

Activity of the dietary antioxidant ergothioneine in a virus gene-based assay for inhibitors of HIV transcription

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Abstract. The “Long Terminal Repeat” (LTR) of HIV-1 is the target of cellular transcription factors such as NF- κ B, and serves as the promoter-enhancer for the viral genome when integrated in host DNA. Various LTR-reporter gene constructs have been used for *in vitro* studies of activators or inhibitors of HIV-1 transcription, e.g., to show that antioxidants such as lipoic acid and selenium inhibit NF- κ B-dependent HIV-1 LTR activation. One such construct is the pHIVlacZ plasmid, with the HIV-1 LTR driving expression of the lacZ gene (encoding β -galactosidase, β -gal). Typically, for inhibitor screening, cells transfected with pHIVlacZ are activated using tumor necrosis factor- α (TNF- α), and the colorimetric o-nitrophenol assay is used to assess changes in β -gal activity. A variant of this assay was developed as described here, in which LTR activation was induced by pro-fs, a novel HIV-1 gene product encoded via a –1 frameshift from the protease gene. Cotransfection of cells with pHIVlacZ along with a pro-fs construct produced a significant increase in β -gal activity over controls. L-ergothioneine dose dependently inhibited both TNF- α -mediated and pro-fs-mediated increases in β -gal activity, with an IC₅₀ of about 6 mM. Thus antioxidant strategy involving ergothioneine derived from food plants might be of benefit in chronic immunodeficiency diseases.

1. Introduction

Gene expression in retroviruses such as HIV-1 is regulated at multiple levels. Transcriptional regulation takes place predominantly via the interactions of cellular RNA polymerases, TATA binding protein, and transcription factors such as NF- κ B, that bind to specific target DNA sequences in the viral “long terminal repeat” (LTR), which serves as the promoter-enhancer for the viral genome when integrated in host DNA.

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Gene expression can be further regulated by co-translational processes such as ribosomal frameshifting, which permits the expression of certain overlapping genes, and various types of readthrough suppression, a process by which a stop codon is translated as an amino acid, leading to the formation of alternate protein isoforms (one example being insertion of selenocysteine at the UGA stop codon) [22].

The LTR of HIV-1 is about 600 base pairs long, with a TATA box near its 3' end. The LTR is highly responsive to changes in cellular redox potential [7]. This is largely mediated via redox-sensitive transcription factors such as AP-1 and NF- κ B [6,11,14,18], which in turn are regulated by cellular reducing agents such as thioredoxin, glutathione, and the selenoproteins thioredoxin reductase and glutathione peroxidase [3,21].

When placed in a suitable plasmid vector upstream of a "reporter" gene, the HIV-1 LTR has proven to be a useful tool for the identification of compounds and cellular factors that can either stimulate or inhibit viral transcription [5]. The reporter gene should code for a protein that can be easily assayed, such as luciferase, or β -galactosidase (β -gal), which is encoded by the lacZ gene. The pHIVlacZ plasmid contains the complete HIV-1 LTR upstream of lacZ. In cells transfected with such a plasmid, inhibitors or stimulators of the regulatory element (in this case viral LTR) yield measurable changes in the level of the reporter gene product. Typically, for inhibitor screening, cells transfected with pHIVlacZ are activated using TNF- α , and the colorimetric o-nitrophenol assay is used to assess changes in β -gal activity. Using similar reporter gene systems, antioxidants such as lipoic acid and selenium have been shown to inhibit the TNF- α -mediated increase in β -gal activity [11,13].

In this paper, we report the development of a variant of this assay, in which, rather than using TNF- α as an activator, LTR activation is induced by co-transfection with pro-fs, a novel HIV-1 gene product which was previously identified in the -1 reading frame overlapping the protease gene, and hence named pro-fs, for protease frameshift. Pro-fs contains a well conserved peptide sequence with local similarity to the DNA binding loop of NF- κ B [21], which is also the target site of thioredoxin binding to NF- κ B [12]. The basis of the current study is the observation that in cells transfected with pHIVlacZ, co-expression of the pro-fs gene produces a multi-fold increase in β -gal activity over controls. This activating effect of pro-fs on the viral LTR can be abolished by specific mutations to either pro-fs or to the two NF- κ B binding sites in the LTR [19]. The pro-fs gene product can mimic the thioredoxin binding ability of NF- κ B, as high affinity specific binding between recombinant pro-fs and thioredoxin has been demonstrated in vitro by several independent methods [20]. Notably, pro-fs is a putative selenoprotein, as it has two sites with the potential to incorporate Se as selenocysteine (i.e., 2 in-frame UGA codons in the mRNA). The first (most 5') of these potential SeCys residues of pro-fs aligns with an essential Cys residue (Cys62) in the DNA-binding loop of NF- κ B [21], which, as well as being directly involved in sequence-specific recognition of DNA, interacts directly with an active site Cys residue of thioredoxin during reductive activation of NF- κ B [12,14,16]. The ability of pro-fs to mimic NF- κ B may be explained at least in part by its demonstrated thioredoxin binding ability [20]. Using fluorescent protein conjugates, pro-fs has been shown to localize in the nuclei of transfected cells, which is also consistent with a potential role in NF- κ B activation in the nucleus [20].

L-ergothioneine (2-mercaptohistidine trimethylbetaine) (Fig. 1), is a naturally occurring dietary antioxidant whose name derives from the fact that it was first identified in the sclerotia of the ergot fungus. Ergothioneine is found in most plants and animals, with availability in humans only coming from absorption through diet. Ergothioneine levels in the blood of healthy human males in the Western province of Saudi Arabia have been estimated to be in the order of 100 μ M [10]. Ergothioneine has been shown to inhibit cell death induced by H₂O₂, and to inhibit DNA oxidation by peroxyxynitrite, making it a potentially interesting antiviral antioxidant [1,2]. Ergothioneine inhibits both H₂O₂ and

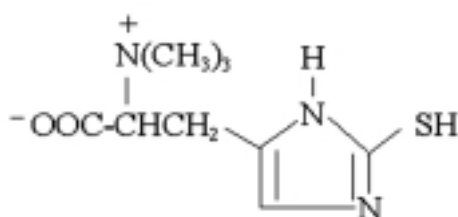


Fig. 1. Structure of ergothioneine.

TNF- α -mediated activation of NF- κ B, as assessed by measurements of NF- κ B-dependent IL-8 release from A549 cells [17], modulates oxidative stress induced by ferric nitrilotriacetic acid [4] and exhibits neuroprotective potential by modulating toxic effects of amyloid beta in PC12 cells [8] and inhibiting retinal ganglion cell death induced by N-methyl-D aspartate [15]. Here we show that ergothioneine dose-dependently inhibits both TNF- α -mediated and pro-fs-mediated increases in HIV-1-LTR-driven β -gal activity.

2. Materials and methods

2.1. Construction of HIV-1 pro-fs (protease frameshift) eukaryotic expression vector

The hypothetical HIV-1 protein encoded by pro-fs can only be expressed *in vivo* by -1 ribosomal frameshifting. The primary gene product, a gag-pol-pro-fs polyprotein, is expected to be rapidly cleaved by HIV protease. The targets of the HIV protease include a site at the N-terminal of HIV protease itself, which also serves as the N-terminal of pro-fs (the first 20 residues of which are identical to that of the HIV protease). This low molecular mass (c. 8 kDa) protease-processed isoform of pro-fs was cloned, incorporating a Met start codon as well as an Asp codon 5' to the Pro residue which begins the actual pro-fs sequence (both are underlined in the primer sequence shown below). The Asp codon was included in order to ensure that the start codon context meets the Kozak +4 rule [9]. This in turn will ensure efficient initiation of translation, which would otherwise be violated by the first C base of the protease N-terminal Pro codon following the engineered AUG. The DNA fragment corresponding to the region of interest of HIV-1 protease was PCR-amplified from the pBH10 clone of HIV-1, obtained from the NIH AIDS Research and Reference Reagent Program, Germantown, MD. The PCR primers used to obtain the complete native HIV-1 protease fragment were:

5' sense, 5' TTGCTAGCATGGACCCTCAGATCACTCTTTGGC 3', containing a NheI site, and 3' reverse complement, 5' GGAAGCTTGTCTCAATAGGGCTAATGGG 3', containing a HindIII site.

To create a construct that would efficiently express the pro-fs protein, it was necessary to introduce a mutation at the predicted frameshift site that would bring the N- and C-terminal regions of pro-fs into the same reading frame. To generate the complete 69 residue pro-fs trans-frame protein sequence, as predicted by Taylor et al. [23], an extra A was added at the frameshift site, and an in-frame TAA near the 3' end of the pro-fs sequence was mutated into CAA (see reference [23] and Table 1 therein). Using the pBH10 plasmid as native pro-fs template, these two mutations were introduced by mega-primer PCR mutagenesis with the primers mentioned above and the following two oligonucleotide primers: sense 5' AGAAATATGTGGACACAAAGCTATAGGTACAGT 3' (underlined C resulting from the UAA to

CAA codon mutation) and antisense 5' CCTGTATCTAATAGAGCTTCCTTTTAGTTGCCCCCCTAT 3' (underlined T corresponding to the A base inserted for the required frameshift mutation).

Because the mechanisms and location of downstream RNA structure elements that HIV-1 may use for recoding the UGA stop codon as a sense codon for Sec are still unknown [22], the pro-fs expression construct was designed to include a mammalian selenocysteine insertion sequence (SECIS) element, from the rat 5'-deiodinase (5'-DI) gene, in order to ensure the translation of the two in-frame UGA codons of the HIV-1 pro-fs sequence as Sec during expression in mammalian cells. A PCR fragment spanning the SECIS element from the 3'-UTR of the rat type I 5'-DI gene was obtained as described previously [24].

To generate the eukaryotic expression vector pPro-fs, the PCR products for the pro-fs coding region and the 5'-DI SECIS element were subcloned into the pEGFP-C1 vector (Clontech) sequentially, using the following cloning sites: NheI, HindIII (coding sequence) and HindIII, BamHI (SECIS), followed by the PCR mutagenesis using the 2 primers described above. A similar (negative control) vector with the SECIS element but without the pro-fs coding sequence was constructed and named p2. The sequence of the entire transcribed region of the resultant plasmids pPro-fs and p2 were verified by DNA sequencing. Sequencing and oligonucleotide syntheses were performed at the University of Georgia Molecular Genetics Instrumentation Facility. Restriction enzymes were obtained from Promega (Madison, WI); other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

2.2. Cell culture and transfection

The MDCK (canine kidney) cell line was a gift from Dr. Fengxiang Gao, Centers for Disease Control and Prevention, Atlanta GA. Cells to be used for the transfection studies were grown under 5% CO₂ at 37°C, in Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 2 mM L-glutamine, 5 µg/ml gentamycin, 0.5 mM sodium pyruvate, 1.2 g/L sodium bicarbonate and 10% calf serum (Atlanta Biologicals). These cells were used directly for subsequent transfections with various plasmids.

2.3. Generation of pProfs and P2 stably transfected MDCK cell lines (Profs-MDCK, P2-MDCK)

MDCK cells were cultured and transfected with either pPro-fs or P2 using Lipofectamine-2000 (GIBco) in DMEM medium supplemented with 10% calf serum. Briefly, 2.5×10^5 cells/well were seeded in 6-well plates the day before transfection. When cells attained 90% confluency, 3 µg plasmid DNA complexed with 6 µg Lipofectamine-2000 was added to each well and left overnight. After 24 hours, cells were diluted 100-fold and transfectants were selected with 500 µg/ml G418 (GIBco), by changing G418-containing medium every 3 days for 4–6 weeks, after which the positive clones were pooled.

2.4. Transient transfection with pHIV-lacZ LTR-reporter construct

The pHIVlacZ plasmid was obtained from the NIH AIDS Research & Reference Reagent program (Cat# 151). To study the effects of pro-fs and TNF-α on HIV LTR promoter activity, and potential inhibitors thereof, the corresponding stably transfected cells (native MDCK, Profs-MDCK, or P2-MDCK) were transiently transfected with pHIVlacZ using lipofectamine-2000, on 6-well plates, with each well seeded with 2.5×10^5 cells and 3 µg of pHIVlacZ plasmid DNA.

2.5. Measurement of HIV-1 LTR activation in pHIVlacZ transfected cells by β -gal reporter gene activity

Immediately following transfection, cells were grown in medium containing 20 nM sodium selenite for 48 hours and then harvested for the β -gal activity assay. For inhibition studies, various concentrations of L-ergothioneine were added one hour after transfection. For studies using TNF- α to activate the LTR, TNF- α to a final concentration of 9 ng/ml was added either 3 hrs after transfection, or 2 hrs after the addition of ergothioneine in inhibition experiments. Approximately 48 hours later, cells were washed with PBS, then harvested by trypsin digestion, centrifuged at 12,000 g, and washed once with PBS. Then 400 μ l cell lysis buffer (Promega) was added to each tube, and vortexed for 30 seconds, allowed to stand for 15 min, then vortexed again. After centrifugation at 12,000 g for 10 minutes, the supernatants were collected and assayed for β -gal activity using Promega's assay kit, according to the manufacturer's instructions. The assay is based on spectrophotometric monitoring of the enzymatic conversion of the substrate o-nitrophenyl- β -D-galactopyranoside (colourless) to galactose and o-nitrophenol (bright yellow in alkaline buffer). Briefly, 150 μ L of cell extract supernatant for each sample was mixed with the same volume of 2X assay buffer, vortexed, and incubated at 37°C for 30 min. Then 500 μ l of 1 M aqueous Na₂CO₃ was added to stop the reaction, and absorption at 420 nm was determined. Measurements were standardized relative to protein concentration, and expressed as OD420 absorption per mg protein. Lysate protein concentration was determined by the Lowry method, using 15 μ l of cell extract for each sample.

2.6. Statistical analyses

Data are expressed as mean \pm S.E.M; when shown, error bars in figures also correspond to S.E.M. The significance of differences between values was assessed by an unpaired t-test.

3. Results

3.1. Pro-fs activation of the HIV-1 LTR

The putative HIV-1 pro-fs protein has significant sequence similarity to the NF- κ B/Rel family of transcription factors [21], and a demonstrated capacity for functional mimicry of the thioredoxin binding ability of NF- κ B [20]. To test whether the expression of pro-fs has any effect on the HIV-1 LTR, MDCK cells stably transfected with either pro-fs (Pro-fs-MDCK) or the control plasmid p2 (P2-MDCK) were transiently transfected with pHIVlacZ and assayed for β -gal enzyme activity as described in the Materials and Methods section. The results of a typical triplicate experiment are shown in Fig. 2.

In pro-fs transfected cells, there was a 3.5 fold increase ($p < 0.001$) in the induction of β -gal enzyme activity (7.26 ± 0.38 , expressed as OD420 absorption per mg protein) relative to that measured in the control cells (2.08 ± 0.15 OD420/mg). This ability of pro-fs to induce the expression of genes driven by the HIV-1 LTR promoter is consistent with the prediction by Taylor et al. that pro-fs may function via mimicry of NF- κ B [21], and was the basis of the use of pro-fs as an alternative to TNF- α for activation of pHIVlacZ.

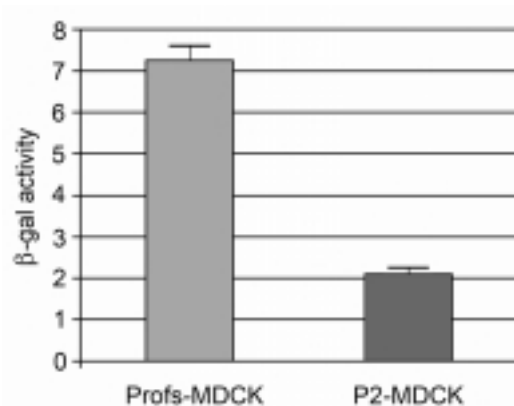


Fig. 2. HIV-1 pro-fs is a potent activator of HIV-1 LTR. MDCK cells stably transfected with either a pro-fs expression construct (Pro-fs-MDCK) or an “empty” control vector (P2-MDCK) were transiently transfected with the pHIVlacZ plasmid, which contains the HIV-1 LTR promoter-enhancer driving expression of the lacZ gene. β -gal enzyme activity is expressed in units of OD420 absorption per mg protein. In pro-fs transfected cells, there was a 3.5 fold increase in the induction of β -gal enzyme activity relative to the control cells ($p < 0.001$).

3.2. TNF- α concentration-response curve

In order to determine an appropriate concentration of TNF- α to be used to activate the HIV-1 LTR, a dose-response curve for TNF- α was obtained. First, MDCK cells were transiently transfected with pHIVlacZ as described in Methods. Three hours after transfection, various concentrations of TNF- α were added to the cell culture medium, to final concentrations of: 0, 0.5, 1, 2, 3, 4, 5, 10, 20, and 40 ng/ml. After 48 hrs, the supernatants were assayed for β -gal enzyme activity, normalized for protein levels; the results of a typical experiment are shown in Fig. 3. Between the concentrations of approximately 5 to 10 ng/ml of TNF- α , and extending up to about 20 ng/ml, the β -gal activity becomes saturated and appears to reach a stable plateau, which is not increased with higher TNF- α concentrations. Therefore, in subsequent experiments with inhibitors, a working concentration of 9 ng/ml was used as the stimulatory concentration of TNF- α , with a range of inhibitor concentrations. While β -gal activity is maximal at a slightly lower TNF- α concentration (about 4 ng/ml), there is more variability around that concentration, which could be a source of experimental error; thus the higher TNF- α concentration of 9 ng/ml from the plateau region of the curve was chosen as the standard for subsequent LTR-stimulation experiments.

3.3. Ergothioneine inhibition of HIV-1 LTR activation by either pro-fs or TNF- α

Using either pHIVlacZ-transfected MDCK cells stimulated with 9 ng/ml of TNF- α , or pHIVlacZ-transfected pro-fs-MDCK cells, various concentrations of ergothioneine were assessed for their ability to inhibit either pro-fs or TNF- α driven HIV-1 LTR activation of β -gal expression. First, the upper limit for the concentration of ergothioneine was determined, in order to ensure that apparent inhibition was not due to reduced β -gal production resulting from cytotoxicity. This was assessed microscopically in pHIVlacZ-transfected pro-fs-MDCK cells, using a range of ergothioneine concentrations up to 50 mM. Significant cell damage was visible only above 25 mM, which was taken as the upper limit in these experiments. This is consistent with a prior report that high concentrations of ergothioneine (5 mM) were well tolerated by cultured N-18-RE-105 cells [1]. In the viral LTR inhibition assays, ergothioneine showed comparable potency as an inhibitor independent of whether pro-fs or TNF- α was used as an

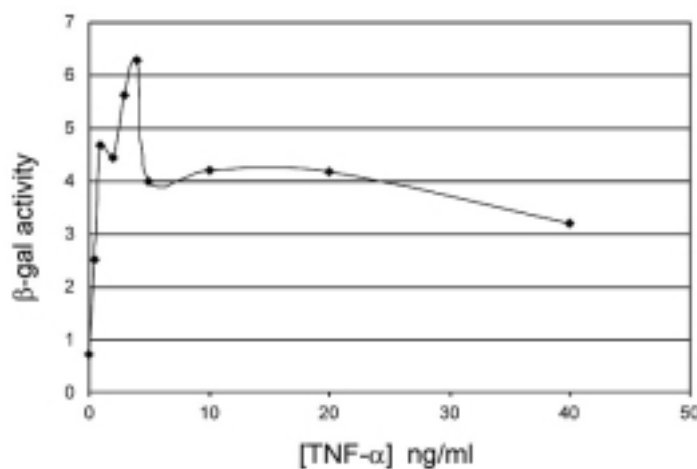


Fig. 3. Determination of TNF- α concentration for activation of LTR. Relative β -Gal enzyme activity induced by various TNF- α concentrations stabilizes and plateaus above about 5 ng/ml. A 9 ng/ml concentration of TNF- α was chosen as the standard concentration for activating pHIVlacZ in subsequent experiments.

activator (Fig. 4). The (maximal) β -gal activity obtained by activation in the absence of inhibitor was defined as 100% in each case. The amount of inhibition was statistically significant for all of the ergothioneine concentrations tested (Fig. 4). At 6 mM, ergothioneine inhibited about 50% of β -gal production independent of the activating agent.

4. Discussion

The ability of recombinant pro-fs to induce HIV-1 LTR-driven gene expression in transfected cells has been studied in detail by using site-directed mutagenesis; the pro-fs gene activates HIV-1 LTR in a structurally specific and NF- κ B-dependent manner, as the activating effect is abolished by specific mutations to either pro-fs or to the two NF- κ B binding sites in the LTR (L. Xiao, L. Zhao and E.W. Taylor, unpublished results). Hence, the pro-fs-pHIVlacZ co-transfection assay described here will be primarily useful in identifying HIV-1 LTR inhibitors whose mode of action is mediated by NF- κ B. However, because pro-fs is an HIV-encoded gene, pro-fs-mediated activation of HIV-1 transcription via the LTR probably comprises a viral autoregulatory mechanism, not all the elements of which are understood at this time, other than that the effects are ultimately mediated via NF- κ B, and may involve the interaction of pro-fs with thioredoxin [20]. Nonetheless, because of its unique viral mechanistic component, this assay may enable the identification of potential inhibitors that work via mechanisms that might not be revealed by standard assays for NF- κ B inhibitors. Thus, the assay system presented here should complement, rather than substitute for, the traditional assay based upon the use of TNF- α or other activators of NF- κ B, which may act upstream of pro-fs.

As observed for ergothioneine in this system (Fig. 4), it is not uncommon that 100% inhibition of HIV-1 LTR activity is not achieved even at the highest inhibitor concentrations, which may reflect the fact that multiple cellular factors can activate transcription via the LTR, and inhibitors tend to work via specific pathways, such as NF- κ B inhibition; this can result in less than complete blockade of transcription because of basal stimulation by other cellular factors. HIV inhibition by ergothioneine in the pro-fs-LTR

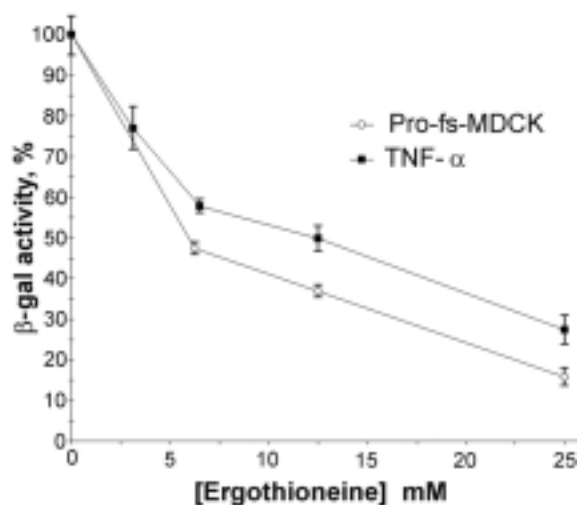


Fig. 4. Ergothioneine inhibition of HIV-1 LTR activation by either pro-fs or TNF- α . Ergothioneine showed comparable potency as an inhibitor independent of whether pro-fs or TNF- α was used as an activator of the viral LTR. The amount of inhibition (compared pairwise to the 100% activity point with no inhibitor) was statistically significant for all of the ergothioneine concentrations tested. With pro-fs as activator, $p < 0.001$ for all ergothioneine concentrations; with TNF- α as activator, $p < 0.005$ for all ergothioneine concentrations, except for the lowest concentration (3.25 mM), for which $p < 0.05$.

cotransfection system has been achieved in the millimolar concentration range (IC_{50} of ~ 6 mM). In regard to the use of TNF- α as an activator of the viral LTR, when TNF- α is stored for some period of time, even at -70°C , its activity can decrease significantly. It is therefore recommended that each time before TNF- α is used as an activator, its activity over a range of concentrations should be measured in order to determine the appropriate TNF- α concentration, as exemplified here (Fig. 3).

The practicality and potential utility of the pro-fs-pHIVlacZ co-transfection assay needs to be explored further. Principally, we have observed that, particularly after some passaging, MDCK cells that are transfected with pro-fs sometimes do not grow as well as the parent cell line, and may show evidence of abnormal nuclear morphology, which may be the result of some cumulative action of pro-fs (e.g., possibly in modulating thioredoxin-related pathways). Future development could involve the identification of other cell lines that may be more permissive for pro-fs expression, hence enabling the high throughput screening of potential inhibitors of HIV transcription. In that regard, the development of a pro-fs transfected T cell line might be most relevant for mechanistic studies of pro-fs, and for the identification of inhibitors of HIV-1 transcription, since T cells are targeted in HIV infection, and also because the redox regulation of NF- κB can vary in different cell types.

In conclusion, using a novel viral gene product, pro-fs, as the transcriptional activator, a variant of the standard HIV-1 LTR reporter gene assay was used to investigate its potential for assessing the inhibitory effects of antioxidants and phytochemicals on HIV-1 transcription. The diet-derived antioxidant ergothioneine showed consistent inhibitory activity on the HIV-1 LTR assessed using either TNF- α or pro-fs as the activator.

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