGCCUGGGAGCUCUCUGCC. 4 μM of the oligo was labeled with 30 μCi of γ-^{32}P-ATP using 10 units of T4-polynucleotide kinase enzyme in a 30 μl reaction. 100 μg of rTat was incubated with 41 μg of Cel (1:10 molar ratio) in 100 μl of 50 mM Tris-Cl, pH 7.5, prepared in nuclease-free water. They were incubated together at 37 °C for 3 h. Buffer only, Cel only and native Tat were used as controls. After incubation, the reaction mixtures were incubated with a HisPur Cobalt (Co^{2+}) Resin (Thermo Scientific) for 3 h on ice on an orbital shaker. The resin was washed 4 times with 50 mM Tris-Cl, pH 7.5 to remove unreacted Cel. The control and sample resins were incubated with 20000 cpm of labeled oligo for 1 h in 50 mM Tris-Cl, 20 mM potassium chloride, 0.1% triton X-100, pH 7.4 buffer (prepared in nuclease-free water) at room temperature. The resins were again washed with 50 mM Tris-Cl, pH 7.5 5 times to

remove unbound oligo. The resins were boiled with 1X SDS gel loading dye and subject to scintillation counting on a Beckman LS6000IC.

Circular dichroism (CD) spectroscopy – Samples were prepared by incubating rTat (100 μg, 60.60 μM) alone or rTat and different concentrations of Cel (60.60 μM, 303.00 μM and 606.00 μM) in 150 μl of buffer (50 mM Tris-Cl, pH 7.5) at 37 °C for 3 hours. Buffer alone and Cel only at the above concentrations were used as controls. The volume was made up to 2 ml with buffer after incubation (final concentrations: rTat 4.55 μM, and Cel 4.55 μM, 22.75 μM, or 45.50 μM). These samples were subjected to CD spectroscopy using a Jasco Corp. spectrophotometer, J-810 with a cuvette (Hellma, USA) path length of 10 mm. The temperature was 25 °C. The spectrum was recorded between 220 nm and 450 nm at the rate of 2 sec per nm.

Quantitative PCR (qPCR) – U937 cells were infected with HIV and treated with Cel as above. At the end of the treatment, the cells were harvested in Trizol (Invitrogen), and total RNA was isolated. cDNA was prepared from 2 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). 5 ng of the cDNA were used in SYBR Green qPCR using the PerfeCTa SYBR Green SuperMix, ROX kit (Quanta Biosciences, Gaithersburg, MD, USA) with primers designed to detect elongated transcripts of HIV (~ 5 kb downstream of the LTR) (Zhang *et al.* 2007). Forward and reverse primers were: 5’ ACTCGACAGAGGAGAGCAAG and 5’ GAGTCTGACTGTTCTGATGA, respectively. PCR conditions used were: Initial denaturation at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 15 sec and

annealing an extension at 60 °C for 1 min. Conditions for studying dissociation curves were: 95 °C for 15 sec, 60 °C for 30 sec, and 95 °C for 15 sec.

Incubation of rTat with TR1 and Trx – Recombinant Tat (1.8 μM) was incubated with purified rat liver TR1 (0.1 μM. Sigma), in the presence of NADPH (0.5 mM), EDTA (5 mM), and 0.1 mg/ml bovine serum albumin, in 100 mM phosphate buffer (pH 7.0) for 1 h at room temperature. To this mixture was added 15d-PGJ_2-biotinamide (18 μM) in DMSO (final concentration of DMSO in the mixture was 4%). The mixture was incubated at 37 °C for 3 h. The mixture was then separated by SDS-PAGE and analyzed by immunoblotting with a streptavidin-HRP conjugate and anti-HIV Tat antibodies (Abcam).

Statistical analysis – When necessary, data are expressed as means ±sd. Student's *t* test was used in statistical analysis for comparison, and $p < 0.05$ was used as the criterion for statistical significance.

Results

Michael acceptor electrophiles (MAEs) inhibit Tat-mediated HIV transcription and HIV replication – To examine the effect of 15d-PGJ_2 on HIV transcription, promonocytic U937 cells were transfected with an HIV LTR-Luc reporter and Tat expression vector (pCITat), followed by treatment of cells with different concentrations of 15d-PGJ_2. As a control, we used 9,10-dihydro analog of 15d-PGJ_2 that lacks the ability to bind to protein thiols (see structures in Fig. 18). 15d-PGJ_2 inhibited the Tat-dependent transactivation of the reporter in a dose-dependent manner, inhibiting LTR activity by ~80% at the highest concentration tested, while the 9,10-dihydro-15d-PGJ_2 displayed

only minimal activity in repressing Tat-dependent LTR activity (Fig. 19A). Treatment of U937 cells with 15d-PGJ_2 or 9,10-dihydro-15d-PGJ_2 did not cause any significant changes in cell viability. Because the major difference between 15d-PGJ_2 and 9,10 dihydro-15d-PGJ_2 is the α,β-unsaturated enone moiety in 15d-PGJ_2, these data suggest that the reactive moiety is required for 15d-PGJ_2-dependent inhibition of Tat-dependent transcription. In addition, we examined the role of PGD_2, PGE_2, and $PGF_{2\alpha}$ on the transactivation function of Tat. Results shown in Fig. 19B clearly indicate the inhibitory activity is specific to 15d-PGJ_2. To determine whether 15d-PGJ_2 inhibited HIV replication in infected cells, we exogenously added 15d-PGJ_2 at concentrations of 0.8 and 3.1 μM to HIV-infected U937 macrophages 24 h post-infection. HIV replication was monitored by measuring the release of the capsid protein, p24, using ELISA. Treating infected cells with 15d-PGJ_2 decreased p24 levels 3 d postinfection by greater than 75% when compared with cells treated with the vehicle control (DMSO) (Fig. 20A). 9,10-Dihydro-15d-PGJ_2 did not cause any significant reduction in p24 levels again, implicating the importance of the enone moiety in 15d-PGJ_2 as being necessary for the antiviral property.

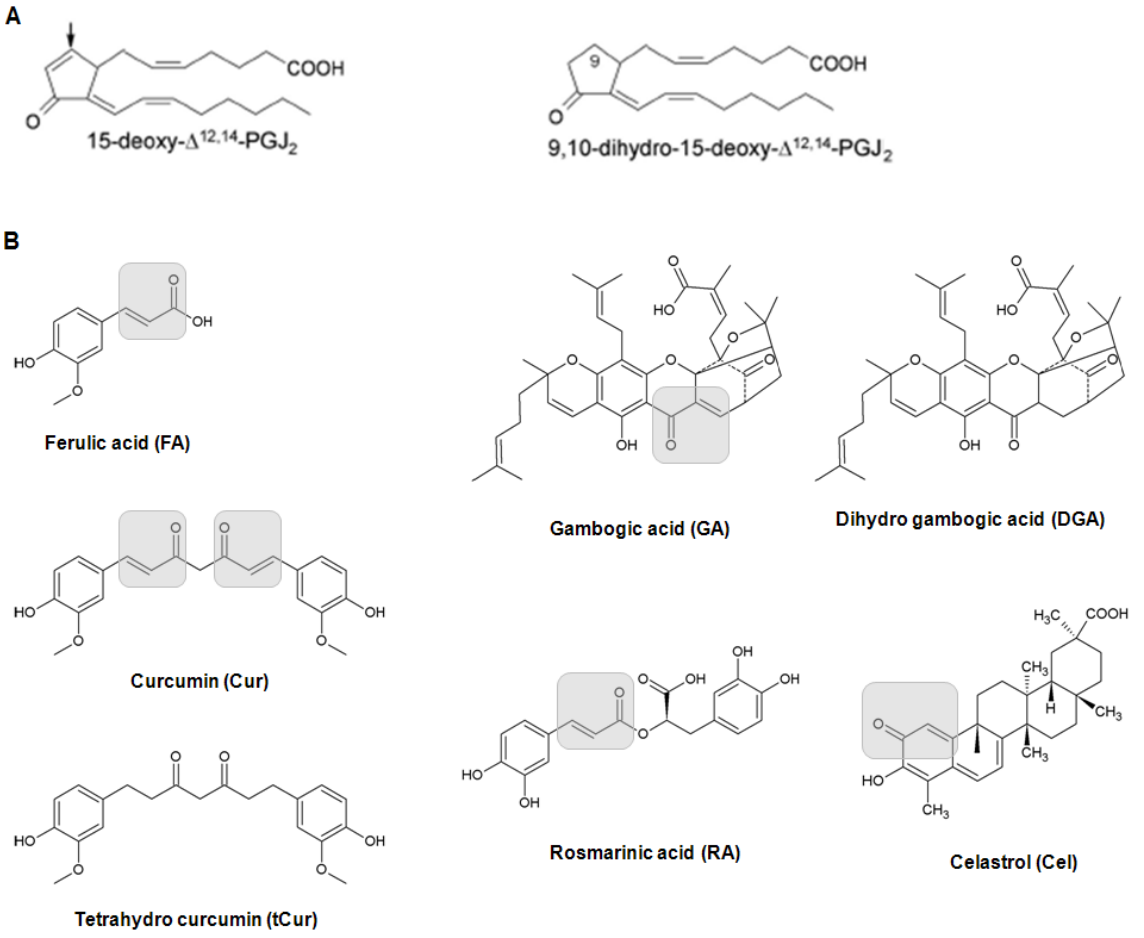

FIGURE 18. **Structures of different MAEs.** A, Arrow indicates electrophilic carbon at C-9 in 15d-PGJ_2. B, Gray highlighting denotes the α,β-unsaturated enone. 9,10-dihydro-15d-PGJ_2, DGA and tCur are inactive analogs of 15d-PGJ_2, GA and Cur, where the α,β-unsaturation has been reduced.

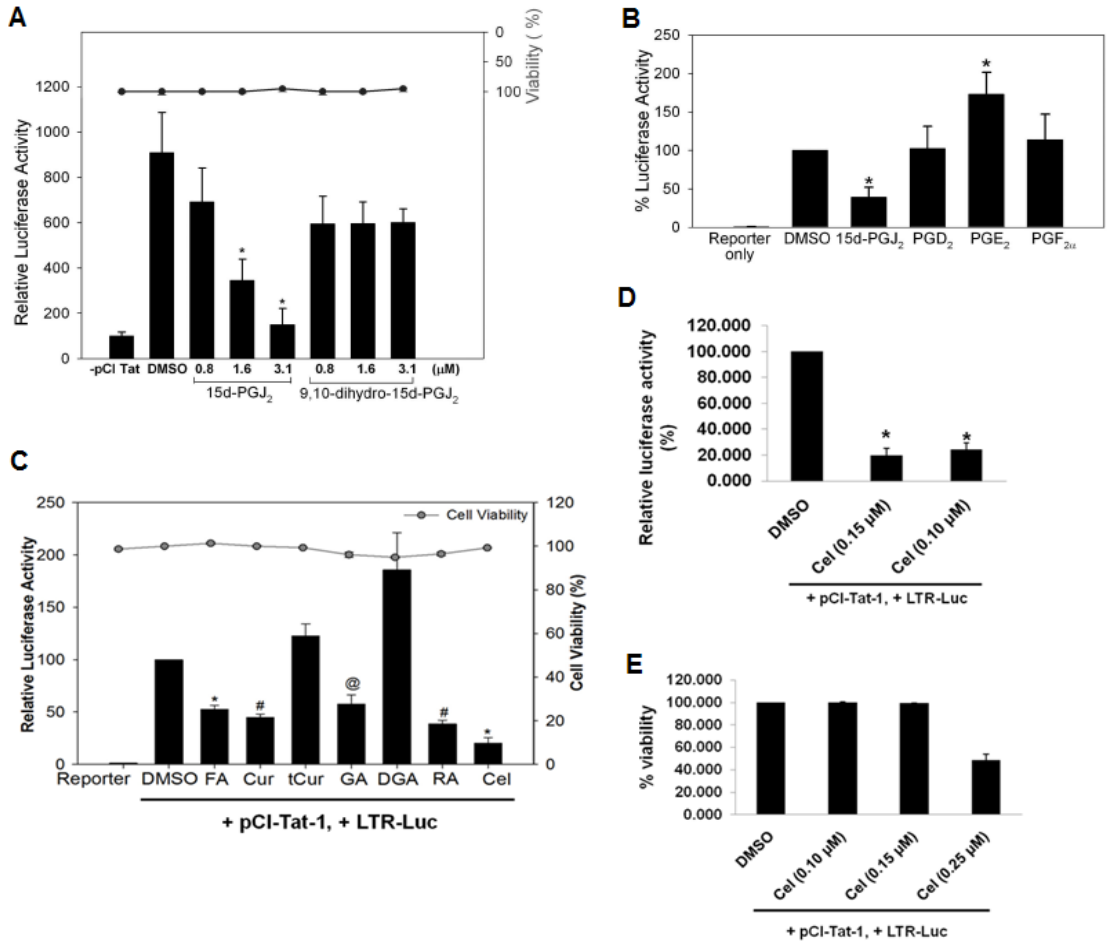

FIGURE 19. **Michael acceptor electrophiles (MAEs) inhibit Tat-mediated HIV transcription.** A, U937 cells were transfected with HIV-LTR-Luc and pCI-Tat using the Jurkat cell transfection reagent (Mirus). Transfected cells were treated with 0.8, 1.6, and 3.1 μM 15d-PGJ_2 or 9,10-dihydro-15d-PGJ_2 for 24 h. Luciferase activities in lysates were measured after 24 h and normalized to total protein. Lanes 1 and 2 represent LTR-Luc + pCI empty vector and LTR-Luc + pCI-Tat in the presence of 0.1% (v/v) DMSO, respectively. Line and circles represent cells treated with PGs or vehicle control subjected to cell viability assays using CCK-8 kit (Dojindo). B, Transiently transfected U937 cells from the above experiment were treated with 3 μM of 15d-PGJ_2, PGD_2, PGE_2, or $PGF_{2\alpha}$. Changes in luciferase activity with each treatment were compared with pCI-Tat transfected cells treated with DMSO. *; $p < 0.05$. C, U937 cells were transfected with LTR-Luc only or LTR-Luc and pCI-Tat or pCI vector. These cells were treated with the 3 μM of FA, Cur, tCur and RA, 0.25 μM of GA and DGA, 0.15 μM of Cel, or 0.1% DMSO as the vehicle control. Luciferase activities from the cell lysates were normalized to protein and compared to the activity of the vehicle treated cells, which was set at 100%. Viability of transfected cells when treated with compounds at the indicated concentrations was analyzed using the CCK-8 kit. @; $p < 0.005$ *; $p < 0.00005$, #; $p < 0.000005$. D, Dose dependent inhibition of Tat activity by Cel. *; $p < 0.00005$. E, Viability of transfected U937 cells at the indicated concentrations of Cel.

Similarly, to examine the effect of the natural compounds on Tat-dependent HIV transcription, human promonocytic U937 cells were transfected with the HIV LTR-Luc reporter and Tat expression vector (pCI-Tat-1) or vector control (pCI), followed by treatment with the five MAE compounds shown in Fig. 18B. It was observed that FA, Cur, GA, RA and Cel inhibited Tat-dependent transcription by ~ 50%, ~ 55%, ~ 40%, ~ 60%, and ~ 80% respectively. Analogs of Cur and GA, tetrahydro curcumin (tCur) and dihydro gambogic acid (DGA) (where the α,β-unsaturated ketone moiety is reduced; Fig. 18B) were used as negative controls and did not inhibit Tat-dependent transcription (Fig. 19C). Viability of transfected U937 cells upon treatment with the compounds at all the concentrations tested was not affected (Fig. 19C). Because Cel had the largest impact on Tat-mediated transcription, we focused our studies on this compound. We used the highest concentration of Cel (0.15 μM) which had no effect on cell viability in our assays, and which showed the most inhibition of Tat-mediated transcription (Fig. 19D and 19E).

To determine if Cel inhibits HIV replication in infected U937 cells, 0.15 μM Cel was added exogenously to U937 cells infected with HXB.2 as described. The extent of HIV replication was measured by p24 ELISA every 24 h for 5 d. We observed that treating infected U937 cells with Cel slowed HIV replication significantly when compared to vehicle treated cells (Fig. 20 B).

We also analyzed Tat-mediated transcriptional elongation using SYBR Green quantitative PCR (qPCR) from virus infected U937 cells treated with Cel. Infected U937 cells were treated with 0.15 μM Cel for 5 d as before. After 5 d, the total RNA from cells was harvested with Trizol (Invitrogen) and elongated transcripts were examined by qPCR

with prevalidated primers. We observed that Cel significantly inhibited Tat-mediated transcriptional elongation in infected cells (Fig. 20C).

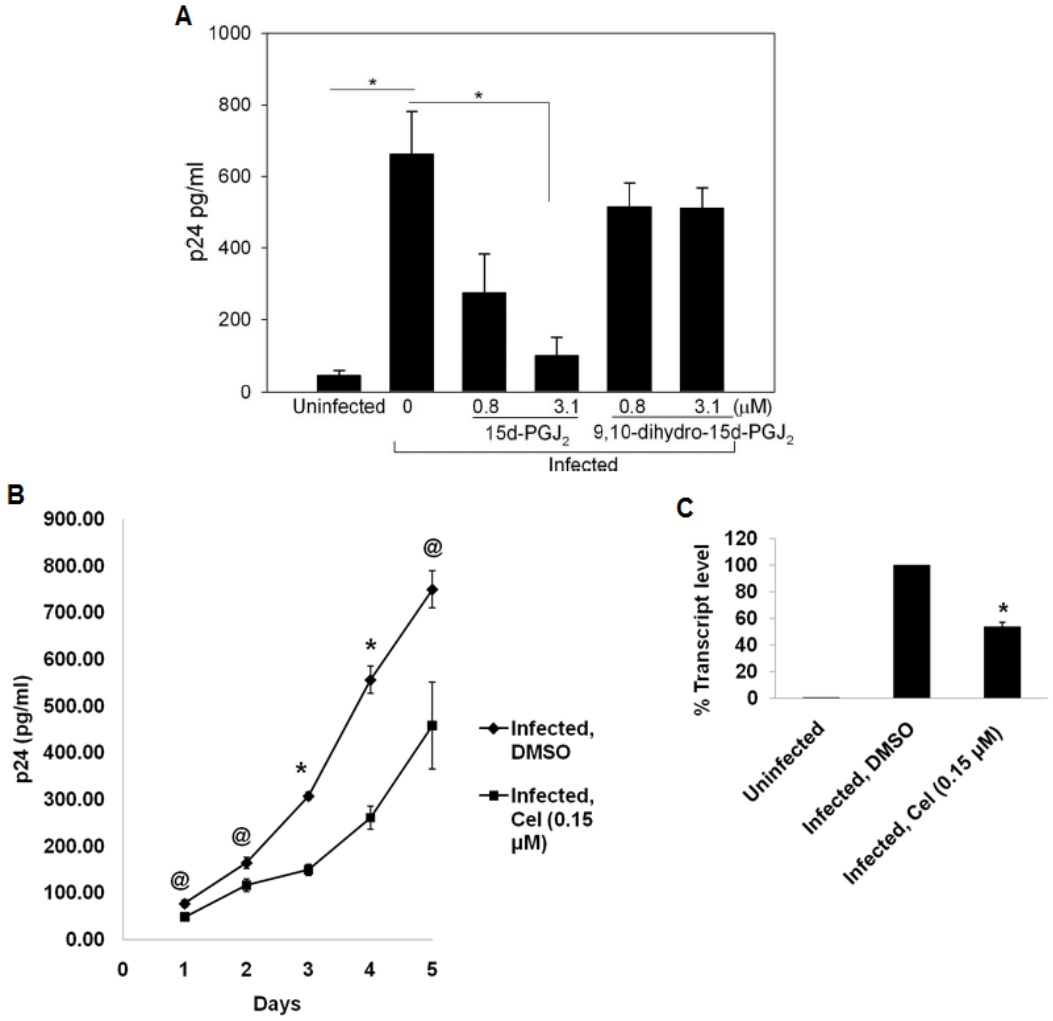

FIGURE 20. **MAEs inhibit HIV replication *in vitro*.** A, U937 cells were infected with HIV-1 and treated with indicated amounts of 15d-PGJ_2 or 9,10-dihydro-15d-PGJ_2. Three days postinfection, supernatants were collected and assayed for viral replication by p24 ELISA. DMSO was added to infected cells in the absence of the CyPGs as a negative vehicle control. *; $p < 0.05$. B, U937 cells were infected with HXB.2 for 5 d followed by treatment with 0.15 μM of celastrol for 72 h with a fresh dose every 24 h. Supernatants were collected every 24 h post treatment and were assayed for p24 levels by ELISA. @; $p < 0.05$, *; $p < 0.005$. C, RNA isolated from the cells were used for qPCR analysis of elongated viral transcripts. *; $p < 0.0005$.

Inhibition of Tat-dependent transcription is independent of the NF-κB pathway – Given that 15d-PGJ_2 and Cel inhibit multiple steps of the NF-κB signaling pathway and that NF-κB activation positively affects HIV transcription, we decided to examine the contribution of NF-κB inhibition in MAE-dependent modulation of Tat function. To address the role of NF-κB in HIV provirus transcription, we used an LTR-Luc reporter that lacked NF-κB sites (ΔκBLTR-Luc). As shown in Fig. 21A, even in the absence of NF-κB binding sites, there was ~90% decrease in Tat-dependent LTR activity, suggesting that 15d-PGJ_2 was not inhibiting the Tat activity through an NF-κB-dependent mechanism. 15d-PGJ_2 had no effect on the luciferase reporter activity in the absence of Tat expression.

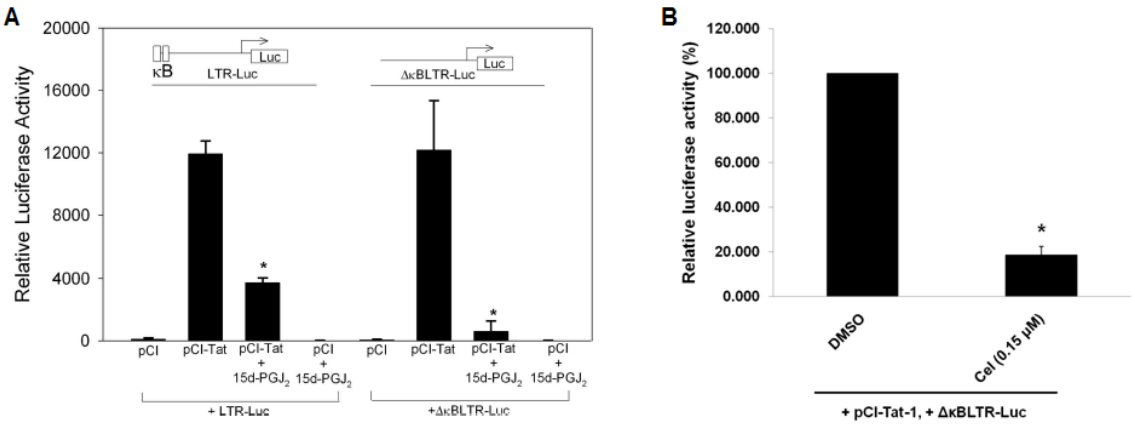

FIGURE 21. **Inhibition of Tat-dependent transcription by MAEs is independent of the NF-κB pathway.** A, U937 cells were transfected with 1 μg of LTR-Luc or ΔκBLTR-Luc and 2 μg pCI vector or pCI-Tat for 24 h. Transfected cells were incubated with 15d-PGJ_2 at a final concentration of 3 μM for 24 h. Luciferase activities in lysates were measured as described in text. *; $p < 0.05$. B, U937 cells were transfected with ΔκBLTR-Luc only or ΔκBLTR-Luc and pCI-Tat-1 or pCI vector. Cells were treated with 0.15 μM Cel or DMSO (final concentration 0.1%) as the vehicle. Luciferase activities from the lysates of the cells were normalized to protein and compared to the activity of the vehicle treated cells, which was set at 100%. *; $p < 0.005$.

Similarly, even in the absence of NF-κB binding sites, there was a significant decrease (~80%), in Tat-dependent LTR activity in cells treated with Cel, suggesting that Cel inhibits HIV transcription via a NF-κB-independent mechanism (Fig. 21B)**.**

Taken together, these results confirm previous reports that MAEs inhibit HIV transcription, and further demonstrate the results of earlier studies by showing that inhibition of NF-κB is not the operative mechanism for repressing Tat-dependent HIV transcription.

Interaction of 15d-PGJ_2 and 9,10-dihydro-15d-PGJ_2 with rTat *in vitro* – To address the possibility of a direct interaction of 15d-PGJ_2 with the Cys residues, and thus affecting the biological activity of Tat, we first examined whether 15d-PGJ_2 interacted with rTat. Given that purified rTat is not commercially available, we used a bacterial expression system to express rTat. Western blot analysis with anti-Tat monoclonal antibodies confirmed that the bacterially expressed, affinity purified, and oxidatively refolded protein indeed was Tat (Fig. 22A). Furthermore, to demonstrate that the rTat expressed in a bacterial system was biologically active, we utilized the U937 cells transfected with the LTR-Luc reporter. The ability of ectopically added rTat to activate the HIV LTR-Luc reporter and the iNOS promoter in U937 cells suggested that the rTat was biologically active (Fig. 22B, C). Further biochemical characterization of rTat using Ellman's reagent (dithionitrobenzoic acid; DTNB) and redox Western immunoblotting techniques demonstrated that purified and refolded rTat contained 3 free thiols/mol protein (data not shown), which is consistent with that reported earlier (Koken *et al.* 1994).

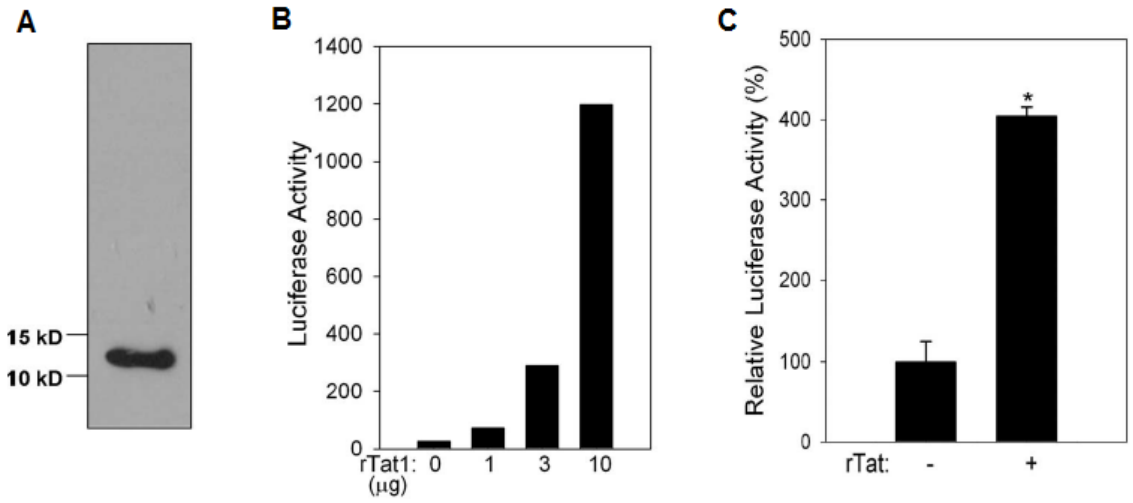

FIGURE 22. **Bacterial expression and biological activity of rTat.** A, Western blot showing the immunoreactive band corresponding to purified and refolded rTat. B, rTat activates LTR-Luc in U937 cells. Cells were transfected with LTR-Luc reporter, and then treated with increasing amounts of rTat. C, Ability of rTat to activate iNOS promoter. U937 cells were transfected with 2 μg iNOS-Luc for 24 h. Transfected cells were then treated with or without refolded rTat protein (180 nM). Luciferase activity was measured 48 h post-rTat treatment. *; $p < 0.05$.

This bacterially expressed active rTat was used to prepare a complex with 15d-PGJ_2 or 9,10-dihydro-PGJ_2 in an *in vitro* reaction. The reaction mixture was subjected to gel-filtration chromatography using a Biogel P6 spin column, to separate the unreacted 15d-PGJ_2 or 9,10-dihydro-PGJ_2. Tat activity in the eluate was measured by adding eluate to U937 cells transfected with LTR-Luc. As a control, the flow-through fraction of rTat protein without 15d-PGJ_2 caused a significant increase in the luciferase activity when added exogenously to cells; while DMSO-treated buffer, processed through the column, had no effect on the luciferase activity (Fig. 23). Previous studies have shown that rTat is rapidly taken up by serum-starved cells to transactivate the HIV LTR (Henderson *et al.* 1995). Interestingly, treatment of cells with the 15d-PGJ_2-rTat complex caused a significant inhibition of LTR-Luc activity, while the rTat incubated with 9,10-dihydro-15d-PGJ_2 still retained 80% of the activity. rTat that was not incubated with CyPG but

passed through the Biogel P6 column, was used as a positive control. These data suggest the possibility of alkylation of Tat by 15d-PGJ_2 as a likely mechanism of inactivation. The physical interaction between 15d-PGJ_2 (*via* electrophilic carbon-9) with the free thiol groups in Tat was more rigorously examined using an *in vitro* binding assay with purified rTat and biotinylated 15d-PGJ_2 (Cayman Chemicals). rTat was treated with excess of 15d-PGJ_2 (7 mol 15d-PGJ_2/mol rTat, based on a total of 7 cysteine residues in completely reduced Tat) in PBS for 3 h at 37°C. Samples were separated by PAGE followed by Western blot analysis with streptavidin-HRP. As shown in Fig. 26A, 15d-PGJ_2 covalently bound rTat; while 9,10-dihydro analog failed to interact with rTat. Furthermore, increasing the thiol reactivity of rTat with the reducing agent DTT (1 mM) enhanced the binding of 15d-PGJ_2, suggesting that all seven cysteines were likely alkylated.

To confirm that Tat and 15d-PGJ_2 physically interact in the presence of other cellular proteins, U937 cells were cultured with or without rTat, and cell lysates were prepared and incubated with 15d-PGJ_2-biotinamide. Tat-15d-PGJ_2 complexes were pulled down using a Neutravidin-pull down assay, separated on PAGE, and Tat was detected by immunoblotting. As shown in Fig. 24B, rTat interacted with 15d-PGJ_2-biotinamide and specifically pulled down rTat from cell extracts, providing additional evidence that 15d-PGJ_2 directly interacts with HIV-1 Tat.

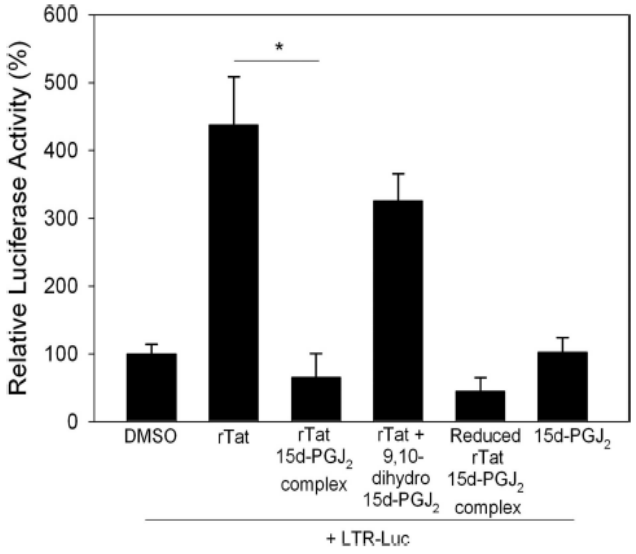

FIGURE 23. **15d-PGJ2 inhibits rTat activity.** rTat was incubated with 15d-PGJ2-biotinamide (1:7 mol/mol) or 9,10-dihydro-15d-PGJ2 (1:7 mol/mol). Some rTat samples were reduced with DTT (1 mM) for 30 min prior to incubating CyPGs. Reaction mixtures were passed through a Biogel P column; flow-through fraction was added to serum-starved U937 cells transfected with the LTR-Luc reporter vector. Cells were lysed 48 h post-addition, and luciferase activity was measured. rTat that was not incubated with CyPG, but passed through the Biogel P column, was used as a positive control. *; $p < 0.05$.

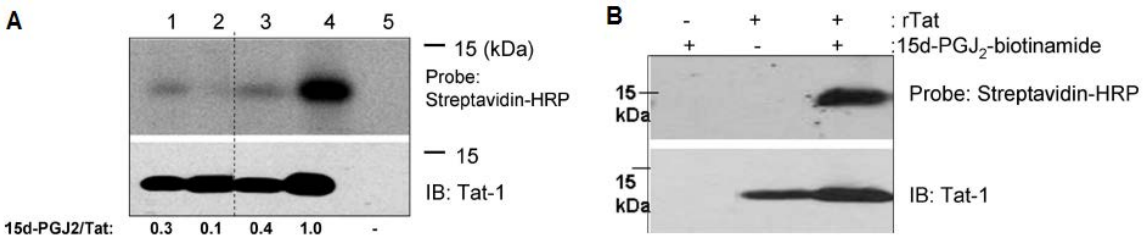

FIGURE 24. **15d-PGJ_2 interacts with rTat.** A) Purified rTat was incubated with 15d-PGJ_2-biotinamide (1:7 mol/mol) or 9,10-dihydro-15d-PGJ2 (1:7 mol/mol) before and after reduction with DTT (10 M and 1 mM) for 30 min. Interaction was analyzed on an SDS-PAGE gel followed by Western blot analysis with streptavidin-HRP. Concentration of rTat was 180 nM. Membrane was stripped and reprobed with anti-Tat-1 monoclonal antibody. Densitometric evaluation of the bands is shown at bottom. Lanes 1–5 represent rTat 15d-PGJ2-biotinamide, rTat 9,10-dihydro-15d-PGJ_2-biotinamide, rTat reduced with 10 M DTT 15d-PGJ2-biotinamide, rTat reduced with 1 mM DTT 15d-PGJ_2-biotinamide, and 15d-PGJ_2-biotinamide alone, respectively. B) U937 cells were incubated with rTat (180 nM) for 12 h. Cells were washed with PBS and lysed using M-PER. 15d-PGJ_2-biotinamide was added to lysate at a final concentration of 1 M and incubated on ice for 3 h. Biotinylated proteins were isolated using neutravidin beads, and interaction was analyzed on an SDSPAGE gel followed by Western blot analysis with streptavidin-HRP. Blot was stripped and reprobed with anti-Tat monoclonal antibody.

Interaction of Cel with rTat *in vitro* – To address the possibility that a direct interaction of Cel with the Cys residues in Tat affects its biological activity, we performed binding analyses with celastrol biotinamide (bCel; synthesized in the lab; Fig. 25A) and rTat. We bacterially expressed rTat. rTat was incubated with bCel at a molar ratio of 1:10 (rTat:bCel) in 50 mM Tris-Cl buffer, pH 7.5 for 3 h at 37°C. Samples were separated by PAGE followed by Western blot analysis with streptavidin-HRP. As shown in Fig. 25B, bCel covalently bound rTat. As a control, we incubated bCel with GSH prior to incubating it with rTat. GSH will form an adduct with bCel across the α,β-unsaturated moiety. The binding of such a GSH modified bCel to rTat was found to be much reduced. We used Cel to compete with bCel for rTat binding, which resulted in reduced biotinamide signal as observed by probing with streptavidin-HRP indicating specific binding. Using AMS (an iodoacetamide derivative) to alkylate the Cys-thiols in rTat prior to treating it with bCel, we showed that the binding of Cel to Tat occurs specifically with the Cys. Furthermore, using UV-Vis spectroscopy, we demonstrated that Cel binds to Tat. As in Fig. 25B, rTat (0.9 μM) and Cel (9.0 μM) were incubated with each other at a molar ratio of 1:10 at 37 °C. The reaction mixture was subjected to extraction with ethyl acetate five times to remove any unreacted Cel, and the resulting aqueous phase was used to perform UV/Vis spectroscopy. As a control, 9.0 μM of Cel alone was incubated in buffer under the same conditions, and then extracted with ethyl acetate five times. This extracted the entire free (unbound) Cel from the solution, resulting in the sample showing no absorption in the visible range, where Cel displays a λ_{max} at 419 nm. As seen in Fig. 25C, rTat showed no absorption in the visible range; however, Cel-modified rTat exhibited a λ_{max} at 404 nm. The increase in absorbance of rTat-Cel complex (modified

rTat) in the visible region, as compared to rTat alone, accompanied by a hypsochromic shift in the λ_{max} of the compound is suggestive of an interaction of Cel with rTat.

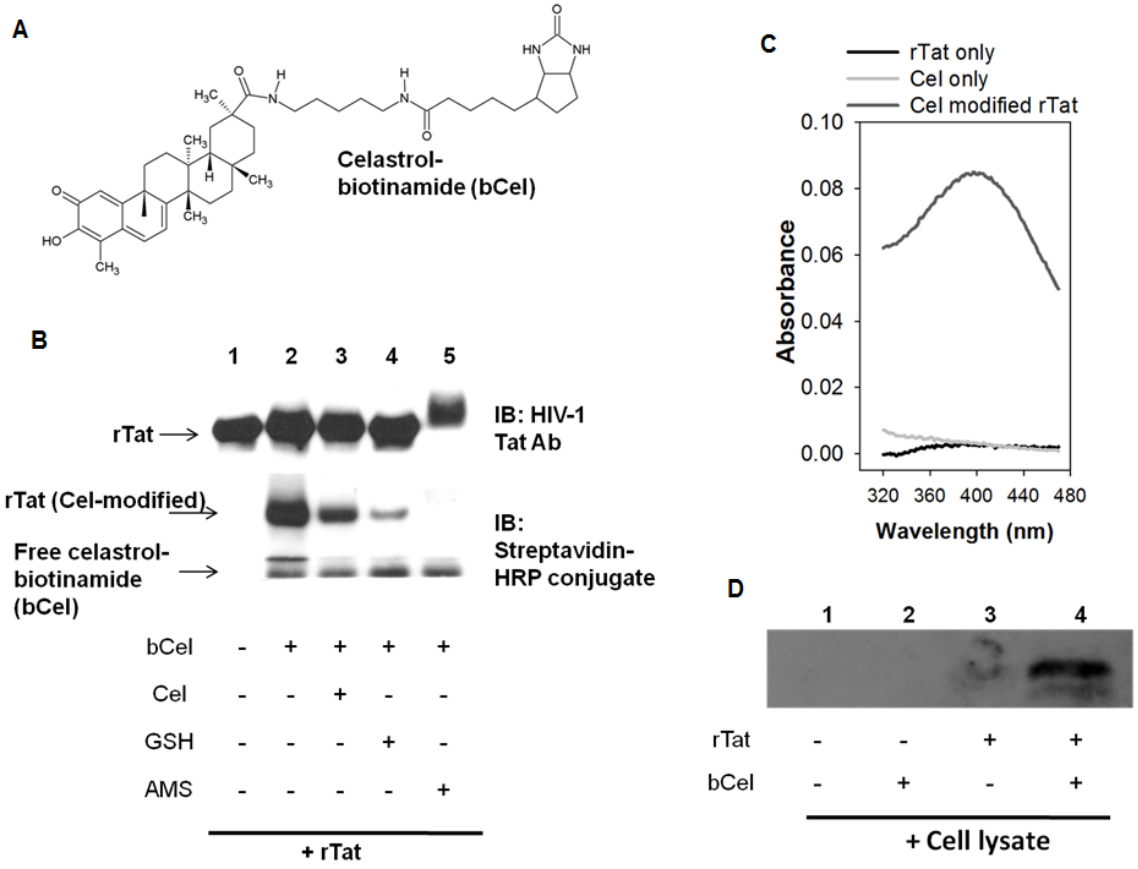

FIGURE 25. **Celastrol interacts with rTat**. A, Structure of celastrol-biotinamide (bCel). B, 0.9 μM of rTat was incubated with 9.0 μM bCel (lane 2), both Cel (9.0 μM) and bCel (lane 3), and bCel conjugated with 90.0 μM GSH (lane 4). The Cys thiols in rTat were modified with 9.0 μM AMS, and this rTat was incubated with bCel (lane 5). The mixtures were incubated at 37°C for 3 hours. Interaction was analyzed by SDS-PAGE followed by Western transfer and probing the membrane with streptavidin-HRP conjugate and anti-Tat antibodies. C, UV/Vis analysis of the interaction of Cel and rTat. D, Interaction of bCel and rTat in the presence of U937 cellular proteins (rTat:bCel = 1:10). Representative of n = 3 shown.

As a proof of principle, we incubated rTat and bCel in the presence of U937 cell lysates to determine if Cel can interact with Tat in the presence of cellular proteins. rTat (250 ng, 0.023 nmol) and bCel (173 ng, 0.23 nmol) were incubated with each other in a molar ratio of 1:10 in the presence of 100 μg of U937 cellular protein in a total volume of 100

µl. The mixture was subjected to pull-down by neutravidin-agarose beads. Immunoblotting clearly demonstrated that that bCel could interact with Tat even in a cellular milieu (Fig. 25D).

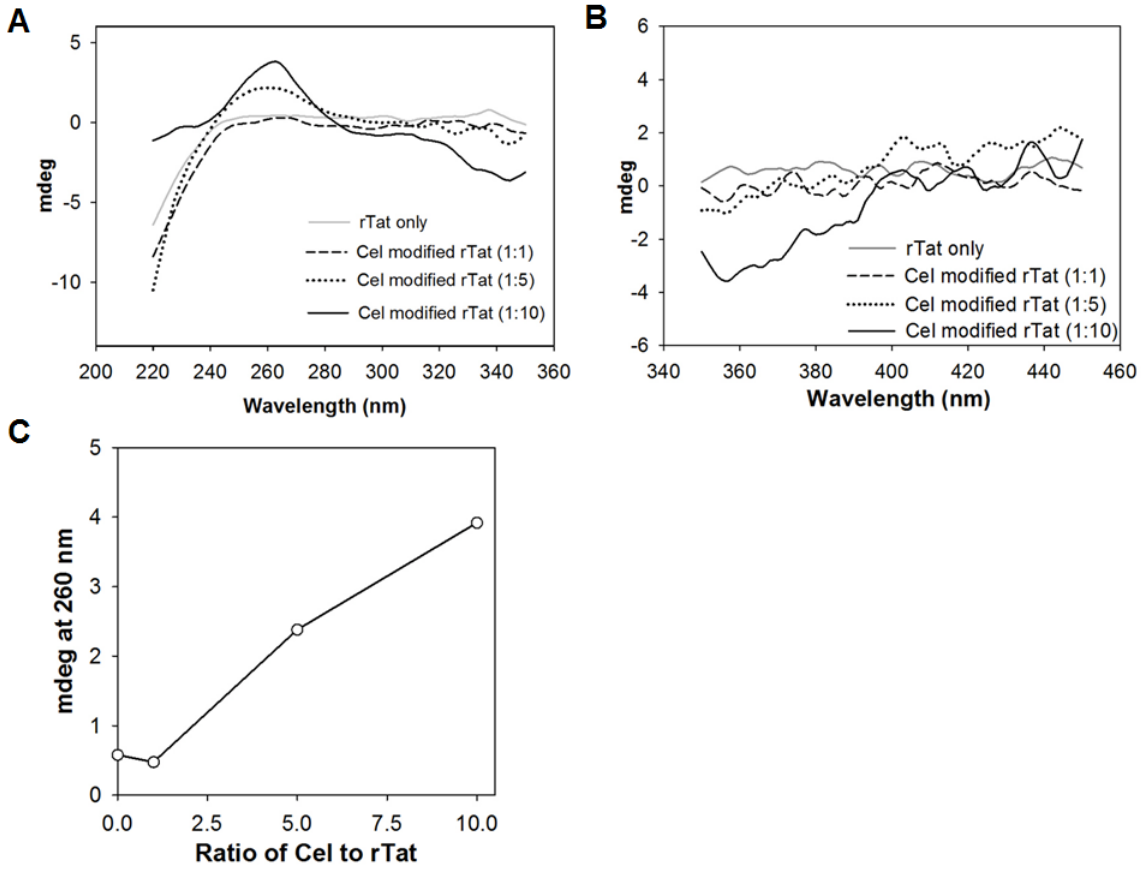

FIGURE 26. **Circular Dichroism analysis of rTat and modified rTat.** A, Near-UV and far-UV CD spectra of rTat and rTat modified with various concentrations of Cel. B, CD spectra in the visible range of the same samples. C, Increase in the ellipticity of rTat at 260 nm when treated with increasing concentrations of Cel. Representative of n = 3 shown.

Inhibition of Tat-dependent transcription may be due to structural changes in Tat – Cel is a MAE that would be expected to modify the Cys rich domain of Tat promoting a conformational change inactivating Tat. To formally test this possibility we performed circular dichroism (CD) spectroscopic studies on free and modified rTat, to detect any

changes in the structure of modified Tat when compared to the native form. rTat was incubated in buffer alone, or with Cel at different molar ratios (1:1, 1:5, 1:10, the concentration of rTat was kept constant at 50 μg/ml), for 3 h at 37°C, and subsequently the samples were subjected to CD spectroscopy. The CD bands of proteins in the far-UV region (220 – 260 nm) are due to the amide linkages in the protein backbone, and these are sensitive to the conformations of the same. A change in this region signifies a shift in the conformation of the protein backbone. The bands in the near-UV region (260 – 350 nm) in the CD spectrum of proteins are due to aromatic amino acids and prosthetic groups, like the thiol-group in cysteines. As seen in Fig. 26A, a shift in the spectrum of Cel-modified Tat as compared to native Tat in the near-UV and far-UV regions was clearly evident. While the spectrum of native rTat was constant around zero in the near-UV region, it became negative in the far-UV region. When the molar concentrations of both rTat and Cel were equal, the spectra of native rTat and modified rTat were similar. As the concentration of Cel in the reaction was increased, the spectra of modified rTat became more positive in both the far-UV and near-UV regions (between 260 – 280 nm). At 10 times the molar concentration of Cel as compared to rTat, the spectrum of modified rTat became slightly more negative in the 320 – 350 nm range of the near-UV region. The spectra of the various concentrations of Cel, used as controls, were increasingly negative in the far-UV region, while they became increasingly positive in the 320 – 350 nm region of the near-UV region (data not shown). CD spectra in the visible range of native and Cel-modified rTat were not significantly different (Fig. 26B). The change in the far-UV region could be attributed to a change in the secondary structure of the Tat protein, while the modification of Cys thiols in Tat could explain the shift in the near-UV

spectrum of modified Tat as compared to native Tat. On examining the ellipticity of rTat at 260 nm, we found that it increased steadily as the concentration of Cel increased in the reaction mixture (Fig. 26C).

Modification of Tat by Cel does not affect its interaction with the HIV TAR element – To try and pinpoint the step that Cel inhibits upon modifying Tat, we designed a binding assay to test the interaction of Cel-modified Tat with a 29 bp RNA ^{32}P-labeled oligonucleotide representing the HIV TAR element (Hamma *et al.* 2003). We found that modification of Tat with Cel does not affect the binding of Tat with the TAR RNA oligo in our system (Fig. 27).

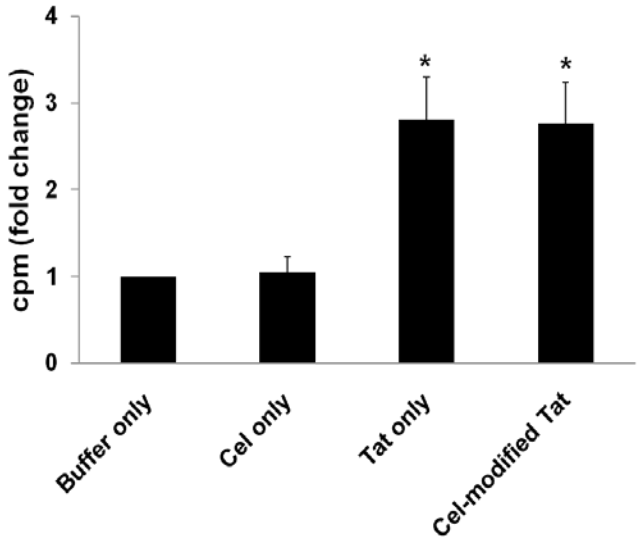

FIGURE 27. **Binding of HIV TAR RNA fragment to Cel-modified Tat.** 100 µg of His-tagged Tat protein was incubated with 41 µg of Cel (1:10 molar ratio) at 37 °C for 3 h, and bound to a cobalt resin. ^{32}P-labeled HIV TAR RNA oligonucleotide was then incubated with the resin bound modified Tat for 1 h at room temperature. The resin was washed with 50 mM Tris-Cl, pH 7.5 following which, the resin was then subjected to scintillation counting. Buffer incubated resin, later incubated with the labeled oligo, was used as a control, and fold increases in cpm were determined. Native Tat was used as a positive control.

Synergistic action of the thioredoxin system and MAE modification on Tat – As has been shown before; reduction of Tat by DTT increases the number of free thiols which can be alkylated by MAEs (Fig. 24A). We performed an *in vitro* experiment to investigate whether the thioredoxin system can in conjuction with MAEs to "over" acetylate HIV Tat. Tat was incubated with TR1 in the presence of the cofactor NADPH, and subsequently incubated with 15d-PGJ_2-biotinamide. The mixtures were separated by SDS-PAGE and analyzed by western blotting. As seen in Fig. 28, treatment of rTat with TR1 or reduced Trx cause more binding of 15d-PGJ_2-biotinamide per molecule of Tat.

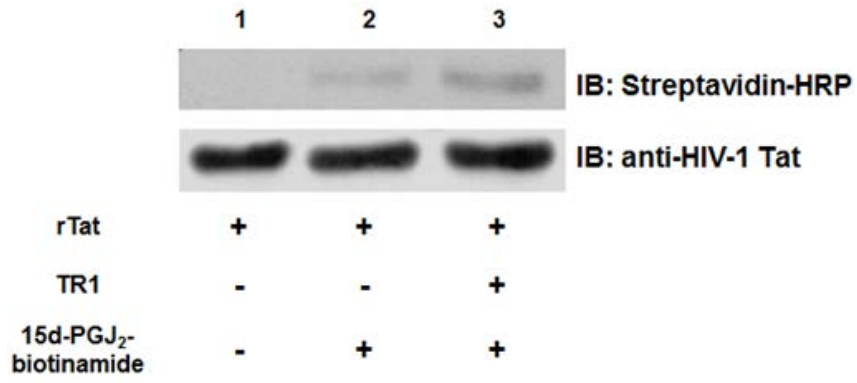

FIGURE 28. **Enhanced binding to 15d-PGJ_2 to Tat after action of TR1.** A. rTat was incubated with or without TR1, and then subsequently incubated with 15d-PGJ_2-biotinamide. The mixtures were analyzed by immunoblotting with streptavidin-HRP conjugate and anti-Tat antibodies. Representative of n = 2.

Discussion

We have demonstrated that MAEs have the ability to inhibit Tat-mediated transactivation of HIV LTR activity. Our studies, for the first time, demonstrate that the inhibitory property of 15d-PGJ_2 and Cel towards Tat involves an intriguing mechanism of modifying Cys thiols in Tat in a covalent reaction.

The binding of Tat to the TAR element is a key event that recruits the positive transcription elongation factor P-TEFb to the HIV promoter (Karn 1999). pTEFb causes the hyperphosphorylation of the CTD of RNAPII, which enhances the processivity of RNAPII and increases HIV proviral transcription. We used a reporter construct consisting of the LTR region of HIV coupled to luciferase gene to specifically address the role of MAEs on transcription. We cotransfected this construct with pCI-Tat-1 into U937 cells, then treated these cells with MAEs to determine if the compounds had any inhibitory activity towards Tat. While our studies demonstrated that the MAEs had inhibitory activity against Tat, the analogs of the MAEs (lacking the α,β-unsaturated carbonyl functionality) had no inhibitory effect. Prostaglandins that lacked the cyclopentenone enone, such as PGE_2, $PGF2_{2\alpha}$, and PGD_2, also did not affect the activity of Tat, establishing the importance of the α,β-unsaturated carbonyl moiety for the inhibitory mechanism. The MAEs inhibited the transactivation activity of Tat by varying degrees. This suggests that there is some level of specificity to the interaction of the compounds with Tat, despite containing the reactive electrophilic carbon. Indeed, it is interesting to note that in Trx, which contains two thiol/disulfide motifs (Watson *et al.* 2003), 15d-PGJ_2 modifies only one Cys in each of these motifs (Shibata *et al.* 2003).

Michael acceptor electrophiles have been shown to inhibit replication of multiple RNA and DNA viruses, including poxviruses (Santoro *et al.* 1982) paramyxoviruses (Amici *et al.* 1991), togaviruses (Mastromarino *et al.* 1993), rhabdoviruses (Santoro *et al.* 1983) and HIV (Rozera *et al.* 1996), although the mechanisms by which MAEs target these viruses are still unclear. It is assumed that some of the antiviral activity of CyPGs and other MAEs is associated with their ability to suppress NF-κB signaling. 15d-PGJ_2 has

been shown to covalently modify cysteines in the p50 and p65 subunits of NF-κB, as well as a Cys in the activation loop of IKKβ (Rossi *et al.* 2000). Binding of 15d-PGJ_2 to reduced Cys residues in p50, p65, and IKKβ inhibits the activation and consequent DNA recognition and binding of NF-κB (Stamatakis *et al.* 2006). NF-κB is also a critical cellular transcription factor that binds to the HIV LTR and promotes efficient HIV transcription (Nabel *et al.* 1988, Kretzschmar *et al.* 1992). Inhibitors of NF-κB signaling decrease HIV provirus transcription and replication (Williams *et al.* 2006). Despite the interaction of 15d-PGJ_2 with IKKβ in the U937 monocytic system (data not shown), the ability of 15d-PGJ_2 to inhibit HIV transcription did not appear to require NF-κB binding to the LTR since HIV-LTR reporter lacking the two NF-κB binding sites was still repressed by 15d-PGJ_2. Similarly Cel, while acting as an antioxidant by affecting the NF-κB pathway, inhibited Tat activity even in the absence of NF-κB activity at the HIV promoter. However, these data do not exclude a role for NF-κB in setting the intracellular oxidative tone that could influence early Tat-independent transcription initiation, and regulation of HIV provirus.

These studies extend original observations by Rozera *et al.* (Rozera *et al.* 1996) that conclusively demonstrated the inhibitory role of CyPGs on HIV replication and suggested that the effect was possibly mediated by targeting proviral transcription. Studies by Hayes *et al.* (Hayes *et al.* 2002) have indicated the role of CyPG-activated PPARγ to cause inhibition of proviral transcription. Here, we demonstrate that 15d-PGJ_2 inhibit HIV transcription and replication by targeting thiols in Tat. We have also extended this mechanism of inhibition to Cel, thus introducing the possibility of using this and similar compounds as leads for the development of anti-HIV drugs.

Tat plays an important role in increasing the processivity of RNAPII to enable the production of elongated viral transcripts. In the absence of Tat, the formation of these elongated transcripts is reduced (Feinberg *et al.* 1991, Marciniak *et al.* 1991). Treatment of infected cells with Cel inhibited the formation of elongated viral transcripts, as tested by SYBR Green qPCR. Based on the inability of rTat-Cel complex to drive the -205LTR-Luc expression, and the fact that Tat is a critical player in recruiting host transcription machinery to LTR, we believe that Tat, once covalently modified, is unable to bind to the HIV LTR and recruit pTEFb and RNAPII efficiently to the HIV promoter. Furthermore, CD analyses of the interaction of Cel and rTat confirmed changes in structure of rTat. The spectrum of native rTat is indicative of a random coil structure. A steady positive shift in the spectra of Cel-modified rTat might indicate that the protein structure is altered to a more "ordered" form, presumably an α-helix. We speculate that such a change in the structure was likely responsible for its impaired transactivational property.

The ability of MAEs to inhibit Tat was dependent on the presence of the α,β-unsaturated enone moiety since the structural analogs did not significantly inhibit HIV transcription. Furthermore, on the basis of the binding studies, it is clear that the α,β-unsaturated group is absolutely essential for its interaction with the Cys residues. It is also clear that this interaction is specific for cysteines as modifying the cysteines covalently, completely abrogated the interaction. Reduction of rTat with DTT prior to incubating with 15d-PGJ_2 increased the binding of 15d-PGJ_2 to Tat. This observation indicates that in a reduced state all the Cys in Tat can potentially bind MAEs. Furthermore, DTT has been shown to inhibit Tat activity, suggesting a need to maintain the integrity of disulfide bonds in Tat (Koken *et al.* 1994). The results showing an increased interaction of 15d-PGJ_2 with Tat is

different from the selective interaction of 15d-PGJ_2 with Trx, where 15d-PGJ_2 modifies two Cys residues from each of the two dithiol/disulfide motifs (Shibata *et al.* 2003, Watson *et al.* 2003). In contrast to Trx, it appears that MAEs do not discriminate between different Cys thiols for alkylation in Tat, which may be attributed to the structural difference between the two proteins, particularly within the thiol/disulfide motifs. It should be noted that the interaction of MAEs with protein thiols depends on complementary interactions between the protein thiol and its surrounding environment (Pande *et al.* 2005). Taken together, these findings support the premise that binding of MAEs to Tat brings about a structural change in Tat, which ultimately leads to inhibition of proviral transcription.

We have previously shown that TR1, in selenium supplemented macrophages, reduces the disulfides in Tat to potentially increase the free thiols to seven, from three. This increases the number of potential targets for alkylation by MAEs. As shown in Fig. 33, the TR1 and reduced Trx are separately able to cause an increase in the binding of 15d-PGJ_2 to Tat. This introduces the possibility of a synergistic effect of selenium supplementation and MAE treatment on retarding HIV replication. Further studies need to be performed to investigate this possible phenomenon.

We also investigated the effect that such a modification of Tat by MAEs would have on its interaction with the HIV TAR RNA, to try and identify the step that is inhibited in viral transcription. Using an oligonucleotide-binding assay, we observed that Cel modification did not affect the interaction of Tat with the TAR element. Thus, modification of Tat probably affects its interaction with host proteins such as pTEFb,

affecting its ability to recruit host transcription factors to the HIV promoter. Such studies are currently being performed in our laboratory.

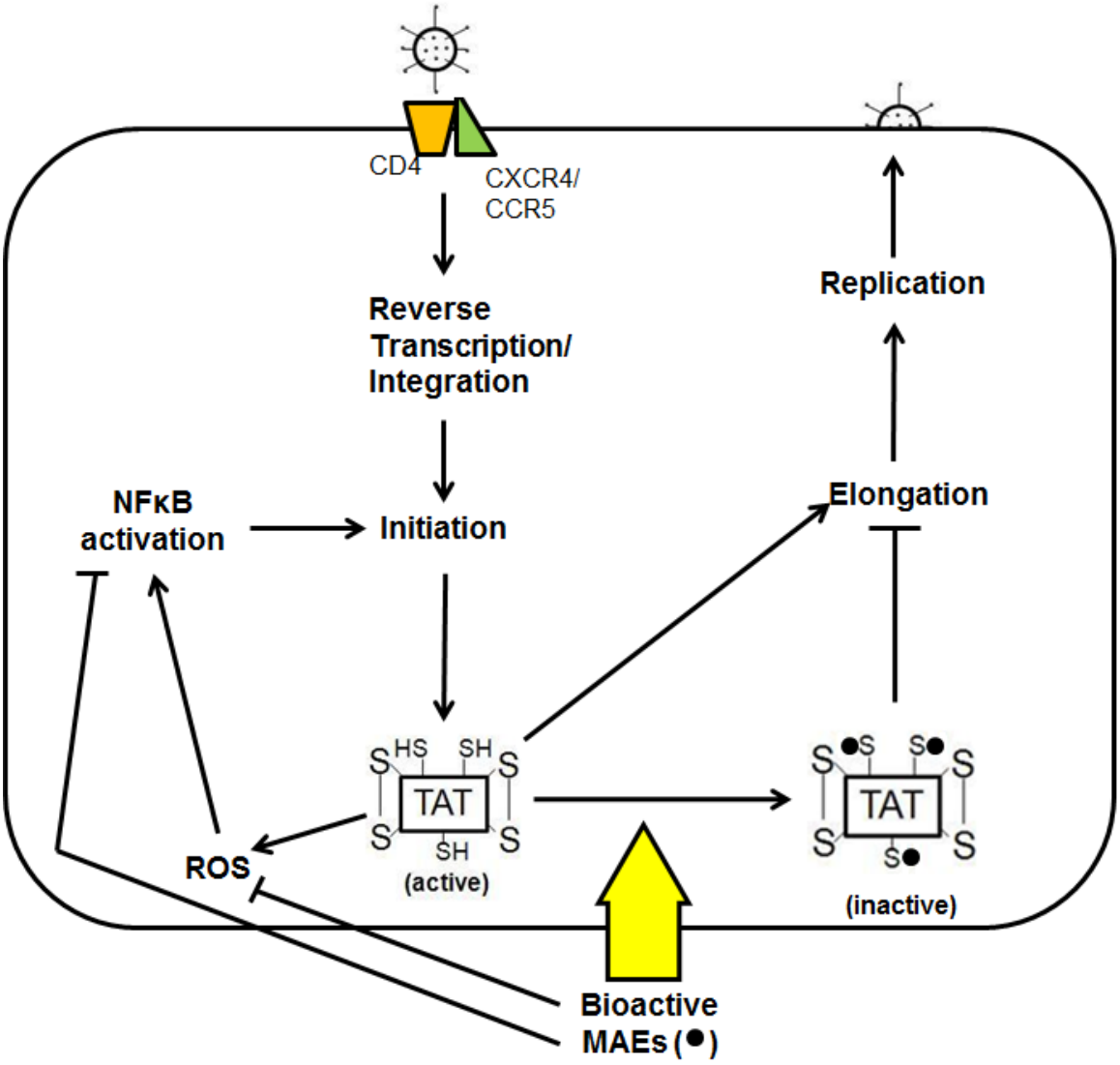

FIGURE 29. **Bioactive MAEs inhibit HIV transcription and replication.** Bioactive MAEs like 15d-PGJ_2 and Cel can, on account of their antioxidant potential, inhibit ROS formation and NFκB activation during HIV infection. In addition to this, they can target the disulfide bonds in the HIV protein Tat, and inhibit its transactivation activity, thereby inhibiting viral transcription and replication.

In summary, we have demonstrated the ability of MAEs to modify free thiols in Tat, leading to a transcriptional block. This activity of MAEs is in addition to their larger role

as antioxidants and inhibitors of the NF-κB pathway (Fig. 29). These findings open new avenues for the understanding of the antiviral effects of MAEs and may help define their therapeutic potential. More importantly, treatment with MAEs might complement other antiretroviral strategies by targeting key Cys residues in Tat that are conserved in HIV strains (both HIV-1 and HIV-2) worldwide (Fig. 30). Further work will be necessary to explore this intriguing possibility.

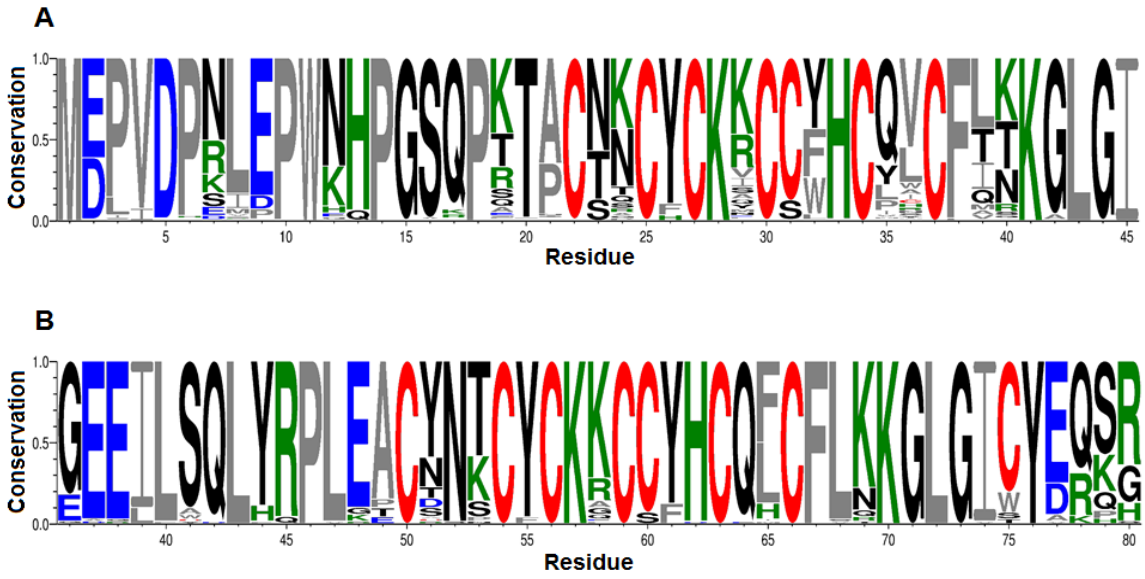

FIGURE 30. C**onservation of the Cys domain in Tat.** The HIV-1 (residues 1 – 45) (A) and HIV-2 (residues 36 – 80) (B) Tat amino acid sequences published in the 2011 compendium by the Los Alamos National Laboratory were compiled and analyzed for sequence conservation, using the WebLogo 3.3 program (http://weblogo.threeplusone.com/create.cgi). The cysteine domain in both HIV-1 and HIV-2 Tat (which are important for their function) are highly conserved across the different strains.

Chapter 4: Targeting p300 HAT activity to modulate post-translational modifications in histones and HIV-1 Tat

Part of the data presented in this chapter has been published –

Kodihalli C. Ravindra, Vivek Narayan, Gerald H. Lushington, Blake R. Peterson, and K. Sandeep Prabhu. **Targeting of Histone Acetyltransferase p300 by Cyclopentenone Prostaglandin Δ^{12}-PGJ_2 through Covalent Binding to Cys^{1438}.** *Chemical Research in Toxicology* 25(2):337-347, February 20, 2012 © American Chemical Society.

(Contributions: Dr. Ravindra Kodihalli and Vivek Narayan generated figures 32, 34, 36, 37, 38 and 39 for the manuscript. Vivek Narayan generated figures 35, 40 and 41. Dr. Gerald Lushington performed the molecular modeling of p300 with the different prostaglandin molecules.)

Abstract

Dietary selenium has been known to modulate epigenetic events like DNA methylation and histone acetylation by inhibiting DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) respectively. Acetylation of the HIV-1 Tat protein by the histone acetyltransferase (HAT) p300 is critical in its function as a transcriptional enhancer of the HIV genome. Inhibitors of HATs are perceived to treat diseases like cancer, neurodegeneration, and AIDS. Selenium has been shown to regulate the production of anti-inflammatory CyPGs by modulating the arachidonic acid – COX – H-PGDS pathway. We demonstrate here the ability of selenium to modulate the activity of p300 in a mouse macrophage model of inflammation, and also a human macrophage model of

HIV infection. Such a modulation of p300 activity by selenium may be via the production of the CyPGs. Based on previous studies, we hypothesized that Cys^{1438} in the substrate binding site of p300 could be targeted by Δ^{12}-prostaglandin J_2 (Δ^{12}-PGJ_2), a CyPG derived from PGD_2, and whose production is enhanced by selenium. A cell-based assay system clearly showed that the α,β-unsaturation in the cyclopentenone ring of Δ^{12}-PGJ_2 was crucial for the inhibitory activity; while the 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2, which lacks the electrophilic carbon (at carbon 9), was ineffective. Molecular docking studies suggested that Δ^{12}-PGJ_2 places the electrophilic carbon in the cyclopentenone ring well within the vicinity of Cys^{1438} of p300 to form a covalent Michael adduct. Site-directed mutagenesis of p300 HAT domain, peptide competition assay involving p300 wild type and mutant peptides followed by mass spectrometric analysis confirmed the covalent interaction of Δ^{12}-PGJ_2 with Cys^{1438}. Using biotinylated derivatives of Δ^{12}-PGJ_2 and 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2, we demonstrate the covalent interaction of Δ^{12}-PGJ_2 with the p300 HAT domain, but not the latter. In addition, Δ^{12}-PGJ_2 also inhibited the acetylation of the HIV-1 Tat by recombinant p300 in *in vitro* assays. This study demonstrates, for the first time, that Δ^{12}-PGJ_2 inhibits p300 through Michael addition, where α,β-unsaturated carbonyl function is absolutely required for the inhibitory activity.

Introduction

The action of cyclooxygenase (COX) enzymes on arachidonic acid, followed by isomerization of the product PGH_2 by hematopoietic PGD synthase (H-PGDS) or lipocalin PGDS (L-PGDS) leads to the formation of PGD_2, which undergoes dehydration to form CyPGs, PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2 (Smith *et al.* 2000). These CyPGs are implicated in a wide variety of diverse functions, such as anti-inflammatory, antiviral,

antitumor and cytoprotective effects via multiple mechanisms, including the modulation of transcription factors such as NF-κB, Nrf-2, and PPARγ (Fukushima *et al.* 1989, Kliewer *et al.* 1995, Rossi *et al.* 2000, Straus *et al.* 2001, Lee *et al.* 2003, Itoh *et al.* 2004, Gandhi *et al.* 2011). Recently we have demonstrated the essential role of selenium, via selenoproteins, in the expression of H-PGDS leading to enhanced production of CyPGs (Gandhi *et al.* 2011). Interestingly, CyPGs interact covalently with nucleophilic Cys thiolate anion in proteins via the two electrophilic carbons at positions 9 and 13 (Atsmon *et al.* 1990, Suzuki *et al.* 1997, Shiraki *et al.* 2005, Stamatakis *et al.* 2006). CyPGs form Michael adducts with nucleophiles such as the free sulfhydryl group of Cys residues located in reduced glutathione (GSH) or many cellular proteins, including thioredoxin, p50, Ras, p53, Keap-1, IKK2, and HIV-1 Tat (Atsmon *et al.* 1990, Noyori *et al.* 1993, Rossi *et al.* 2000, Oliva *et al.* 2003, Shibata *et al.* 2003, Itoh *et al.* 2004, Gayarre *et al.* 2005, Kalantari *et al.* 2009, Kobayashi *et al.* 2009, Kim *et al.* 2010). As a result, modification of functionally important sulfhydryl groups in many proteins often results in the modulation of their biological activities, leading to changes in the transcription of several downstream gene targets (Cernuda-Morollon *et al.* 2001).

The DNA is packaged as chromatin in the nucleus of eukaryotes by both histone and non-histone proteins (Batta *et al.* 2007). Chromatin plays a pivotal role in transcription, DNA repair, and replication (Wei *et al.* 1999, van Attikum *et al.* 2005). The basic unit of chromatin is the nucleosome, which is composed of dimers of histones H2A, H2B, H3, and H4 around which 147 base pairs of DNA are wrapped. The N-terminal tails of histones are exposed to the surface of the nucleosome, which serve as the main sites for post-translational modifications. Among the different post-translational modifications,

reversible acetylation of histones plays an important role in maintaining the structure of the chromatin (Grant *et al.* 1999, Roth *et al.* 2001). Therefore, histone acetylation plays an essential role in epigenetic regulation. Histone acetyltransferases (HATs) refers to the class of enzymes that catalyze the acetylation reaction, which transfers the acetyl group from acetyl-CoA to the amino tail of histones and other proteins at specific lysine residues. Thus, HATs are also referred to as lysine acetyltransferases (KATs). These enzymes and the associated acetylation events have been implicated in a wide variety of physiological and diseases like neurodegeneration, cancer, HIV-AIDS, and inflammation (Pumfery *et al.* 2003, Iyer *et al.* 2004, McKinsey *et al.* 2004, Barnes *et al.* 2005). Histone acetylation is catalyzed by five different classes of HATs (Sterner *et al.* 2000). Among them, the best studied are p300 (also referred to as KAT3B) and its close analogue CBP (KAT3A).

Histone acetyltransferase p300 is involved in various cellular events. The increasing evidence of p300 HAT activity with cancer causation and progression has made it to be targeted for the development of anticancer therapeutics. Many of the inhibitors of these enzymes are peptide conjugates of CoA or natural products and their derivatives (Lau *et al.* 2000, Balasubramanyam *et al.* 2003, Balasubramanyam *et al.* 2004, Balasubramanyam *et al.* 2004, Mantelingu *et al.* 2007, Ravindra *et al.* 2009). All these inhibitors provide a valuable tool for analyzing the structure and function of these enzymes, although their potential for development as clinical drug candidates still remains to be determined. However, given the presence Cys^{1438} in the substrate-binding site of p300 HAT domain that is critical in the binding and stabilization of the substrate,

we hypothesized that CyPGs are likely to target this reactive Cys residue to inhibit the enzymatic activity of p300 (Liu *et al.* 2008, Bowers *et al.* 2010).

HIV-1 Tat is acetylated by p300 at Lys^{50} (Kiernan *et al.* 1999, Ott *et al.* 1999, Col *et al.* 2001), and this event appears to be critical as antibodies targeting this acetylated Lys^{50} in cells suppress its transactivation activity (Kaehlcke *et al.* 2003). Lys^{50} acetylation is thought to result in the neutralization of the charge in the highly basic ARM, which obviates the electrostatic interactions between Tat and the TAR element (Ott *et al.* 2011). This terminates the TAR-dependent phase of transactivation by Tat, and leads to its dissociation from the TAR RNA and P-TEFb to return to the "active" Tat pool in the infected cell. The abundance of the Tat protein is known to be limiting in infected cells and thus targeting the Lys^{50} acetylation of Tat by inhibiting the p300-dependent acetylation of Tat by a naturally occurring endogenous metabolite may provide yet another level of therapeutic control over HIV transcription and replication.

In this study, we describe the ability of selenium, via the production of CyPGs, to inhibit the activity of p300 *in vitro* and in hepatocytes and inflamed macrophages. Our studies comparing the various CyPGs clearly shows an intriguing structure-activity relationship in PGs indicating the importance of the α,β-unsaturated carbonyl function to be essential to inhibit the activity of p300 HAT. Molecular modeling studies, showing the interaction of biotinylated derivatives of CyPGs, further lend credence to the idea that these reactive metabolites could impact epigenetic and gene regulatory functions.

Materials and methods

Materials – The following PGs: Δ^{12}-PGJ_2, PGJ_2, PGD_2, PGE_2, PGK_2, PGA_2, PGB_2, 9,10-dihydro-15d-PGJ_2, and 13,14-dihydro-PGD_2 were purchased from Cayman Chemicals (MI, USA). All compounds were of high purity and were used without further purification. [Acetyl-1-^{14}C]-CoA (60 mCi/mmol) was purchased from Perkin-Elmer Life Sciences (Waltham, MA). Whatman P81 chromatography paper was obtained from Fisher Scientific Chemicals (Pittsburgh, PA). HeLa core histones and antibodies that recognize specific acetylated lysine residues in histone H3 (K9 and K14) and total histone H3 were obtained from Active motif (Carlsbad, CA). GST-tagged recombinant p300 HAT domain corresponding to amino acids 1066-1707 of human p300 expressed in *E.coli* was obtained from Millipore (Billerica, MA). Similarly, GST-tagged recombinant PCAF (p300/CBP-associated factor; 165 amino acids; corresponding to residues 503-651) was purchased from Cayman Chemicals. HIV-1 Tat protein was expressed in a bacterial system and purified using His-affinity chromatography in our laboratory as described previously (Kalantari *et al.* 2009).

Histone acetyltransferase assay – HAT assays were performed as described previously with some modifications (Kundu *et al.* 2000). p300 or PCAF were incubated with or without molar equivalents of PGs at room temperature for 3 h. 0.8 μg of highly purified HeLa core histones were incubated in HAT assay buffer (50 mM Tris–HCl, pH 8.0, 1 mM PMSF, 0.1 mM EDTA, and 10% v/v glycerol) at 30 °C for 10 min, with or without p300 or PCAF (PG treated and untreated). The reaction mixture was incubated for an additional 10 min upon addition of 0.25 μl of 60 mCi/mmol [^{14}C] acetyl CoA in a total volume of 30 μl. The reaction mixture was then blotted onto a P-81 filter paper

(Whatman), washed with carbonate buffer (0.2 M Na_2CO_3 and 0.2 M $NaHCO_3$), and counts were recorded in a Beckman liquid scintillation counter. All assays were performed in triplicate.

Viability assay – All PGs were tested for toxicity at 10 μM. The viability of cells after various treatments was estimated in terms of their ability to reduce the dye (3,4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (CCK-8 kit, Dojindo, Gaithersburg, MD) to blue purple formazan crystals, as per the manufacturer's instructions.

Analysis of histone acetylation in HepG2, RAW264.7 and U1/HIV-1 cells – Human liver hepatoblastoma (HepG2) cells (ATCC; HB-8065) (1 X 10^6 cells per 60 mm^2 dish) were seeded overnight and treated with various PGs at indicated concentrations or vehicle (DMSO, 0.1 % v/v) the following day for 24 h. Total histones were extracted from vehicle and compound-treated cells as described previously (Mantelingu *et al.* 2007). Equal amounts of the protein samples were run on a 12 % (%T) SDS-polyacrylamide gel and the separated histones were electro-transferred onto a nitrocellulose membrane. The membranes were probed with specific anti-(K9/K14) acetyl H3 and anti-H3 C-terminal antibodies (Active motif). Detection was performed with goat anti-rabbit secondary antibody (Thermo Pierce, Rockford, IL), and bands were visualized using the ECL detection system (Thermo Pierce). RAW264.7 macrophages [cultured in DMEM (Invitrogen) supplemented with 5 % FBS (ATCC), 2 mM L-glutamine, and antibiotics] were treated with 50 ng/ml LPS for 2 h, following which they were incubated with different concentrations of selenium for 72 h, with or without indomethacin (10 μM, COX inhibitor) or HQL-79 (25 μM, H-PGDS inhibitor). Histones were isolated from

these cells and analyzed for their histone acetylation status using anti-(K12/K16) acetyl H4 antibodies. U1/HIV-1 cells, obtained from the NIH AIDS Research and Reference Reagent Program, were cultured in RPMI 1640 supplemented with FBS (10 % v/v) and stimulated for 12 h with 20 ng/ml phorbol myristic acid (PMA; Sigma) and subsequently treated with vehicle or Δ^{12}-PGJ_2 for 24 and 48 h. To test the effect of selenium supplementation on histone acetylation, U1/HIV-1 cells were stimulated with 20 ng/ml PMA for 4 h and subsequently treated with different concentrations of selenium (as selenite) for 72 h. Histones were isolated from the cells and their acetylation status was analyzed as described earlier.

Analysis of p65 acetylation – RAW264.7 macrophages were treated with 50 ng/ml LPS for 2 h, followed by incubation with different concentrations of selenium. These cells were harvested and the nuclear and cytoplasmic fractions were separated. The nuclear fractions were analyzed by immunoblotting for the acetylation status of p65 at Lys^{310}.

Site-directed mutagenesis of p300 HAT domain – The p300 HAT cDNA (region corresponding to amino acid residues 1066 to 1707) was cloned from HEK293T cDNA using the following primers: Forward – 5’ TACCGTCAGGATCCAGAATCCCTTCCC 3’ and Reverse – 5’ TTTCTCCATTTTGTGGTCATGGTTTTTAGTGTTATAGC 3’. EcoRI and EagI restriction endonuclease sites (in bold) were added to the p300 cDNA using the primers: Forward – 5’ **GAATTC**TACCGTCAGGATCCAGAATCC 3’ and Reverse – 5’ CCATAG**CGGCCG**CTTTCTCCATTTTGTGGTCATGG 3’. The p300 cDNA was subcloned into EcoRI and EagI sites in the pET41c(+) vector (Novagen). Such a pET41c-p300 plasmid was used as the template to generate the p300C1438A mutant using the Stratagene site-directed mutagenesis kit according to the manufacturer’s

instructions. The primers used for the mutagenesis were: Forward – 5' GCATATTTGGGCA**GCA**CCACCAAGTGAG 3' and Reverse – 5' CCTCACTTGGTGG**TGC**TGCCCAAATATG 3' (underlined codons in bold represent the mutated Cys residue).

Reaction of p300 HAT domain and biotinylated PGs – The ability of these compounds to bind p300 HAT domain was examined *in vitro*. Δ^{12}-PGJ_2 and 9,10-dihydro-15d-PGJ_2 were biotinylated using EZ-link-5-(biotinamido)pentylamine (Thermo Pierce) in presence of EDC (1-ethyl-3-(3-diaminomethylaminopropyl) carbodiimide hydrochloride) as the coupling agent as described earlier in Chapter 3. Briefly, the biotinylated PGs were purified on a preparative silica gel 60 column (70-230 mesh; Sigma Chemicals, St. Louis, MO) developed with ethyl acetate followed by elution with ethyl acetate:methanol (8:2). The eluates were run on a thin layer chromatography using AL SILG/UV plates (Whatman, 250 μm layer; Kent, UK). Fractions containing biotinylated PGs were pooled and confirmed by mass spectrometry (*m/z* of 645.44 and 627.78 for Δ^{12}-PGJ_2-biotinamide and 9,10-dihydro-15d-PGJ_2-biotinamide, respectively) and a dot blot probed with streptavidin-HRP. The p300 HAT domain (10 pmol/30 μl reaction volume), biotinylated Δ^{12}-PGJ_2 (10 pmol), and 9,10-dihydro-PGJ_2 (10 pmol) were incubated in HAT assay buffer for 3 h (at 25 °C) with 10 μg cell nuclear extract from U937 monocytic cells. The latter was subjected to pull-down with neutravidin-agarose beads followed by Western blot analysis by with anti-GST antibody or neutravidin-HRP conjugate to examine the interaction between p300 and Δ^{12}-PGJ_2.

Binding studies with C1438A mutant – BL21 cells were transformed with native pET41c-p300 or pET41c-p300C1438A plasmids. The transformed cells were grown in

Terrific Broth to log phase and induced with 1 mM IPTG overnight. 0.2 nmol of the native or mutant p300 protein were bound to HisPur resin (Pierce) according to the manufacturer's instructions. 0.2 nmol of Δ^{12}-PGJ_2 biotinamide solution prepared in 1% DMSO (in 50 mM Tris-Cl, pH 8.0) was reacted with the resin-bound protein for 3 h at room temperature on an end-to-end shaker. Following washes with 50 mM Tris-Cl, pH 8.0, the resin was boiled with 1X SDS gel loading buffer. HisPur resin alone, 0.2 nmol of Δ^{12}-PGJ_2 biotinamide solution bound to resin, native p300, and p300C1438A protein bound to the resin were used as negative controls. The samples were analyzed by PAGE and Western transfer followed by immunoblotting with the indicated reagents.

Interaction of Δ^{12}-PGJ_2 with p300 peptide – The peptide sequences ^{1433}GHIWA**C**PPSEG and its mutant ^{1433}GHIWA**A**PPSEG were purchased from GenScript Inc., (Piscataway, NJ). The peptides (0.3 μM) and Δ^{12}-PGJ_2 (0.3 μM) were incubated for a 3 h in HAT assay buffer, in a reaction volume of 30 μl. The reactions were analyzed by infusion, and using an LC-MS/MS system comprising Shimadzu LC20AD UFLC pumps, a Luna phenyl-hexyl column (150 x 2 mm, 3 μm; Phenomenex), and ABI2000 triple quadruple mass spectrometer with an electrospray ionization probe set to positive mode at 250 °C for the confirmation of the product ion peak. The solvent system used was methanol:H_2O (70:30), with 0.1% acetic acid, at a flow rate 0.15 ml/min.

Peptide competition assay – The peptides (10 pmol) were added to p300 HAT domain (10 pmol) along with biotinylated Δ^{12}-PGJ_2 (10 pmol and 20 pmol) for 3 h at 25 °C, in a

reaction volume of 30 µl followed by Western blot analysis with neutravidin-HRP or anti-GST.

p24 Quantitation – The U1/HIV-1 cell line was cultured in RPMI-1640 media (Cellgro) supplemented with 10 % heat inactivated fetal calf serum (Hyclone), 2 mM L-glutamine (Invitrogen), Penicillin (0.5 units/ml) and Streptomycin (0.5 µg/ml). The cells were stimulated with 20 ng/ml of PMA for 12 h. Δ^{12}-PGJ_2 in DMSO (0.1% v/v) was added to the stimulated cells, and the cultures were incubated at 37 °C for 24 and 48 h. Unstimulated cells were used as a negative control. Culture supernatant was sampled every 24 h for p24 analysis. The quantity of p24 in the samples was measured with a commercial kit following the manufacturer's instructions (CosmoBio, Japan). All the assays were performed in triplicate.

***In vitro* acetylation of HIV-1 Tat protein by p300 HAT** – Ten pmol of p300 HAT domain was incubated in the presence or absence of 30 pmol of Δ^{12}-PGJ_2 for 3 h at room temperature in 30 µl. This mixture was then incubated with 350 pmol His-tag labeled recombinant Tat (rTat) protein and 0.1 µCi of [acetyl-1-^{14}C] CoA for 1 h at room temperature. The rTat protein was subjected to pulldown using HisPur resin, which was boiled, centrifuged, and the supernatant subjected to scintillation counting.

Molecular docking studies – To develop a prospective pharmacophore for ligand interactions with the Cys^{1438} of p300 HAT domain, a two-stage modeling protocol was performed in collaboration with Dr. Blake Peterson and Dr. Gerald Lushington, University of Kansas: first the following three fragment species were docked: a) 4-allyl-5-methylenecyclopent-2-enone, b) (4R,5R)-4-methyl-5-vinylcyclopentane-1,3-dione, c)

(R)-3-allyl-2-methylenecyclopentanone into p300-HAT via the Surflex program, as guided by specifying a protomol construct based solely on the position and character of Cys^{1438} (residue-based protomol generated automatically in Surflex according to default parameters). Docking proceeded according to default parameters settings with the exception that the number of initial starting conformations was increased to 20, and the number of requested poses was set to 50. From the resulting docking simulation, the bound conformations were examined in order to find the top scoring pose that positioned either a sp2 hybridized ring carbon or oxygen within 4 Å of the Cys sulfur; in each case either the very highest or the second highest scoring pose satisfied this criterion. The fragment poses selected in this first stage were then merged into a single MOL2 file to serve as the base for a ligand-based protomol generation (performed automatically in Surflex according to default parameters) for the second stage. In the second stage, PGJ_2, Δ^{12}-PGJ_2, PGK_2 and 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 were docked to p300-HAT via Surflex via the above fragment-based protomol and according to the same docking protocol as described in the first stage. Bound conformations were again examined to identify the top scoring pose that positioned either a sp2 hybridized ring carbon or oxygen as described earlier. This spatial orientation criteria was again satisfied either by the single top scoring pose (as was the case for PGJ_2, PGK_2 and 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2) or by the second-best pose (Δ^{12}-PGJ_2). Pose clustering analysis revealed that the single most populous conformational family resolved for each ligand corresponded to poses satisfying the Cys^{1438} spatial proximity criterion, and in each case more than half of all poses placed either the ring oxygen or electrophilic carbon within a somewhat more lenient criterion of 5.0 Å distance from the sulfur.

Chromatin immunoprecipitation (ChIP) – RAW264.7 cells were cultured in 20 nM trichostatin A (TSA) with 0 nM or 250 nM sodium selenite for 72 h, followed by stimulation with 50 ng/ml LPS for 2 h. These cells were harvested post-stimulation and used for crosslinking ChIP (X-ChIP). Briefly, the cells were treated with 1% formaldehyde for 10 m. They were then treated with 125 mM glycine to stop the crosslinking reaction. The cells were then washed with ice-cold 1X phosphate buffered saline (PBS) thrice. They were then harvested in PBS; 10 million cells were counted and centrifuged at 2000g for 5 min at 4°C to pellet them. The cells were lysed with ChIP lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS), and subjected to sonication using a water-bath sonicator (Diagenode Bioruptor; 30 s ON, 60 s OFF for 20 cycles) to degrade the chromatin. 25 µg of the sonicated chromatin, diluted to 1 ml with the ChIP dilution buffer (0.5% triton-X 100, 2mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl) was pre-treated with 20 µl of protein A/G agarose (Santa Cruz) for 30 m. The agarose beads were spun down by centrifugation at 1500g for 5 m at 4°C. The chromatin was now incubated with antibodies to H4K12 or H4K16 (2 µl) and 20 µl of protein A/G agarose overnight at 4°C. 25 µg of pooled chromatin from the samples were also subjected to IP with antibody against HIV-1 Tat-1 (Abcam). The beads were then washed with 500 µl of ChIP wash buffer (0.1% SDS, 1% triton-X 100, 2 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl) thrice, followed by 500 µl of Tris-EDTA buffer (1 mM Tris, pH 8.0, 0.1 mM EDTA) twice. The immunoprecipitated chromatin was eluted off the beads using the ChIP elution buffer (1% SDS, 0.1 M $NaHCO_3$). The samples were now subjected to reverse-crosslinking by treating them with 300 mM NaCl, 0.5 µg of RNase A (Sigma), and 3 µl of Proteinase K (New England Biosciences) per ChIP reaction, and

incubating at 65°C in a water-bath for 4-6 h. The DNA was now isolated and concentrated using the PCR purification kit (Promega) using the manufacturer's instructions. The DNA was eluted in 30 µl of nuclease-free water (Promega).

Results

Selenium supplementation inhibits histone acetylation *in vitro* – Histone acetyltransferases like p300 and PCAF are recruited to the HIV LTR during viral transcription. These HATs acetylate the histones forming the nucleosomes at the HIV LTR, thus de-repressing the LTR and allowing transcription to be initiated. To investigate the effect of selenium supplementation on the histone acetylation status of HIV-infected cells, we cultured PMA-stimulated U1/HIV-1 cells (a model for chronically infected cells) with different concentrations of selenium for 72 h. These cells were then harvested and the histone fractions were isolated. The histones were then analyzed by immunoblotting to examine their acetylation status. We observed that selenium supplementation of stimulated U1/HIV-1 cells caused an inhibition in the acetylation of histone H3 at position K9 when compared to cells deficient in selenium, indicative of an inhibition in viral replication (Fig. 31).

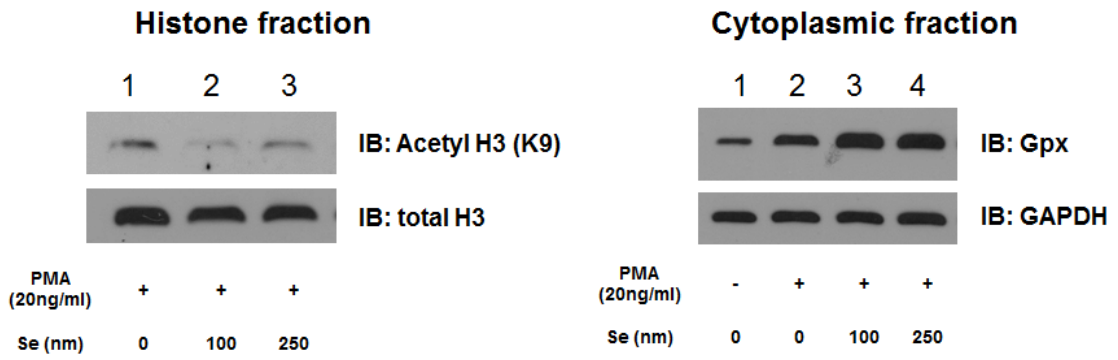

FIGURE 31. **Selenium supplementation of stimulated U1/HIV-1 cells inhibits histone H3 acetylation.** U1/HIV-1 cells were treated with 20 ng/ml PMA for 4 h. These cells were then washed and incubated with sodium selenite at different concentrations for 72 h. The cells were then harvested and histone fractions were isolated. The histones were analyzed by immunoblotting with anti-K9 acetyl H3 antibodies.

We tested our hypothesis regarding the inhibition of HAT activity in inflammatory conditions upon selenium supplementation in a murine macrophage model of LPS-induced inflammation. RAW 264.7 macrophages were treated with LPS, following which they were incubated with selenium for 72 h at the indicated concentrations. Histones were isolated from these cells and the acetylation status of histone H4 at positions K12 and K16 were analyzed by immunoblotting. As shown in Fig. 32, the acetylation of histone H4 at positions K12 and K16 in inflamed macrophages was reduced upon selenium supplementation, which is indicative of a reduction in the inflammatory status of the cell.

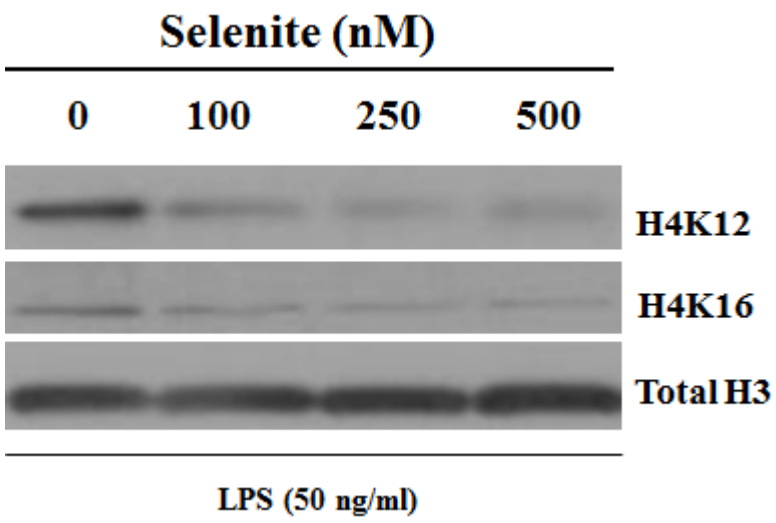

FIGURE 32. **Selenium supplementation of LPS-stimulated RAW264.7 cells inhibits histone acetylation.** RAW cells were treated with 50 ng/ml LPS for 2 h, followed by incubation with selenium for 72 h. Histones were isolated from these cells, and analyzed by immunoblotting. Representative of n = 3 shown.

To investigate if selenium supplementation of inflamed macrophage cells could epigenetically regulate inflammatory gene expression, we analyzed the promoter regions of two proinflammatory genes, COX-2 and TNFα, by ChIP for changes in histone acetylation status. To capture these gene-specific early acetylation events, we incubated RAW macrophages with 250 nM of sodium selenite and 20 nM trichostatin A (TSA; HDAC inhibitor) for 72 h, following which they were treated with 50 ng/ml LPS for 2 h. The cells were fixed and were subjected to a ChIP assay with anti-K12 acetyl H4 and anti-K16 acetyl H4 antibodies. We observed that the acetylation of histone H4 at the promoters of COX-2 and TNFα in inflamed macrophages was inhibited when they were incubated with selenium (Fig. 33).

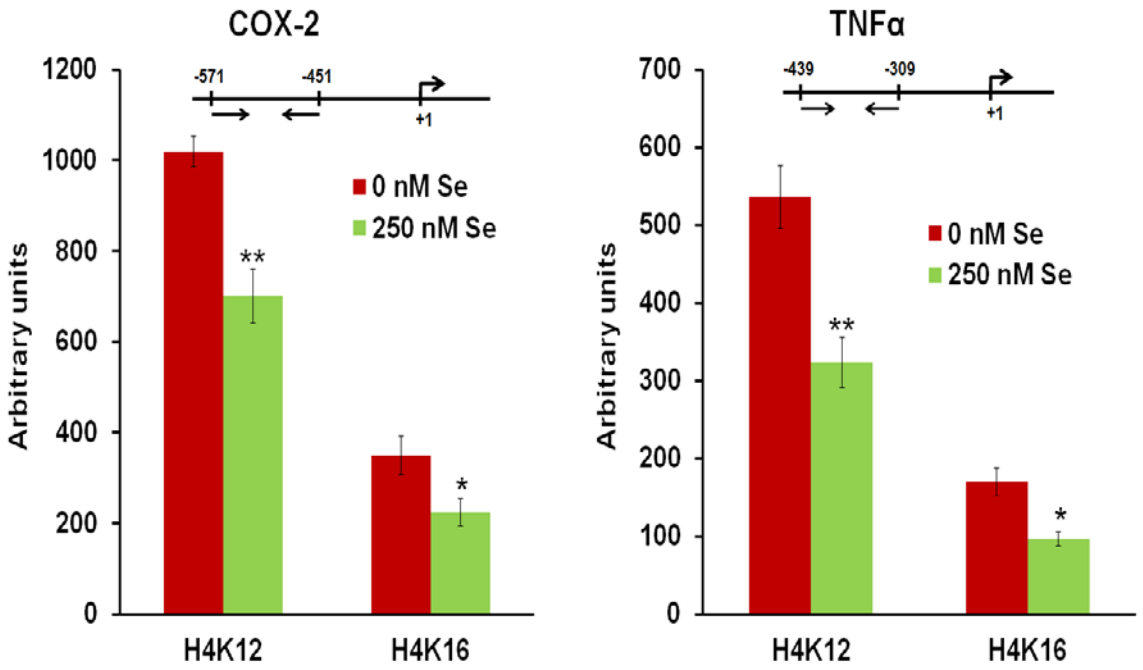

FIGURE 33. **Epigenetic regulation of proinflammatory gene expression by selenium.** RAW264.7 macrophages were maintained on 20 nM TSA, with or without selenium as sodium selenite, for 3 d. The cells were then treated with 50 ng/ml LPS for 2 h. These were then fixed with formaldehyde, lysed, and sonicated to fragment the chromatin. The chromatin was then subjected to immunoprecipitation with antibodies to acetylated histone H4 at positions K12 and K16. The immunoprecipitated chromatin was then analyzed by SYBR green qPCR using primers for the promoter regions of the COX-2 and TNFα genes. Results shown as mean ± SE of three independent experiments. *; $p < 0.05$, **; $p < 0.005$.

The inhibitory effect of selenium on HAT activity may be established via the production of anti-inflammatory CyPGs – Our group has shown that selenium supplementation leads to the preferential upregulation of anti-inflammatory CyPGs in inflamed macrophages (Gandhi *et al.* 2011). To examine the possibility that the observed inhibition of HAT activity by selenium supplementation is regulated by the selenium-dependent shunting of the AA-COX pathway towards production of CyPGs of the J_2 series, we incubated LPS-treated RAW264.7 cells with selenium in the presence of indomethacin (COX inhibitor) or HQL-79 (H-PGDS inhibitor), to inhibit the production

of the J_2 series of CyPGs. The data suggests that inhibition of the AA – COX – H-PGDS pathway reverses the inhibition of HAT activity afforded by selenium in these cells (Fig. 34) suggesting that the inhibitory effect may be CyPG dependent. We observed a similar effect in U1/HIV-1 cells, although it was not consistent (data not shown).

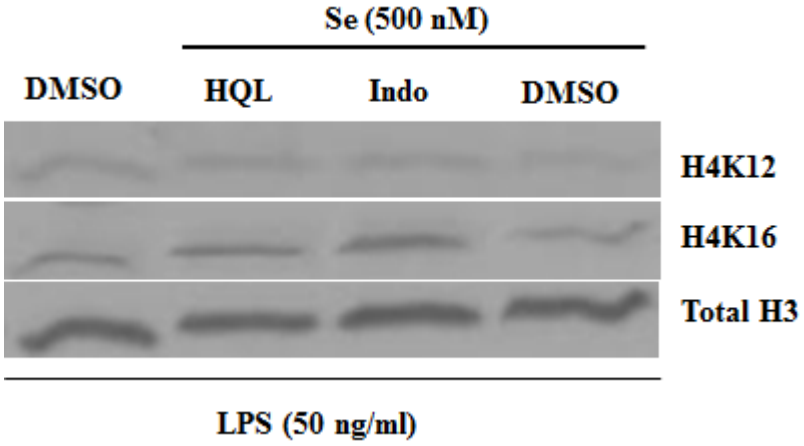

FIGURE 34. **Inhibition of COX or H-PGDS enzymes reverses the inhibitory effect of selenium.** RAW cells were incubated with inhibitors 30 min prior to treatment with 50 ng/ml LPS for 2 h. The cells were washed to remove LPS and then incubated with selenium in the presence of the inhibitors for 72 h. Histones were isolated from these cells, and analyzed by immunoblotting. Representative of n = 3 shown.

Δ^{12}-PGJ_2 inhibits the acetylation of HIV-1 Tat protein by p300 HAT – It has been previously shown that the HIV Tat protein, which serves as a substrate for p300, is acetylated at K^{50} and K^{51}, and that this acetylation is important for its activity (Kiernan *et al.* 1999, Ott *et al.* 1999). To examine if Δ^{12}-PGJ_2-dependent inhibition of p300 HAT activity had any effect on the acetylation of Tat, p300 HAT domain was incubated in the presence or absence of Δ^{12}-PGJ_2 (1:3) for 3 h at room temperature. Recombinant Tat protein (His-tagged) and [acetyl-1-^{14}C] CoA were added to the p300 HAT-Δ^{12}-PGJ_2 complex and the reaction mixture was incubated for an additional 1 h at room

temperature. rTat was subjected to "pull-down" with HisPur resin, washed with PBS, and the beads were boiled with SDS-PAGE gel loading buffer. Radioactivity in the supernatant was counted by scintillation counting. As expected, rTat was acetylated by p300 HAT domain that was not preincubated with Δ^{12}-PGJ_2 (Fig. 35A). However, p300 HAT domain that was alkylated (carbonylated) by Δ^{12}-PGJ_2 exhibited significantly low acetylation activity towards rTat. It was observed that the acetylation of rTat by p300 was inhibited upon incubation of the HAT domain with Δ^{12}-PGJ_2 (Fig. 35A). Furthermore, we extended the analysis to examine the effect of Δ^{12}-PGJ_2 treatment of U1/HIV cells (human monocytic cells chronically infected with HIV-1) that were previously stimulated with PMA to activate the expression of the integrated provirus. Treatment of such cells with Δ^{12}-PGJ_2 (2 μM) for 24 and 48 h clearly showed differences in the levels of acetylation of H3 (at K9 and K14), as a function of time (Fig. 35B). While the decrease in Ac-H3 on day 1 was not substantial (Fig. 35B; compare lane 1 versus 2), the decrease in Ac-H3 on day 2 post treatment was greatly decreased (Figure 34B; compare lane 3 versus 4). Treatment with 2 μM of Δ^{12}-PGJ_2 for 2 days did not cause any toxicity in these cells. Levels of p24, a component of the HIV virus capsid in the supernatant of these PMA-stimulated cells also showed a significant (~ 70 %) decrease upon treatment with Δ^{12}-PGJ_2, particularly at day 2 post-treatment (Fig. 35C). Taken together, these studies indicate that Δ^{12}-PGJ_2 is a potent inhibitor of p300-dependent acetylation of Tat as well as H3 in HIV-infected cells, which contributes, in part, to the reduction in HIV proviral expression.

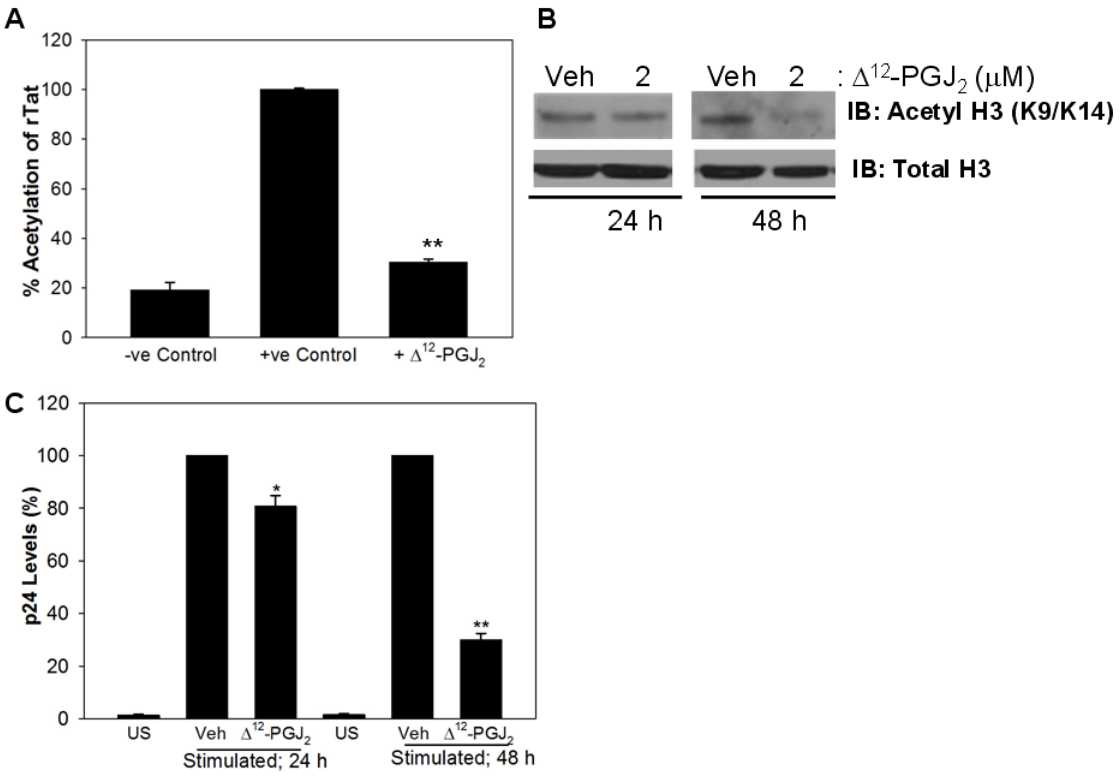

FIGURE 35. **Δ^{12}-PGJ_2 inhibits p300-dependent of acetylation of HIV-1 Tat.** (A) rTat was incubated with Δ^{12}-PGJ_2 treated and untreated p300 HAT domain and [acetyl-1-^{14}C] CoA. rTat was pulled down using HisPur resin. The resin was washed with PBS, boiled with SDS-PAGE gel loading buffer, and subjected to scintillation counting. Negative and positive controls correspond to: p300 + [acetyl-1-^{14}C] CoA and p300 + [acetyl-1-^{14}C] CoA + rTat, respectively. Mean ± SEM of three independent experiments shown. (B) U1/HIV cells were stimulated and treated with Δ^{12}-PGJ_2 at 2 μM for 24 h and 48 h. Day 1: DMSO treated (lane 1), 2 μM Δ^{12}-PGJ_2 (lane 2); Day 2: DMSO treated (lane 3), 2 μM Δ^{12}-PGJ_2 (lane 4). Representative blots of n = 3 shown. (C) Δ^{12}-PGJ_2 decreases p24 levels in chronically HIV-1 infected human monocytes. The viral structural protein p24, secreted into the culture medium was estimated by using an antigen-capture assay. The difference between cells treated with control and 2 μM concentration of Δ^{12}-PGJ_2 was found to be statistically significant. Error bars represent SEM of three independent experiments. *, $p < 0.05$, **, $p < 0.005$. US, Unstimulated cells; Veh, vehicle (DMSO).

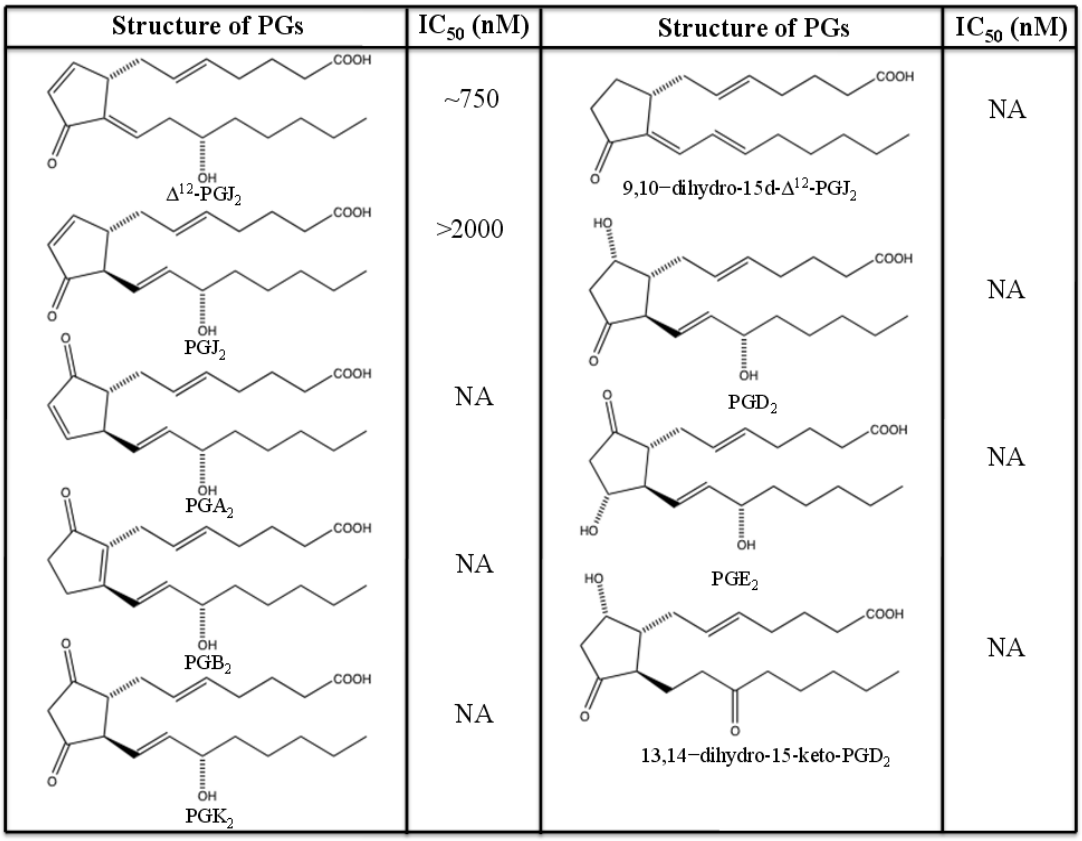

FIGURE 36. **Structure of PGs along with their inhibitory activity towards p300.** Each compound was tested for inhibitory activity of p300 from 0.1-5 μM in *in vitro* assays and IC_{50} values were calculated. Average of three independent assays shown. NA, no activity.

Inhibition of p300 HAT activity by CyPGs – *In vitro* p300 HAT assays using [acetyl-1-^{14}C] CoA and HeLa core histones with the recombinant p300 HAT domain that was preincubated with various concentrations (100 nM - 5μM) of PGs clearly indicated an interesting pattern. While PGD_2, PGE_2, PGK_2, PGB_2, and PGA_2 failed to inhibit the HAT activity of p300, Δ^{12}-PGJ_2 and PGJ_2 (Δ^{13}-PGJ_2) inhibited the activity of p300 significantly. The IC_{50} values with Δ^{12}-PGJ_2 and PGJ_2 were calculated to be ~750 nM and >2 μM, respectively (Fig. 36). Furthermore, Δ^{12}-PGJ_2 and PGJ_2, being positional isomers, showed differences in their reactivities towards the inhibition of p300 activity. However,

Δ^{12}-PGJ_2 failed to inhibit recombinant PCAF activity even at 5 μM (data not shown). PGD_2, PGE_2, PGK_2 and 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 lack an unsaturation at carbon-9 did not exhibit any inhibitory properties. It should also be noted that PGD_2, the precursor for CyPGs of the J_2 class, did not affect the HAT activity; while the dehydration product (Δ^{12}-PGJ_2) was effective towards HAT p300 suggesting that metabolism to CyPGs is essential for activity.

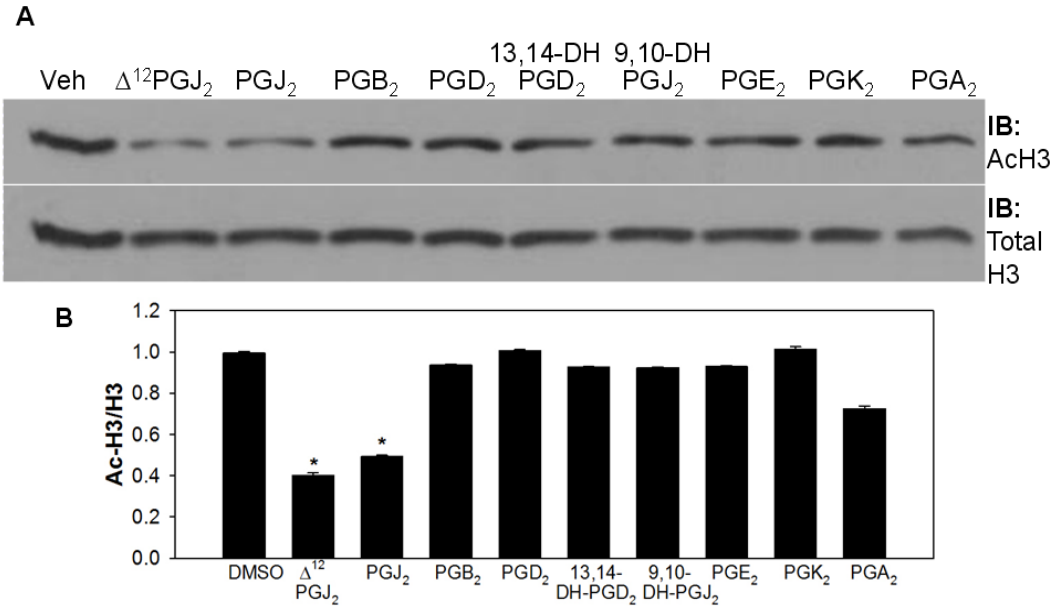

FIGURE 37**. Inhibition of H3 acetylation by PGs in HepG2 cells.** (A) HepG2 cells were treated as indicated for 24 h; histones were isolated from DMSO treated cells (lane 1). PGs-treated cells at 10 mM concentration (lanes 2-10). The histone acetylation was analyzed by immunoblotting using acetylated H3 antibody and histone H3 as a loading control. (B) Densitometric analysis of total H3 versus acetylated H3. Mean ± SEM of three independent assays shown. *, $p < 0.00005$. 13,14-DH-PGD_2 and 9,10-DH-PGJ_2 represent 13,14-dihydro-15-keto-PGD_2 and 9,10-dihydro-15d-PGJ_2, respectively.

The ability of these PGs to inhibit cellular p300 HAT activity was tested in HepG2, a human hepatocarcinoma cell line, where histones (H3 and H4) are known to be hyper acetylated (Bai *et al.* 2008, Ravindra *et al.* 2009). As a preliminary screen, HepG2 cells

were treated with 10 μM of each of the PGs. No significant toxicities were observed. As shown in Fig. 37, treatment with Δ^{12}-PGJ_2 and PGJ_2 showed significant reduction in the histone H3 acetylation level. (Fig. 37A; lanes 2 and 3). PGA_2 showed a non-significant decrease (Fig. 37A; lane 10). However, PGE_2, PGB_2, PGK_2, 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2, PGD_2, and 13,14-dihydro-PGD_2 were ineffective at inhibiting acetylation in these cells (Fig. 37A; lanes 4-9).

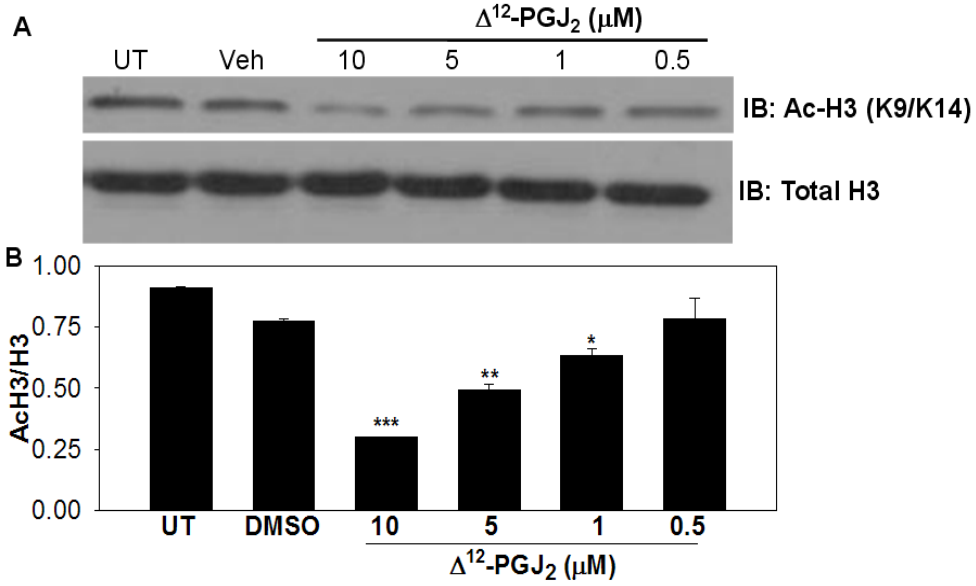

FIGURE 38. **Δ^{12}-PGJ_2 inhibits p300 acetylation of H3 in HepG2 cells.** (A) HepG2 cells were treated with various concentrations of Δ^{12}-PGJ_2 for 24 h and histones were isolated from untreated cells (lane 1), DMSO treated cells (lane 2), Δ^{12}-PGJ_2-treated cells at 10 mM (lane 3), 5 mM (lane 4), 1 mM (lane 5), and 500 nM (lane 6). The histone acetylation was probed by immunoblotting using acetylated H3 antibody and histone H3 as a loading control. (B) Densitometry analysis of total H3 versus acetylated H3. Mean ± SEM of three independent experiments shown. *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0005$.

Based on these results, it became clear that Δ^{12}-PGJ_2 appeared to be a potent inhibitor of p300. Thus, we used Δ^{12}-PGJ_2 as a lead compound for further studies. Treatment of HepG2 cells with increasing concentrations (0.5 - 10 μM) of Δ^{12}-PGJ_2 showed a dose-dependent inhibition of histone H3 K9/K14 acetylation, with more than 90 % inhibition

at 10 μM of Δ^{12}-PGJ_2 compared to DMSO control (Fig. 38A; compare lane 1 versus lane 3). The IC_{50} was calculated to be ~5 μM.

Δ^{12}-PGJ_2 inhibits p300 by Michael addition – Based on the differences in the ability of Δ^{12}-PGJ_2 and 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 to inhibit p300 activity, and the fact that the substrate-binding site of p300 contains a Cys residue (aa 1438), we speculated that p300 perhaps covalently interacted with Δ^{12}-PGJ_2. To examine the interaction of Δ^{12}-PGJ_2, biotinylated Δ^{12}-PGJ_2 and 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 were used. 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 was mainly used as a control. The biotinylated compounds were incubated with the recombinant p300 HAT domain for 3 h and the binding of biotinylated derivatives of Δ^{12}-PGJ_2 or 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 to p300 was analyzed by SDS-PAGE under denaturing conditions followed by Western blotting with neutravidin-HRP and GST. Figure 38A clearly indicates the binding of Δ^{12}-PGJ_2 to p300; while 9,10-dihydro-derivative was ineffective. To further address if this interaction was intact even in the presence of other proteins, nuclear extracts from U937 human monocytic cells were mixed with recombinant p300 to which biotinylated CyPGs were added. These samples were subjected to pull-down with neutravidin-agarose beads overnight. The binding of p300 to biotinylated Δ^{12}-PGJ_2 or biotinylated 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 was analyzed by gel electrophoresis and immunoblotting with anti-GST antibody (Fig. 39A) followed by probing the same membrane with neutravidin-HRP conjugate (Fig. 39A). The results in Figure 38A clearly demonstrate that the presence or absence of nuclear proteins did not affect the interaction of biotinylated Δ^{12}-PGJ_2 with p300; while biotinylated 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 did not interact with p300. Furthermore, in the above *in vitro* reaction, a peptide containing the reactive Cys^{1438} was

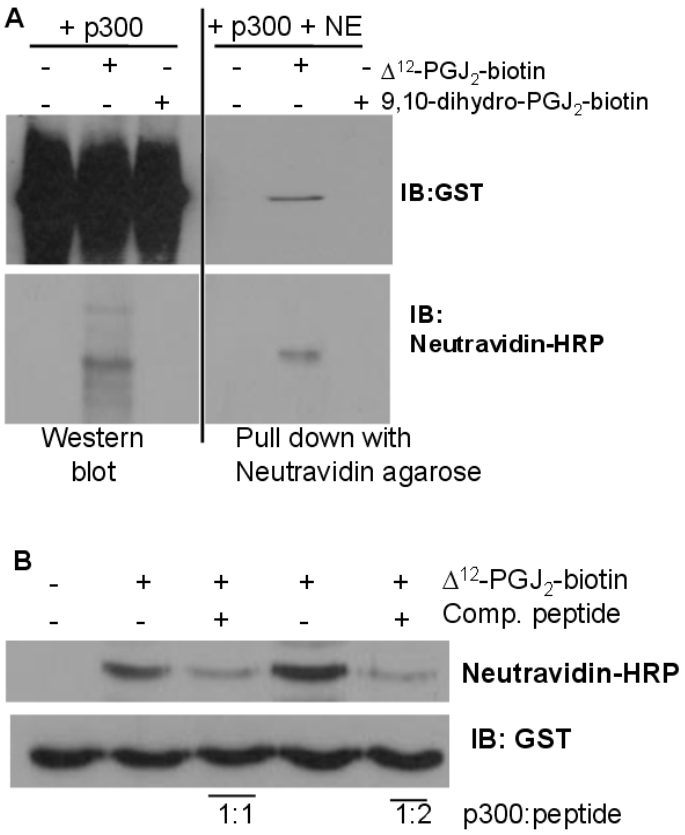

FIGURE 39. **Δ^{12}-PGJ_2 forms a covalent adduct with p300.** (A) *Left panel* (Western blot): p300-GST was incubated with and without biotinylated PGs in an *in vitro* reaction. The reactions were analyzed by immunoblotting. *Right panel* (Pulldown with neutravidin agarose): p300-GST was mixed with nuclear extracts (NE) from U937 monocytic cells and reacted with or without biotinylated PGs. These samples were subjected to pull down with neutravidin-agarose beads and analyzed by immunoblotting. Representative of n = 2 shown. (B) Peptide competition assay. p300 only (lane 1), p300 and biotinylated Δ^{12}-PGJ_2 (lane 2), p300, biotinylated Δ^{12}-PGJ_2 and peptide (1:1 = p300 : peptide; lane 3), p300 and biotinylated Δ^{12}-PGJ_2 (lane 4), p300, biotinylated Δ^{12}-PGJ_2 and peptide (1 : 2 = p300 : peptide; lane 5). As a loading control the blots were probed with GST. Representative of n=2 shown.

co-incubated with p300 HAT domain and Δ^{12}-PGJ_2 at 1:1 and 1:2 molar ratio (p300:peptide) for 3 h followed by immunoblotting with neutravidin-HRP conjugate to examine the biotinylation of p300. As expected, the peptide inhibited the interaction of Δ^{12}-PGJ_2 with p300 at both 1:1 and 1:2 molar ratios (Fig. 39B). Taken together, these results strongly support the ability of Δ^{12}-PGJ_2 to interact with p300 by forming a covalent adduct.

Δ^{12}-PGJ$_2$ interacts covalently with Cys^{1438} in p300 HAT – Given the presence of Cys^{1438} in the active site of p300; we examined its role as a nucleophilic acceptor for the binding of Δ^{12}-PGJ$_2$ in the following studies. First, the p300 peptide (GHIWA**C**PPSEG) or mutant peptide lacking Cys (GHIWA**A**PPSEG) corresponding to amino acids 1433-1443 in p300, were incubated for 3 h with molar equivalents of Δ^{12}-PGJ$_2$ and the complex was analyzed by LC-MS as well as by direct infusion. As shown in Fig. 40A, B, the native peptide and the peptide- Δ^{12}-PGJ$_2$ adduct could be separated by LC. An increase in the *m/z* of molecular ion of the peptide from 1153.2 to 1487.0 clearly indicated that the peptide interacted covalently with Δ^{12}-PGJ$_2$ (Fig. 40C, D). Second, mutagenesis of Cys^{1438} to Ala in the substrate binding site of p300 HAT domain followed by *in vitro* binding studies with Δ^{12}-PGJ$_2$-biotinamide clearly indicated that Cys^{1438} was indispensible for the covalent interaction of Δ^{12}-PGJ$_2$ with p300 (Fig. 41A, B). Incubation of the mutant peptide with the p300 HAT domain and Δ^{12}-PGJ$_2$ demonstrated a clear lack of competition with p300 for Δ^{12}-PGJ$_2$ (Fig. 41B). In addition, incubation of the mutant peptide with Δ^{12}-PGJ$_2$ did not lead to a corresponding increase in the molecular mass of the peptide (Fig. 41C).

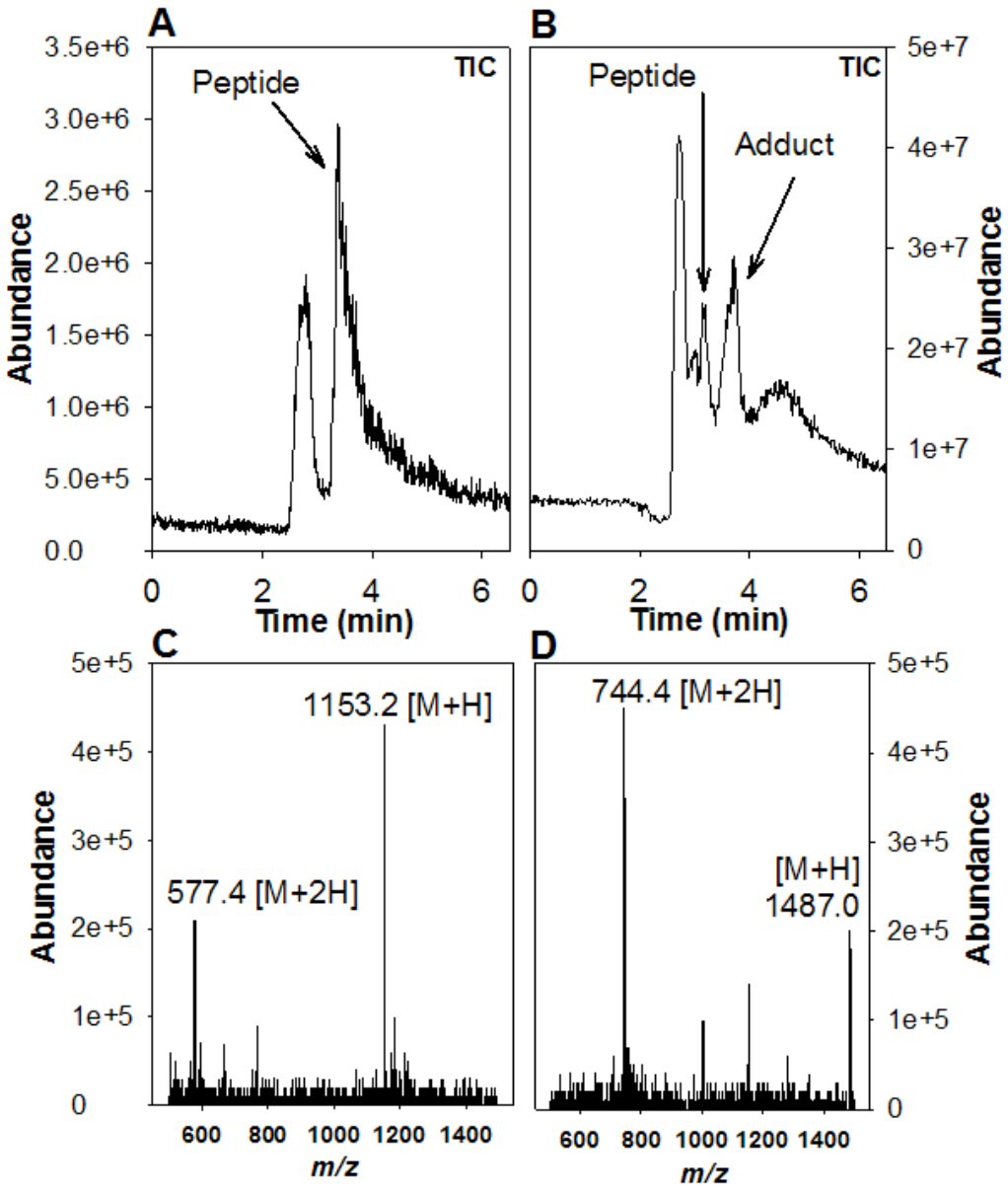

FIGURE 40. **LC-MS analysis of p300 peptide conjugated with Δ^{12}-PGJ$_2$.** The peptides (0.3 μM) and Δ^{12}-PGJ$_2$ (0.3 μM) were incubated for 3 h in HAT assay buffer, in a reaction volume of 30 μl. The reactions were analyzed by LC-MS/MS as described in the "Methods" section. The solvent system used was methanol:H_2O:acetic acid (70:30:0.1) at a flow rate of 0.15 ml/min. Panels A and B represent p300 peptide only and p300 peptide incubated with Δ^{12}-PGJ$_2$, respectively. Panels C and D represent the spectra of the "peptide peak" and "adduct peak" from the TIC in panels A and B, respectively.

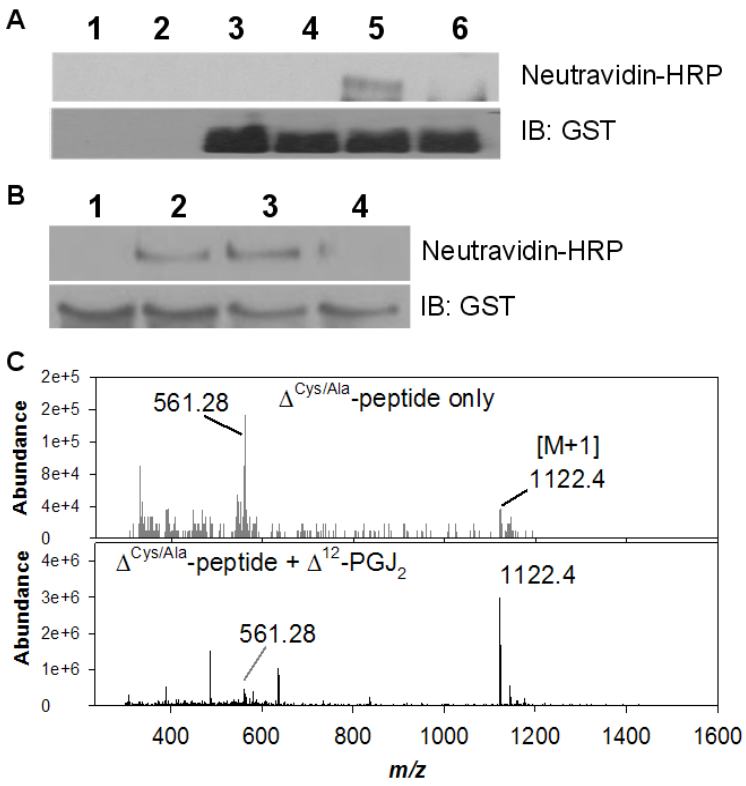

FIGURE 41. **Site-directed mutagenesis of p300 HAT domain.** (A) Interaction of p300C1438A protein with Δ^{12}-PGJ_2. p300 wild type or C1438A mutant proteins were incubated with Δ^{12}-PGJ_2 biotinamide followed by SDS-PAGE analysis and Western blotting with neutravidin-HRP or GST. Lanes 1-6 represent, buffer alone, Δ^{12}-PGJ_2 biotinamide alone, native p300 only, p300-C1438A alone, native p300 + Δ^{12}-PGJ_2 biotinamide, and p300 C1438A + Δ^{12}-PGJ_2 biotinamide, respectively. (B) Peptide competition assay. In the above reaction, wild type or mutant peptides were included followed by Western blotting. Lanes 1-4 represent, p300 only, p300 + biotinylated Δ^{12}-PGJ_2 (1:1 molar ratio), p300 incubated with biotinylated Δ^{12}-PGJ_2 and mutant peptide, p300 incubated with biotinylated Δ^{12}-PGJ_2 and wild type peptide. Since native p300 and p300C1438A were expressed with a GST tag, blots were reprobed with anti-GST to confirm near uniform loading. Representative of n = 2 shown. (C) Mass spectrometric evaluation (by direct infusion) of the interaction of mutant peptide before and after incubation with Δ^{12}-PGJ_2.

To probe this interaction further, we utilized a molecular modeling approach using the crystal structure of the HAT domain of p300 liganded to a synthetic inhibitor, lysyl-CoA, reported recently (Liu *et al.* 2008). In order to gauge the propensity for PGs to bind to p300-HAT via precovalent conformations suitable for covalent reaction with binding site nucleophiles, we examined the model generated from the crystal structure of p300-HAT

for solvent-exposed Cys residues within close proximity of the co-crystallized CoA ligand. Using this structure, molecular modeling studies with different CyPGs were performed. As shown in Fig. 42, all of the PGs tested were seen to bind to p300 HAT. Of these, Δ^{12}-PGJ_2 was found to have the strongest affinity for this putative covalent binding mode (Surflex score = 7.86), followed by 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 (7.18), PGK_2 (6.49) and 15d-PGJ_2 (6.07). Further analysis of the interaction between Δ^{12}-PGJ_2 and Cys^{1438} showed that the electrophilic carbon-9 was positioned within 4 Å of the nucleophilic Cys-S^- to facilitate the formation of an adduct. 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-PGJ_2 also snuggly fit into the substrate binding site, but was not capable of interacting with Cys^{1438} due to the lack of an electrophilic carbon as shown in Figure 7A. Interestingly, the two hydrophobic tails of Δ^{12}-PGJ_2 were positioned around the antiparallel beta-sheets in p300 such that the electrophilic center at carbon-9 in the cyclopentenone was placed in the vicinity of Cys1438 (Fig. 42B-D). However, in the case of PGA_2, PGK_2, and PGB_2, which were ineffective in inhibiting H3 acetylation, the positioning of the α,β-unsaturated reactive center was farther away from the Cys^{1438}, thus making them less likely to interact with the nucleophilic Cys-S^- in the substrate binding site. Taken together, these studies further provide support to the premise that Δ^{12}-PGJ_2 covalently interacts with p300 via the Cys^{1438} in the substrate-binding pocket of p300.

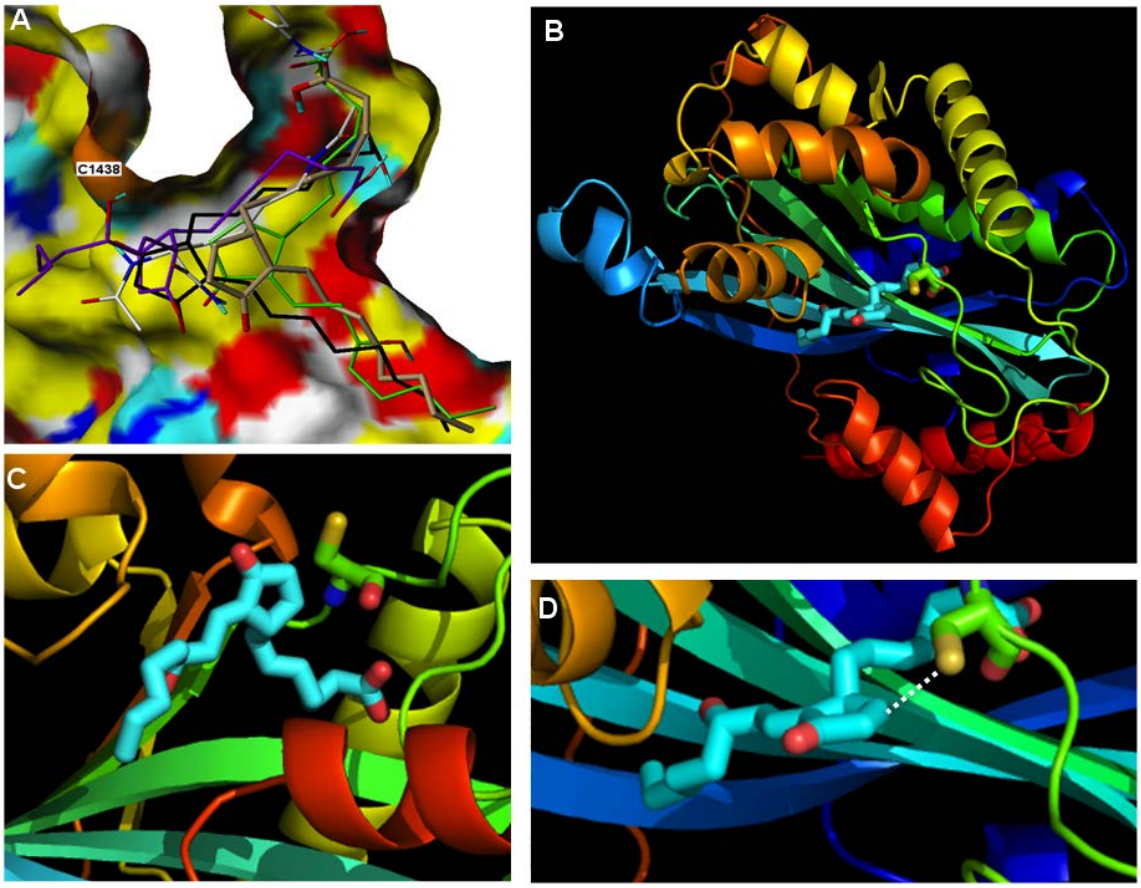

FIGURE 42. **Molecular modeling of CyPGs to p300 HAT domain.** (A) Predicted precovalent conformers for four PGs in the CoA binding site of p300-HAT. Connolly surface color scheme: hydrophobic = yellow; weakly polar alkyl = white; polar O, N, H = red, blue and cyan respectively, and the surface of the putatively reactive sulfur on Cys^{1438} is orange for contrast. Ligands are rendered as sticks with CPK colors, except for carbon atoms which are shown as follows: co-crystallized CoA analog = white; PGJ_2 = green; Δ^{12}-PGJ_2 = brown; PGK_2 = violet; 9,10-dihydro-15d-PGJ_2 = black. (B) Docking study of PGs to p300 HAT domain. (C) Orientation of cyclopentenone ring of PGs towards Cys^{1438}. (D) Formation of covalent bond between carbon 9 of CyPG with Cys^{1438} shown as a dotted line.

Discussion

Dietary selenium is known to regulate epigenetic events by modulating the activity of DNA and histone modifying enzymes. Inorganic selenium has been shown to inhibit the activities of HDAC (causing increased histone acetylation) and DNMT1 (inhibiting DNA methylation) in a prostate cancer cell line, leading to the reactivation of *GSTP1*

expression by de-repressing the promoter (Xiang *et al.* 2008). Organic forms of selenium like methylselenocysteine and selenomethionine can be converted to their α-keto derivatives, β-methylselenopyruvate and α-keto-γ-methylselenobutyrate respectively by the action of β-lyases and γ-lyases, which inhibit HDAC activity in human colon and prostate cancer cell lines. Here, we investigate the effect of selenium supplementation (as inorganic selenite) on HAT activity in macrophages. We show that selenium supplementation inhibits HAT activity in a human cell line model of chronic HIV infection, and a murine macrophage model of LPS-induced inflammation. Our data also suggests that the observed inhibition of HAT activity in these cell lines may be due to the of selenium-dependent upregulation in production of the CyPG, Δ^{12}-PGJ_2, as inhibition of the AA – COX pathway by indomethacin, and of the enzyme H-PGDS (which catalyzes the conversion of PGH_2 to PGD_2) by HQL-79 leads to an abrogation of the inhibitory effect. Many studies have documented an important role for CyPGs as key modulators of gene expression by their ability to modify proteins involved in signaling transduction cascades, chromatin dynamics, and transcription factors (Bai *et al.* 2008). Such an interaction with proteins, mainly through Cys thiols, contributes to the pleiotropic effects of these reactive metabolites of PGD_2. Although beneficial effects of CyPGs are reported in experimental models of inflammation, CyPGs are also known to promote proliferation and angiogenesis (Millan *et al.* 2006, Rajakariar *et al.* 2007). Thus, given the ability of these molecules to act in a context-specific, cell-specific, and concentration-dependent manner, it is very likely that CyPGs may impact many vital cellular processes that continue to unfold. In continuation of our quest into the characterization of the CyPG

interactome, we report here, for the first time, that Δ^{12}-PGJ_2, a metabolic end product of arachidonic acid-derived PGD_2, inhibits the enzymatic activity of p300 HAT.

Site-directed mutagenesis and peptide competition experiments further indicated that the inhibition of p300 HAT activity by Δ^{12}-PGJ_2 occurred via the alkylation of Cys^{1438} in the substrate-binding site. This is in contrast to the role of 15d-PGJ_2, a dehydrated product of Δ^{12}-PGJ_2, which has been shown to inactivate HATs through their insolubilization in HepG2 cells; while Δ^{12}-PGJ_2 did not affect the stability of p300 (Hironaka *et al.* 2009). Furthermore, 15d-PGJ_2 also has been shown to inhibit mammalian class I HDACs by covalent binding to two conserved Cys (Doyle *et al.* 2010). Thus, given these effects of 15d-PGJ_2, we have excluded this molecule from the current studies.

Although the molecular basis of epigenetic regulation of gene expression is complex, there is now a clear understanding that HATs, such as p300 and its paralog CREB-binding protein (CBP), modify the unstructured N-termini of histones (called "histone tails"), and are generally correlated with transcriptional competence and diverse biological processes. However, the ability of p300 to impact the function of many histone and non-histone proteins by acetylation has further invigorated the search for specific inhibitors of this key transferase (Wang *et al.* 2008). The "relaxed" substrate specificity of p300 is attributed due to the lack of a deep substrate-binding pocket that potentially prohibits formation of a stable ternary complex between enzyme and the two cosubstrates (Liu *et al.* 2008). These studies reporting the ability of Δ^{12}-PGJ_2 to inhibit the activity of p300 by a unique mechanism involving the nucleophilic Cys in the substrate binding site opens a new area in the field of eicosanoid-dependent regulation of gene expression, particularly in inflammation and HIV biology.

The ability of CyPGs to interact with nucleophiles, particularly Cys thiols (thiolate anion), provides the basis for the biological effects. However, this depends largely on whether cells can produce such high amounts of free CyPGs in cells. One might speculate that localized concentrations of CyPGs in the high nanomolar range may be possible given the fact that COX isozymes, which are membrane bound, functionally couple with downstream PGDS isozymes, which are mostly cytosolic, to produce PGD_2 that undergoes non-enzymatic dehydration and isomerization to form PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2. In fact, recent reports from our laboratory have demonstrated the role of micronutrient selenium (selenium), in the form of selenoproteins, to shunt pathways of arachidonic acid metabolism from PGE_2 to PGD_2 metabolites in macrophages (Vunta *et al.* 2007, Gandhi *et al.* 2011). Our macrophage model is capable of producing relatively high concentrations of Δ^{12}-PGJ_2. As a result, the production of high levels of extracellular Δ^{12}-PGJ_2 relative to its dehydration product, 15d-PGJ_2, was observed in macrophages supplemented with selenium, which is not surprising given the thermodynamic constraints associated with the final dehydration of Δ^{12}-PGJ_2 to 15d-PGJ_2 (Maxey *et al.* 2000, Gandhi *et al.* 2011). Our results show an interesting structure-function correlation wherein Δ^{12}-PGJ_2 and its positional isomer, PGJ_2 (Δ^{13}-PGJ_2), differ in their ability to inhibit p300, with Δ^{12}-PGJ_2 being more reactive than Δ^{13}-PGJ_2. Comparing the two isomers, the presence of a conjugated diene structure following tautomeric rearrangement of electrons within the cyclopentenone ring possibly makes the carbon 9 more electrophilic; while in the case of PGJ_2, due to the presence of an unsaturation at carbon 13, a relatively less electrophilic nature of carbon 9 may help explain the differences in their inhibitory activities (Atsmon *et al.* 1990). The differences in reactivity of CyPGs

isomers are reminiscent of their interaction with GSH as reported by Atsmon *et al* (Atsmon *et al.* 1990). Based on the molecular modeling studies, all PGs tested, including 9,10-dihydro-15d-PGJ_2, seem to enter the substrate-binding site in p300, where the positioning of the electrophilic carbon 9 in the close vicinity of Cys^{1438} appears to be critical for the inhibitory activity. Along the same lines, PGA_2 and PGB_2, although CyPGs, were ineffective as inhibitors of p300, possibly owing to the position of its electrophilic carbon. Similarly, PGK_2 that lacks an alkylidenecyclopentenone structure did not inhibit the enzyme even though the cyclopentanedione ring was likely to orient π orbitals favorably for S nucleophilic attack. This is in contrast to that in HDACs, where even an unrelated electrophile like 4-hydroxynonenal binds to the Cys residues (Doyle *et al.* 2010). Thus, it appears that binding and positioning of the electrophilic carbon 9 in CyPGs to the nucleophilic Cys^{1438} determines the selectivity. Experiments showing the abrogation of interaction of p300 HAT domain with Δ^{12}-PGJ_2 upon incubation of p300 peptide containing Cys^{1438} further suggest that accessibility of the Cys residue plays an equally important role, which is reminiscent of the interaction of CyPGs with specific Cys residues in thioredoxin, GSTP1-1, ubiquitin carboxyl-terminal hydrolase-1, and H-Ras (Oliva *et al.* 2003, Shibata *et al.* 2003, Koharudin *et al.* 2010, Sanchez-Gomez *et al.* 2010). Along these lines, we have previously described the ability of CyPGs to interact with Cys thiols in HIV-1 Tat, here we demonstrate that the consequence of inhibition of p300 has a major effect on the activation of Tat and HIV replication in general (Figure 36B and 36C), accompanied by a decrease in H3 acetylation levels.

Based on the discussion above, it is clear that the mere presence of an α,β-unsaturated carbonyl structure is not sufficient to inhibit p300 activity . That said, natural products

like curcumin, plumbagin, and garcinol, which also contain α,β-unsaturated carbonyl functionalities, inhibit p300 HAT activity through a different mechanism involving a weak hydrogen bonding with Lys^{1358} (Balasubramanyam *et al.* 2003, Balasubramanyam *et al.* 2004, Balasubramanyam *et al.* 2004, Mantelingu *et al.* 2007, Ravindra *et al.* 2009). Furthermore, oxo-containing metabolites of lipid mediators, such as 17-oxo-RvD1, which are formed endogenously from docosahexaenoic acid, could also modulate HAT activity by forming Michael adducts (Sun *et al.* 2007). Thus, it would be interesting to examine the ability of all these (natural) compounds to interact with Cys^{1438} to better appreciate the stereoselectivity as well as define their role in modulating gene expression. Interestingly, our results are in agreement with those reported with plumbagin with regard to the increased specificity towards p300 rather than PCAF (Ravindra *et al.* 2009). This is not surprising since the active site of PCAF lacks the presence of a nucleophilic residue in the form of a conserved Cys648 residue that is too far from the active site (Clements *et al.* 1999).

In summary, we have identified an oxidized fatty acid metabolite as a p300 HAT inhibitor. Our data supports the selective inhibition of p300 HAT activity only by certain CyPGs, based on their ability to interact covalently with Cys^{1438}, a key residue that is pivotal for the binding of substrates, to form a Michael adduct. Although the observed IC_{50} of Δ^{12}-PGJ_2 towards p300 is higher than the concentration of Δ^{12}-PGJ_2 produced in the macrophage model, the cellular data may not correlate entirely with the *in vitro* analysis due to many factors, such as the use of p300 HAT domain in isolation that could alter the structure of this domain in the absence of other interaction partners, lowering the reactivity and accessibility of the Cys thiol. Needless to say, these studies need to be

evaluated in the light of tissue and cell-specific toxicity of CyPGs. Our studies demonstrating the targeting of p300 with Δ^{12}-PGJ_2 on the acetylation of Tat opens a new window of opportunity to regulate proviral transcriptional replication. Such studies are likely to further expand to examine the effect of inhibition of p300 by Δ^{12}-PGJ_2 on many other transcription factors to provide a better understanding of the role of this class of endogenous metabolites in areas such as resolution of inflammation, where CyPGs are already known to facilitate the process. Although preliminary studies on the inability of Δ^{12}-PGJ_2 to inhibit PCAF suggests some level of selectivity, further studies are required to examine the effect of these CyPGs on other classes of HAT enzymes, where a similar mechanism may be followed (Biel *et al.* 2004, Stimson *et al.* 2005).

Chapter 5: Summary and Discussion

Even after the implementation of HAART 17 years ago, there have been countless deaths due to AIDS, with nearly 2 million deaths worldwide in 2010 alone. The cost of the HAART regime, the adverse side-effects of some of the drugs, the emergence of resistant strains of HIV, all make it a challenge to keep HIV/AIDS at bay. Also, due to the cost and side-effects, many patients are driven towards alternative modes of therapy. These CAM approaches can range from the use of herbal medicine and nutrient supplements, to massage therapy and acupuncture. Many such therapies have been suggested to have no curative effects towards the disease itself, but are used by patients because it makes them feel "better". But some modes of therapy like the use of supplements and herbal medicine have been investigated by researchers to determine if there are any underlying scientific bases for the perceived effectiveness of such treatments.

One such mode of treatment is the increased intake of selenium via supplements. Selenium deficiency has been strongly implicated in the disease progression and rate of mortality in HIV infected individuals, while supplementation with selenium has been shown to decrease the viral load if HIV, improve the $CD4^+$ T-cell count, and also improve the overall health and well-being of HIV infected individuals. Selenium, a known antioxidant which exerts its antioxidant functions by incorporation into selenoproteins (e.g. GPx, TR1), is thought to act by inhibiting the NF-κB pathway; but the exact mechanism by which selenium retards viral replication has not been studied comprehensively.

We have shown that selenium, via incorporation into the selenoenzyme TR1, also exerts its effects directly on the HIV protein Tat to bring about a decrease in viral replication. Our studies suggest that TR1 directly interacts with Tat, reducing one or more of the disulfide bonds required to maintain the structure of Tat. This inhibits the transactivating activity of Tat, thus preventing the transcription of the integrated viral genome. Abrogating the activity of TR1 in selenium supplemented, HIV-infected macrophages by ebselen, or by downregulating TR1 expression by siRNA, reversed the protective effect of selenium seen earlier, confirming that selenium inhibited HIV replication via the activity of TR1. Although our data suggest that TR1 directly interacts with Tat to reduce it, another possibility is that TR1 could act indirectly via its action on Trx. Thioredoxin is the natural substrate of TR1 and when reduced, it can cause the reduction of disulfide bonds in a large number of proteins, thus indirectly increasing the reduction potential of TR1. We have preliminary *in vitro* data suggesting that Trx, when reduced, can cause the reduction of disulfides in Tat. Thioredoxin reduces protein disulfides in a non-enzymatic manner, and thus can increase the reduction potential of TR1 greatly. Studies need to be done to investigate the involvement of Trx in the selenium-mediated regulation of HIV Tat via TR1. Knockdown of Trx in cells will allow us to examine if TR1 can act alone to reduce the disulfides in Tat. Trx is known to translocate into the nucleus in the presence of oxidative stress. This ability of Trx could allow it to reduce the disulfide bonds in Tat proteins in the nucleus which TR1 may not have access to. It would be interesting to study the relative efficiencies with which TR1 and Trx reduce HIV Tat *in vitro* and *in vivo*.

It has been established in literature that CyPGs like 15d-PGJ_2 can inhibit the functions of certain proteins by alkylating critical Cys residues in those proteins. It has been also been suggested that this covalent modification of Cys residues is likely specific to a certain degree depending on the structures of the target protein and the CyPG. Based on observations reported by Rozera *et al* that CyPGs could inhibit HIV replication by possibly causing a transcriptional block, we decided to determine the exact mechanism by which CyPGs exert their effects. Our studies showed that CyPGs like 15d-PGJ_2 (and Δ^{12}-PGJ_2, data not shown) could covalently modify the Cys residues in Tat, forming Michael adducts. We further show that such a modified Tat could not transactivate transcription in our luciferase reporter assays, and that the effect translated into diminished viral replication in human macrophages. We also examined the ability of naturally occurring MAEs, structurally unrelated to the CyPGs but containing the α,β-unsaturation found to be important for CyPG activity, to similarly inhibit Tat activity. We have shown that the inhibitory activity of the MAEs is independent of NF-κB activity. We also have preliminary data suggesting that the covalent modification of the Cys residues in Tat likely changes its structure, possibly contributing to the inhibition of transactivating function, although it doesn't seem to affect the binding of Tat to the HIV TAR RNA.

Our group has shown that selenium supplementation enhances the endogenous production of CyPGs in a murine macrophage model. While it is unlikely that endogenously produced CyPGs would efficiently modify HIV Tat in infected cells (because of the relatively high concentrations suggested in our studies), the synergistic effect of TR1 activity and exogenous MAE treatment on the activity of Tat needs to be

studied further. We have preliminary *in vitro* data that reduction of the disulfide bonds in Tat by the thioredoxin system enhances the binding of 15d-PGJ_2 to Tat. Since Tat can spontaneously oxidize, this effect of TR1 and CyPGs could serve to "lock" Tat in an inactive state.

Recent studies from our laboratory have suggested that selenium supplementation causes a shift in macrophage polarization from pro-inflammatory M1 towards the anti-inflammatory M2 phenotype. We posit that this is due to the enhanced activity of PPARγ, and subsequently the expression of PPARγ-dependent genes, and suppression of NFκB in selenium supplemented macrophages. The enhanced activity of PPARγ is due to the production of ligands like Δ^{12}-PGJ_2 and 15d-PGJ_2 in selenium supplemented cells. PPARγ activation has been shown to decrease HIV replication in macrophages in murine models of encephalitis, brain endothelial cells, and primary blood cells (Skolnik *et al.* 2002, Huang *et al.* 2008, Potula *et al.* 2008). Thus, in addition to targeting Tat via TR1 (in the case of selenium supplementation) and covalent modification of the Cys domain (in the case of MAEs), activation of PPARγ-dependent signaling, and plausible polarization of the infected macrophages towards a M2 phenotype could also contribute to the reduction of HIV replication in our experiments. Although it is generally accepted that HIV infection polarizes macrophages towards a M1 phenotype (Porcheray *et al.* 2006, Brown *et al.* 2008), a recent report has suggested that soluble HIV-1 proteins like Nef, Tat and gp120 may activate the MAPK and NF-κB pathways in M2 macrophages, leading to a pro-inflammatory phenotype (Chihara *et al.* 2012). This occurs independently of viral infection in the macrophages. It would be interesting to see the effect of selenium in such macrophages. It remains to be seen if selenium would

counteract the effect of HIV proteins by activating PPARγ dependent anti-inflammatory genes and repressing the κB pathway accompanied by a switch in the phenotype of macrophages.

We have also described preliminary data suggesting that selenium supplementation could further inhibit HIV replication by inhibiting the activity of lysine acetyltransferase KAT3B (p300). KAT3B activity has been shown to be essential for HIV replication. It acetylates the Lys^{50} residue in Tat, allowing it to be released from the transactivation complex, and thus get recycled into the "active" pool of Tat in the infected cell. KAT3B also acetylates histones in the nucleosome at the nuc-0 and nuc-1 position on the HIV promoter, thus de-repressing the promoter and allowing transcription factors to access the same. It has been shown in literature that inhibition of KAT3B activity severely inhibits HIV replication. Although, selenium has been shown to exert epigenetic control by affecting DNA methylation, limited information exists on its effect on histone acetylation and lysine acetylation in general. We show that selenium supplementation leads to an inhibition in the HAT activity of KAT3B, and that this effect may be partly regulated by the production of anti-inflammatory CyPGs like Δ^{12}-PGJ_2. We have also established that Δ^{12}-PGJ_2 inhibits KAT3B activity by covalently modifying a key Cys residue, Cys^{1438}, in the active site of the enzyme. This effect of selenium and CyPGs may be cell type and context-dependent. Also, although KAT3B (and not PCAF) is inhibited by Δ^{12}-PGJ_2, it does not rule out the possibility that selenium supplementation may be inhibiting other HAT enzymes by different mechanisms. Further work needs to be performed to elucidate these mechanisms, if any.

Studies have also shown that HIV infection leads to oxidative stress in HIV-infected individuals. There is evidence showing that the induction of this oxidative stress may partly be mediated by Tat (Westendorp *et al.* 1995, Aksenov *et al.* 2001). Tat is known to transactivate proinflammatory genes like interleukin (IL)-2, IL-6, tumor necrosis factor (TNF) and lymphotoxin (Buonaguro *et al.* 1994, Scala *et al.* 1994, Westendorp *et al.* 1994). The modulation of cellular proinflammatory genes by Tat during HIV-1 infection results in the production of reactive oxygen species (ROS) in the mitochondrion (Murphy 2009), probably due to the dysregulation of the electron transport chain of the mitochondrion. An important defense against ROS are the superoxide dismutases (SODs), which convert the superoxide radical (O^-) to hydrogen peroxide (H_2O_2) (Murphy 2009). It has been reported earlier that Tat reduces the expression and activity of manganese-dependent superoxide dismutase (Mn-SOD), an SOD enzyme located in the mitochondrion (Westendorp *et al.* 1995). This loss in activity of Mn-SOD results in the generation of oxidative stress, which results in increased viral transcription via the activation of NF-κB. This downregulation of the expression of Mn-SOD by Tat is thought to be mediated by the inhibitory action of Tat on the histone acetyltransferase Tip60 (Creaven *et al.* 1999). Simultaneous expression of both Tat and Tip60 has been shown to downregulate the activity of Tip60 on the promoter of Mn-SOD, causing a decrease in the expression levels of the protein (Creaven *et al.* 1999). The increased oxidative stress caused as a result of the loss of Mn-SOD activity was reflected as a decrease in the amount of total glutathione in cells, and also a decrease in the ratio reduced glutathione to oxidized glutathione (Westendorp *et al.* 1995). Selenium and MAE treatment may function to reverse the oxidative stress due to this activity of Tat. It

is not known if the Cys domain of Tat is essential in its binding to Tip60 (and to other cellular proteins). Studies are being designed to address this question and to determine whether the targeting of the Cys domain by selenium supplementation and/or MAE treatment plays a role in inhibiting the interaction of Tat and its binding partners. Earlier literature suggests that HIV Tat conforms to the structure of its binding partners (Shojania *et al.* 2006, Tahirov *et al.* 2010), thus efficiently recruiting them to the HIV LTR, or inhibiting their activity. Our observation that the structure of Tat changes upon modification of its Cys residues opens the very interesting possibility that such modification may be affecting the ability of Tat to interact with its binding partners, thus making Tat unable to recruit the additional transcription factors needed to enhance the transcription of the HIV genome.

Though a substantial body of evidence supporting the beneficial effects of selenium supplementation on HIV infected cells *in vitro* and in laboratory animal models is available, this has not been the case with clinical trials involving selenium supplementation in HIV infected individuals. Studies have consistently shown that low serum selenium concentrations are associated with increased mortality in HIV infected children and adults (Constans *et al.* 1995, Baum *et al.* 1997, Campa *et al.* 1999, Kupka *et al.* 2004, Kupka *et al.* 2005). Studies have also shown that low serum selenium levels increased the risk of HIV-related diseases, like cardiomyopathy, or Keshan's disease (Kupka *et al.* 2007, Twagirumukiza *et al.* 2007). Selenium intervention has resulted in reduced oxidative stress in infected individuals and reduced cardiomyopathy (Zazzo *et al.* 1988, Batterham *et al.* 2001). Selenium supplementation has also been shown to reduce hospital admission rates and health-related costs (Burbano *et al.* 2002), and also reduce

the risk of diarrheal morbidity (Kupka *et al.* 2009). In a clinical study involving 262 HIV-positive individuals, individuals who "responded" to selenium supplementation showed a significant increase in $CD4^+$ cell counts accompanied by a decrease in viral load compared to individuals in a control group (Hurwitz *et al.* 2007). However, this study was criticized due to several limitations in the study where a third of the participants were lost to the study during follow-up, which reduced the significance of the trial. In addition, the selenium supplemented group was divided into "responders" and "non-responders" based on an increase in serum selenium concentration greater than 26.1 µg/L during supplementation. This division of participants during the post-hoc analyses was not originally a part of the study dampening the significance and effect of selenium.

A randomized clinical trial in Zambia on 106 individuals with HIV diarrhea wasting syndrome assessed the benefits of supplementing the subjects with multivitamins (A, C and E), zinc, and low-dose selenium (150 µg) alongwith abendazole and compared the results with a control group given abendazole and placebo. The trial found that supplementation neither affected morbidity and mortality, nor provided symptomatic relief (Kelly *et al.* 1999). Another trial in Kenya looked at cervicovaginal shedding of HIV virus, and also $CD4^+$ counts and viral load in 400 participants, who received vitamins (B complex, C and E) and selenium over 6 weeks. It was found that genital shedding of the virus was 2.5 fold greater in supplemented individuals, accompanied by an increase in viral RNA in vaginal secretions, and no change in viral load (McClelland *et al.* 2004). However, the study also found that $CD4^+$ and $CD8^+$ cell counts were higher in supplemented participant when compared to placebo controls (McClelland *et al.* 2004).

Based on the outcomes of these studies, it can be inferred that selenium offers some modest benefits in HIV infected individuals. This makes it clear that selenium can in no way replace current anti-retroviral therapies, but could be used as a possible adjunct therapy against HIV infection. Also, recent reports from our lab have suggested that the use of NSAIDs could play a confounding role and negate the beneficial effects provided by selenium supplementation (Gandhi *et al.* 2011, Nelson *et al.* 2011). Future trials may have to be designed with a plan to control NSAID use among participants. It is clear that selenium acts via multiple pathways to bring about its effects, and much of this information is just being discovered. As the scientific community gets closer to understanding the myriad roles played by selenium, future clinical trials with better designs and endpoints may be able to address the current clinical concerns.

In the work presented here, we have shown that selenium supplementation can inhibit Tat-dependent transcription by modulating the redox potential of the cell via its incorporation into the selenoenzyme TR1, which reduces the disulfides required for the transactivation activity of Tat (Fig. 43). Selenium may also, via the production of the anti-inflammatory CyPG, Δ^{12}-PGJ_2, inhibit the post-translation modification of Tat by KAT3B. Selenium, thus, can also exert control over the expression of proinflammatory genes at the epigenetic level of histone modification due to its inhibition of KAT3B activity (Fig. 43). Also, use of endogenous or naturally occurring MAEs also inhibit the activity of HIV-1 Tat by covalently modifying the Cys domain of Tat, thus lending another level of control over HIV replication (Fig. 43). Given the need to investigate and establish new and alternative therapies against HIV infection and AIDS, the efficacy of these therapeutic modalities need to be tested further in animal models of HIV.

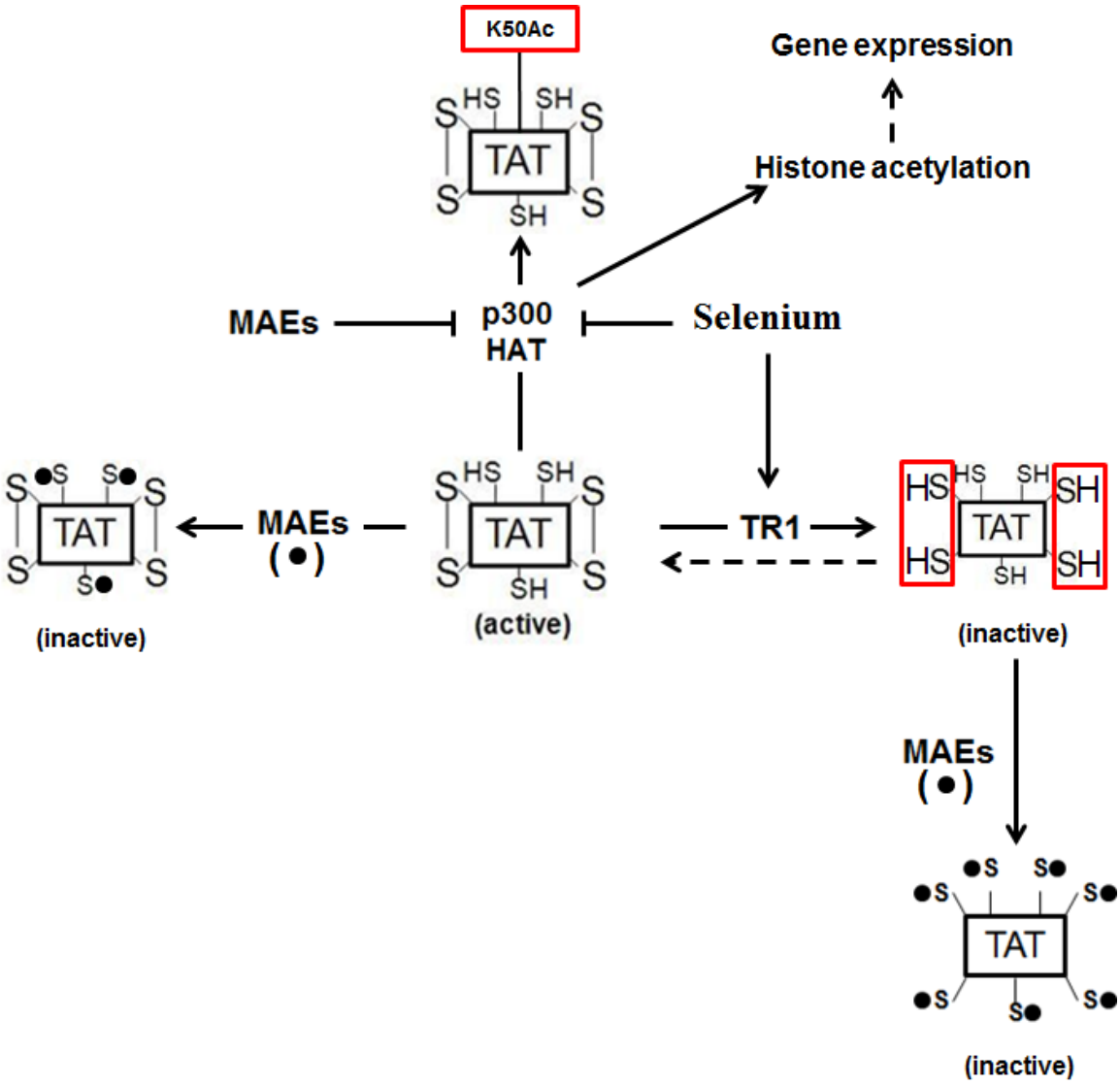

FIGURE 43. **Selenium and MAEs inhibit Tat-mediated HIV replication at multiple levels.** Selenium supplementation, via the production of TR1, can target the disulfides in HIV-1 Tat to inactivate it. MAEs like 15d-PGJ_2 and Cel can covalently modify the free thiols in Tat to inhibit its activity. It may be possible to use Se supplementation and MAEs together to completely modify all the Cys residues in the Cys domain of Tat. Se supplementation and MAEs may also inhibit the acetylation of Tat by inhibiting the activity of the HAT p300. Inhibition of Tat acetylation by p300 results in a decrease in viral replication. Modulating p300 activity may also have implications on the expression of certain genes which are activated during HIV infection and which help in establishing the disease.

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C. Publications.

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