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Nf-κb-Dependent Inhibition of HIV-1 Transcription by Withaferin A

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Abstract

Despite the remarkable progress made in suppressing HIV-1 infection, antiretroviral drugs are still often inaccessible in developing countries, and there is an urgent need for cheaper and alternative drugs. HIV-1 replication is triggered by the activation of the long terminal repeat (LTR) promoter, which contains two binding sites for the transcription factor nuclear factor κB (NF-κB). Withaferin A (WA), a steroidal lactone isolated from the Indian medicinal plant *Withania somnifera*, has been found to have significant pharmacological effects on the regulation of immune response. Recent studies have demonstrated that the anti-inflammatory properties of WA are mainly due to its inhibition of the NF-κB pathway. In the present study, we demonstrate that WA represses HIV-1 LTR transcription and viral replication through NF-κB inhibition.

The human lymphocyte T cell line Jurkat E6.1 was treated with WA and infected with wild-type pseudotyped HIV-1 particles or mutant viruses containing inactive κB sites. Then, the effect of WA on HIV-1 replication was evaluated by the measurement of reporter activity. Electrophoretic mobility shift assays and Western blots analysis were also performed to study the impact of WA on NF-κB. We found that WA inhibited the transcription of wild-type pseudotyped viruses in single-round infection assays, whereas mutant viruses containing inactive κB sites showed a reduced response to WA. Moreover, we found that WA directly or indirectly inhibits NF-κB nuclear translocation, including RelA and p50 subunits. However, the degradation of inhibitory protein IκB-α was not prevented by WA. Taken together, our results suggest that WA inhibits HIV-1 transcription in human Jurkat E6.1 lymphocyte T cells through the NF-κB pathway.

Keywords: T cells; HIV-1; Withaferin A; NF-κB

Introduction

Highly active anti-retroviral therapy (HAART) has revolutionized the treatment of HIV-1 infection by dramatically reducing the mortality and morbidity of infected individuals. However, 95% of people with HIV and AIDS live in developing countries, where access to antiretrovirals is limited. Thus, there is an urgent need for cheaper and alternative drugs [1].

HIV-1 replication is triggered by the interaction of host cellular transcription factors with *cis*-regulatory elements within the viral long terminal repeat (LTR). The HIV-1 LTR promoter contains functional regions required for transcriptional activation, including the core enhancer, which contains two binding sites for nuclear factor κB (NF-κB) that are critical in the regulation of HIV transcription [2-5]. Both sites are activated during transcription; a point mutation at one of the binding sites abolishes gene activation and thereby prevents viral production [2-3,5]. In addtion, the transcription factor NF-κB is a key regulator of cellular processes involved in the immune response, and in cellular differentiation, proliferation, and apoptosis [6-8].

*Withania somnifer*a (also known as Ashwagandha or Indian Winter Cherry) is a traditional medicinal plant that has been safely used for centuries in Indian Ayurvedic medicine for treating various disorders. The roots and leaves of *W. somnifera* are commonly prescribed for tumors, inflammation, arthritis, asthma, and hypertension [9-15]. *W. somnifera* is the source of the largest and structurally most diversified set of withanolides, of which withaferin A (WA) is among the first of the group to be isolated and to show significant pharmacological properties, such as anti-tumor, anti-inflammatory, and immunomodulatory activity [9]. WA exhibits strong inhibitory effects on the growth of several human leukemic cell lines [16], and causes mitotic arrest in human breast cancer cells [12]. Recent studies have shown that *W. somnifera* extract, including its main component WA, inhibits NF-κB activation by blocking the activation of IκB-α, which is in turn induced by tumor necrosis factor-α in several cell types, such as L929 fibrosarcomic cells, A549 lung epithelial cells, the erythroleukemia cell line K562, and HUVEC endothelial cells [9,14,17]. It has also been reported that WA disrupts IκB kinase (IKK) kinase activity by the hyperphosphorylation of its catalytic subunit IKKβ on ser-181, leading to IKK inactivation [15]. To date, the impact of WA on HIV-1 transcription and viral production has never been determined.

Given the central role of NF-κB in the regulation of immune reactions and its implication in the transcriptional activation of HIV-1, we investigated the antiviral activity of WA against HIV-1 in T cells resulting from the inhibition of the NF-κB pathway. We found that WA inhibits HIV-1 transcription in Jurkat cells, and that this inhibition is modulated by the reduced nuclear translocation of the active form of NF-κB, p65/p50. A greater understanding of the interaction between WA and the NF-κB pathway will aid in the development of alternative therapies for controlling HIV-1 replication.

Materials and Methods

Reagents and plasmids

Phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin (PHA), and WA were purchased from Sigma (St. Louis, MO, USA). Recombinant human tumor necrosis factor-α (TNF-α) was purchased from Cederlane (Burlington, ON, Canada). Stock solutions of WA (10 mM) were prepared by dissolving the lyophilized product in DMSO,

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and were stored at -20°C until needed.

The pLTR-luc plasmid contains the luciferase reporter gene, which is under the control of the wild-type (GGGACTTTCC) HIV-1 $_{HXB2}$ LTR (-453 to +80) [18]. The commercial pNFκB-luc molecular construct contains five consensus NF-κB-binding sequences controlling the luciferase gene, along with a minimal promoter. The pκB-TATA-luc plasmid contains the HIV-1 enhancer region (-105 to -70) and a TATA box placed upstream of the luciferase gene. This plasmid is a generous gift from Dr. WC Greene (The J. Gladstone Institutes, San Francisco, CA, USA). Anti-phospho-IκB-α (Ser32/36), anti-IκB-α, and anti-actin antibodies were purchased from Cell Signaling Technology (Whitby, ON, Canada). Anti-NFκB-p50 (H119) and anti-NFκB-p65 (C20) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

To generate the mutant sequence lacking the κB binding sites, the *Xho*I-*Nco*I portion of pNL4.3.lucE$^-$ containing the 3′ LTR of HIV-1 was cloned into a pMCD-HIV1 plasmid [19], and directed mutagenesis was performed by PCR as described previously [20]. Briefly, 200 nM primers containing the desired mutation (sense: 5′-GCT ACA ACT CAC TTT CCG CTG CTC ACT TTC CAG GGA GGC-3′, reverse: 5′-GAG CAG CGG AAA GTG AGT TGT AGC AAG CTC GAT GTC AGC AGT TCT TGA-3′) were used in a 50 µl reaction containing 50 ng of template plasmid, 200 µM dNTPs, and 2.5 U Pfu Turbo enzyme in 1× reaction buffer (New England Biolabs, Ipswich, MA, USA). The reaction was then incubated with 10 U *Dpn*I restriction enzyme at 37°C for 1 h to eliminate the template. The *Xho*I-*Nco*I portion was then reintroduced into pNL4.3.lucE- by standard subcloning.

Cells and culture conditions

The parental lymphoid T cell line, Jurkat E6.1, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), while the 1G5 cell line was supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (Rockville, MD, USA). 1G5 is a clonal cell line derived from Jurkat E6.1 cells that have been stably transfected with a luciferase gene driven by the HIV-1 LTR. The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.22% $NaHCO_3$. The cells were maintained at 37°C in a 5% CO_2-humidified atmosphere.

Transient transfection and luciferase assays

The transfection assays were carried out as described previously [20]. Jurkat E6.1 cells (2.5×10^7) were first washed in a TT buffer (25 mM Tris-HCl, pH 7.4, 5 mM KCl, 137 mM NaCl, 0.6 mM Na_2HPO_4, 0.5 mM $MgCl_2$, and 0.7 mM $CaCl_2$) and then resuspended in 2.5 ml of TT containing 25 µg of the indicated plasmids at a final concentration of 500 µg/ml. The cells/TT/plasmid/DEAE-dextran mixture was incubated for 25 min at 37°C. Thereafter, cells were diluted with complete culture medium supplemented with 100 µM chloroquine (Sigma) to reach a concentration of 1×10^6 per ml. After 45 min of incubation at 37°C, cells were centrifuged and resuspended in complete culture medium and incubated at 37°C overnight. To minimize variations in plasmid transfection efficiencies, transfected cells were pooled after transfection and then separated into various treatment groups as follows. Transiently transfected cells were seeded at a density of 5×10^5 cells per well (100 µl) in 96-well plates. Cells were left untreated or were treated with PMA/PHA (20 ng/ml and 1 µg/ml, respectively) and 0.5 to 5 µM WA in a final volume of 200 µl for a period of 8 h at 37°C. Cells were then lysed and luciferase activity was assessed.

In order to assess whether WA can modulate HIV-1 LTR activity, 1G5 cells or Jurkat E6.1 cells (5×10^5) transfected with different reporter plasmids were either left untreated or treated with PMA/PHA (20 ng/ml and 1 µg/ml, respectively) and 0.5 to 5 µM of WA in a final volume of 200 µl for 8 h at 37°C [21]. Next, 100 µl of cell-free supernatant were withdrawn from each well and 25 µl of cell culture lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 1% Triton X-100, and 10% glycerol) were added before incubation at room temperature, while vortexing for 30 min. One freeze cycle was performed afterwards to facilitate cell lysis. An aliquot of cell extract (20 µl) was mixed with 100 µl of luciferase assay buffer (20 mM Tricine, 1.07 mM $(MgCO_3)_4{\cdot}Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 270 µM coenzyme A, 470 µM luciferin, 530 µM ATP, and 33.3 mM dithiothreitol) and cell lysates were evaluated for luciferase activity by LUMIstar luminometer (BMG Lab Technologies GmbH, Offenburg, Germany) according to the manufacturer's instructions. Values are expressed as RLU (relative light units) as measured by the apparatus.

Western blot analysis

Jurkat E6.1 cells (2×10^6) were treated with PMA/PHA (20 ng/ml and 1 µg/ml, respectively) and 1 µM WA for the indicated time periods. Then, cells were washed with ice-cold PBS and lysed in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 50 mM NaF, and 2 mM sodium orthovanadate, with indicated quantity of protease inhibitor cocktail (Sigma), adjusted to pH 8.0). The lysates (20 µg) were separated by 10% SDS-PAGE and electrotransferred onto a PVDF membrane. Blots were probed using the appropriate antibodies. The immunoreactive protein was detected using enhanced chemiluminescence reagents (GE Healthcare, Aliso Viejo, CA, USA) and analyzed with a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Viral production and HIV-1 infection assays

The wild-type and mutant pseudotyped HIV-1 viruses were produced as described previously [19]. Briefly, 4×10^6 HEK293T packaging cells were seeded into 100 mm dishes in 9 ml complete medium the day before transfection. Calcium phosphate transfection was performed by mixing together 1.5 µg pCMV-VSV-G and 13.5 µg pNL4.3.-lucE$^-$-based constructs in 500 µl of 250 mM $CaCl_2$ and 500 µl HBS 2× (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.08). The precipitates were allowed to form for 2 minutes and immediately added drop-wise on top of the cell culture medium. Two days later, the virion-containing medium was recovered and filtered through a 0.45 µm filter and kept frozen at -80°C. We used qPCR to estimate the required minimal virion quantity for the integration of proviral DNA in each cell. One region of the HIV-1 promoter (sense: 5′-CTG CTG ACA TCG AGC TTT CTA CAA GGG-3′, reverse: 5′-AGG CTC AGA TCT GGT CTA ACC AGA GAG-3′) was used to estimate proviral number, whereas genes coding for β-globin (sense: 5′-AGG GCT GAG GGT TTG AAG TCC AAC TC-3′, reverse: 5′-TGT AAG CAA TAG ATG GCT CTG CCC TGA C-3′) were used to evaluate the number of cells.

The infectious proviral content of cells was quantified with qPCR prior to infection, and a quantity equivalent to 1 virus/cell was used to perform infection assays [19]. Virus stock was added directly to 5×10^5 Jurkat E6.1 cells in the minimal volume of complete medium, and incubated at 37°C for 1 h to allow pre-infection. PMA/PHA (20 ng/ml and 1 µg/ml, respectively) and/or 0.5 µM WA was then added and diluted with culture medium to a concentration of 10^6 cells/ml. Cells were then incubated at 37°C for 48 h. Viral reporter expression was measured as previously described [19]. Briefly, cells were washed twice with ice-cold PBS, then 1/5 of the cells were used for proviral content quantification by qPCR and 4/5 were used for the luciferase assay. For the qPCR, 200 µl of qPCR lysis buffer (10 mM Tris-HCl, pH 8, and

polyoxyethylene 10 lauryl ether 0.1% (v/v) (Sigma)) was added to the cell pellet, followed by proteinase K, for a final concentration of 100 µg/ml. Samples were first incubated at 65°C to allow protein digestion, which was followed by a 15 min incubation at 95°C for Proteinase K inactivation. The extracts obtained were used directly for proviral content quantification with probes coding for one region of the HIV-1 promoter (sense: 5′-CTG CTG ACA TCG AGC TTT CTA CAA GGG-3′, reverse: 5′-AGG CTC AGA TCT GGT CTA ACC AGA GAG-3′). Known numbers of pNL4.3.-lucE$^-$ cells were included in each qPCR as a standard for quantification. For the luciferase assay, cell pellets were resuspended in 250 µl lysis buffer (1×25 mM Tris phosphate, pH 7.8, 2 mM dithiothreitol, 1% Triton X-100, and 10% glycerol). The luciferase activity was measured as described previously and normalized to the proviral content in each sample.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described in [21]. Briefly, Jurkat E6.1 cells (10^6) were either left untreated or treated with PMA/PHA (20 ng/ml and 1 µg/ml, respectively) and/or WA (0.5 µM) for the indicated time at 37°C. The incubation was terminated by centrifugation at 4000 rpm at 4°C for 5 min. Sedimented cells were washed once with ice-cold PBS, then resuspended in 400 µl of cold Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). After 15 min on ice, 25 µl of Nonidet P-40 10% was added, and the lysate was vortexed for 10 s. The samples were then centrifuged for 30 s at 14 000 rpm at 4°C. The supernatant fraction was discarded and the pellet resuspended in 50 µl of cold Buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. Cellular debris were removed by centrifugation at 12 000 g for 5 min at 4°C, and the supernatant fraction was stored at -70°C until used.

We used 2 µg nuclear extracts to perform EMSA. Double-stranded DNA (10 pmol) was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase in a kinase buffer (New England Biolabs, Beverly, MA, USA). This mixture was incubated for 45 min at 37°C and the labeled oligonucleotide was extracted with a G-25 spin column (GE Healthcare). The double-stranded DNA oligonucleotide, which was used as a probe, contained either the home-made NF-κB binding sites (sense: 5'-CGA CAC CCC TCG GGA ATT TCC CCA CTG G-3', reverse: 5'-GGC CCA GTG GGG AAA TTC CCG AGG GGT-3') or sequence corresponding to bases -104 to -78 of the HIV-1 LTR (sense: 5'-AGG GAC TTT CCG CTG GGG ACT TTC CAA-3', reverse: 5'-TTG GAA AGT CCC CAG CGG AAA GTC CCT-3'). Nuclear extracts were incubated for 20 min at room temperature in binding buffer (25×, 100 mM HEPES pH 7.9, 40% glycerol, 10% Ficoll, 250 mM KCl, 5 mM EDTA, 250 mM NaCl, and 10 mM dithiothreitol) mixed with 5 µg of ultrapure non-acetylated BSA (Thermo Fisher Scientific, Waltham, MA, USA), 1 µg of Poly(dI-dC) (Sigma), and 0.2 pmol of ^{32}P-5'-end-labeled double-stranded oligonucleotide in a final volume of 25 µl. For competition assays, 20 pmol of unlabeled competitor oligonucleotide were added to the reaction. For the supershift assays, 1–2 µg of the respective antibody were added to the nuclear extract and incubated on ice for 45 min prior to the addition of labeled oligonucleotide. Samples were then loaded on a native 4.5% polyacrylamide gel, and complexes were separated for 2 h at 200 Volts.

Statistical analysis

All results were calculated as the mean ± SEM of independent experiments. Results were analyzed using the t-test. P values less than 0.05 were considered significant.

Results

Effect of WA on LTR transcription and HIV-1 replication

To study the inhibitory effects of WA on HIV-1 LTR transcriptional activity, we initially tested whether WA could modulate HIV-1 LTR activity using 1G5, a clonal cell line derived from Jurkat E6.1 that contains an integrated construct made of the luciferase reporter gene under the control of the HIV-1 LTR [18]. In these experiments, WA was reconstituted in DMSO to produce a stock solution of 10 mM, which was then diluted in complete culture medium to obtain our working dilutions (0.5–10 µM). Incubation of 1G5 cells with the concentration of DMSO equivalent to 10 µM WA did not induce LTR activation (data not shown). In this experiment, 1G5 cells were treated with PMA/PHA (20 ng/ml and 1 µg/ml, respectively), alone as a positive control, or in combination with increasing concentrations of WA (from 0.5 to 5 µM). PMA is a strong mitogen capable of improving the proliferative response of human T cells when combined with lectins, such as PHA [22-24]. As shown in Figure 1A, we observed a 167-fold increase in luciferase activity in 1G5 cells in the presence of PMA/PHA. Interestingly, we found that WA inhibited the LTR transcriptional activity induced by PMA/PHA in a dose-dependent manner, whereas WA alone did not induce LTR activation (data not shown).

To exclude the possibility that WA repression of HIV-1 LTR is a

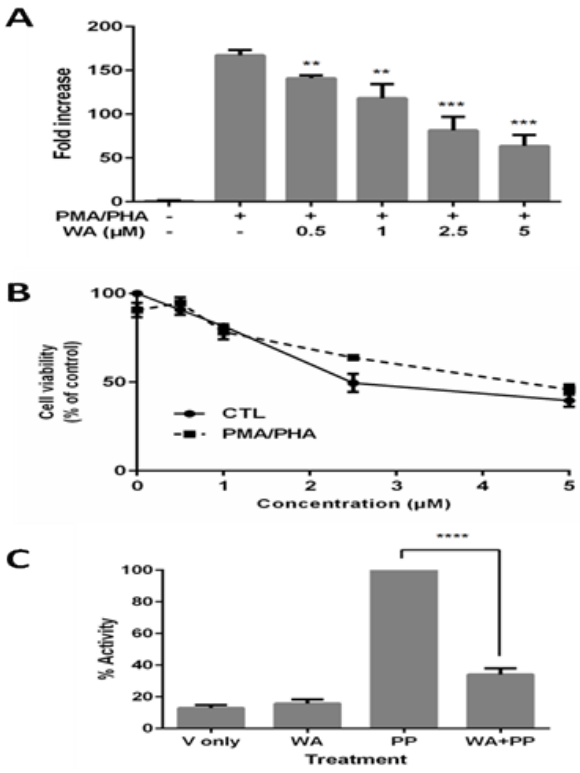

Figure 1: WA inhibits HIV-1 transcription and viral replication in a human T cell line. (A) 1G5 cells were treated with the indicated concentrations of WA in combination with PMA/PHA (20 ng/ml, 1 µg/ml, respectively) for 8 h. The cells were then lysed and luciferase activity was analyzed. Results shown are the means ± SEM of three determinations, and are expressed as fold increases compared with untreated cells. These results are representative of three independent experiments. **P≤0.01; ***P≤0.001 (B) MTT assays were carried out under the same conditions as in (A) to evaluate the effects of WA on cell viability in 1G5 cell lines. (C) Jurkat E6.1 cells were infected with a defined number of pNF4.3.-lucE- pseudotype virus particles to attain one virus integration per cell, and then treated with 0.5 µM WA and/or PMA/PHA for 48 h. Virus-encoded luciferase activity in the cell extracts was then measured. Results represent the mean ± SEM of three independent experiments, considering values obtained with PMA/PHA treated cells to be 100% activation. ****P≤0.0001.

result of its effect on cell viability, 1G5 cells were treated with different concentrations of WA alone or with PMA/PHA for 8 h, followed by MTT assays. As shown in Figure 1B, we found a dose-dependent inhibition of cell viability and by WA, which exhibited a significant level of toxicity at concentrations greater than 2.5 μM. Although PMA/PHA showed a degree of toxicity, no additive effect was observed when combining WA with PMA/PHA. To minimize any nonspecific toxic effects of WA on T cells, we chose to continue our study with the lowest concentrations of WA (0.5 and 1 μM).

To further confirm the anti-HIV activity of WA, we infected Jurkat E6.1 cells with the pNL4.3-lucE$^-$ HIV pseudotyped viral particles in single-round infection assays. This system relies on a luciferase reporter gene that replaces the HIV *Nef* gene and includes a defective *Env* gene. The defective envelope is compensated by the co-transfection of a second plasmid, pCMV-VSV-G, which provides the VSV-G envelope protein and allows viral entry by endocytosis. The luciferase activity is proportionate to the transcriptional activity of the proviral LTR, thus eliminating the interference of non-integrated free viral particles and mimicking an HIV Tat-dependent amplification loop identical to that in a natural infection. The results depicted in Figure 1C show that HIV infection, in the absence of any treatment, results in low virus production. In contrast, treatment with PMA/PHA alone results in more efficient viral replication compared with control cells. Interestingly, the addition of WA (0.5 μM) abrogated the PMA/PHA-induced increase in HIV replication. However, post-treatment with WA 4 h after PMA/PHA treatment did not result in significant inhibition at the concentration tested (data not shown).

Effects of WA on the NF-κB signaling pathway and HIV-1 LTR transcription

To assess the role of transcription factor NF-κB in WA-induced inhibition of the HIV-1 LTR, Jurkat E6.1 cells were transfected with luciferase reporter plasmids containing mutations at the NF-κB binding sites of HIV-1 LTR. We also used the pNF-κB-luc plasmid, which contains five consensus sequences of NF-κB binding sites, thus rendering it highly responsive to NF-κB activation, and the pκB-TATA-luc plasmid, which is made of the minimal HIV-1 NF-κB binding motifs with a TATA box, followed by the luciferase reporter gene. Indeed, the pκB-TATA-luc construct allows HIV-1 activation to occur almost exclusively via NF-κB-dependent mechanisms. The pLTR-luc plasmid was used in parallel to compare how different constructs of NF-κB binding sites affect LTR activation. The transfected cells were incubated with either PMA/PHA or TNF-α, and treated with WA simultaneously for 8 h. TNF-α is known to activate the LTR exclusively through the NF-κB pathway [25,26]. As shown in Figures 2A–2F, WA inhibited LTR activation in all cases, and the inhibition was higher in cases in which more NF-κB binding sites were present (in pNF-κB-luc). Cells transfected with pκB-TATA-luc showed moderate inhibition after treatment with WA. However, it should be noted that the pκB-TATA-luc plasmid resulted in greater induction of luciferase activity upon stimulation of PMA/PHA, as compared with those cells containing the pNF-κB-luc construct, while pLTR-luc induced the lowest level of luciferase activity (data not shown). This observation corroborates the findings of previous studies and can be explained by the absence of other modulatory elements in the HIV-1 LTR that regulate NF-κB's activation potential [27].

To further investigate the role of NF-κB in WA-induced inhibition of the HIV-1 LTR, we generated a mutated virus termed the mκB variant, which bears mutated NF-κB binding sites in the U3 LTR region (three nucleotides have been replaced at both core binding sites:

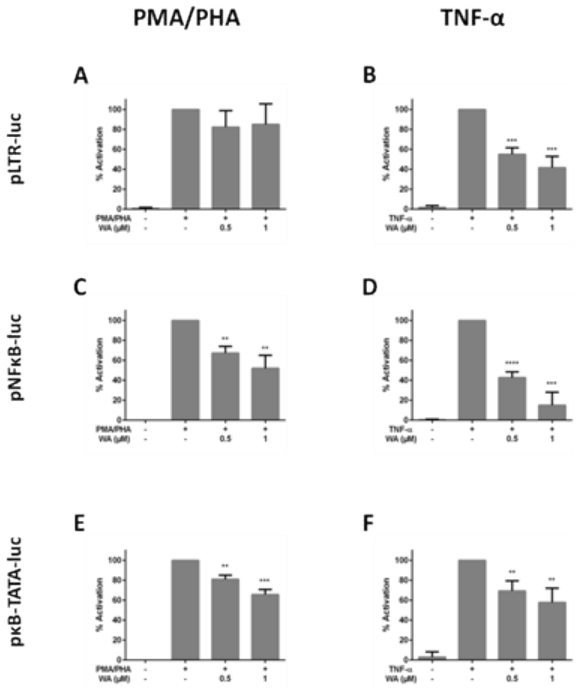

Figure 2: WA inhibits LTR promoter activation through NF-κB signaling. Jurkat E6.1 cells were transiently transfected with pLTR-luc (A, B), pNF-κB-luc (C, D), or pκB-TATA-luc (E, F). After 20 h, cells were incubated with increasing concentrations of WA in the presence of PMA/PHA (20 ng/ml, 1 μg/ml, respectively) or TNF-α (20 ng/ml) for 8 h. Luciferase activity was then analyzed. Results represent the mean ± SEM of three independent experiments, and are expressed as percent activation, considering values obtained with PMA/PHA or TNF-α treatment in the absence of WA to be 100% activation. **p≤0.01; ***p ≤0.001; ****p ≤0.0001.

CTCACTTTCC instead of GGGACTTTCC). The mutation greatly reduced HIV replication; under the same conditions, mκB variant produced 1/10th the number of infectious particles compared with the wild type, as determined by measuring the proviral content with qPCR (data not shown). To avoid discrepancies in viral integration, the proviral content in each condition was quantified by qPCR to normalize luciferase activity. Surprisingly, the mutant virus showed a slightly higher basal activity in unstimulated cells than the wild type, which was unexpected, but which suggests a possible regulatory role of NF-κB in LTR inhibition (Figure 3). The mutation abolished NF-κB induction by PMA/PHA or TNF-α, illustrated by the significant decrease in fold induction (wild-type, PMA/PHA: 21.4, TNF-α: 19.9; mutant, PMA/PHA: 2.5, TNF-α: 3.5). Moreover, treatment with WA did not affect LTR expression in the mutant in the same way as in the wild-type pseudovirus, suggesting that WA requires intact NF-κB binding sites to mediate its effect.

Because it has been previously reported that pretreatment with WA inhibits the activation of NF-κB by preventing the phosphorylation and subsequent degradation of the inhibitory unit IκB-α [15], we next sought to determine whether the effect of WA on HIV-1 LTR expression was caused by the absence of IκB-α degradation. Jurkat E6.1 cells were incubated with 1 μM WA for 5–60 min following PMA/PHA stimulation. As depicted in Figures 4A-4D, we found that PMA/PHA-induced IκB-α phosphorylation on serine 32 and 36, which was followed by its rapid degradation. IκB-α was fully degraded after 20 min, whereas it reappeared after 45 min, suggesting that NF-κB activation induced new synthesis of IκB-α. WA only transiently affected the degradation of IκB-α induced by PMA/PHA (Figures 4B and 4D). However, it clearly

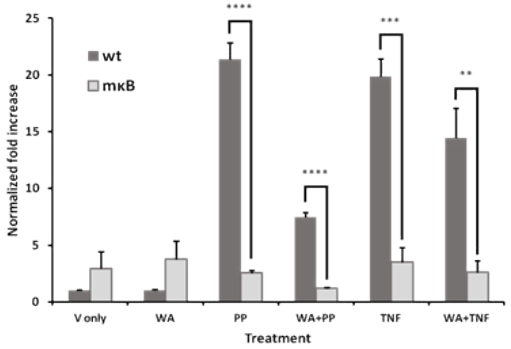

Figure 3: WA inhibits viral transcription through an NF-κB-dependent pathway. Jurkat E6.1 cells were infected with HIV-1 wild-type or mκB mutant (with inactive κB binding sites) pseudotyped virus, and stimulated under the indicated conditions for 48 h. Luciferase activity was measured and normalized to proviral content as measured by qPCR amplification of an LTR promoter sequence. **P≤0.01; ***P≤0.001; ****P≤0.0001.

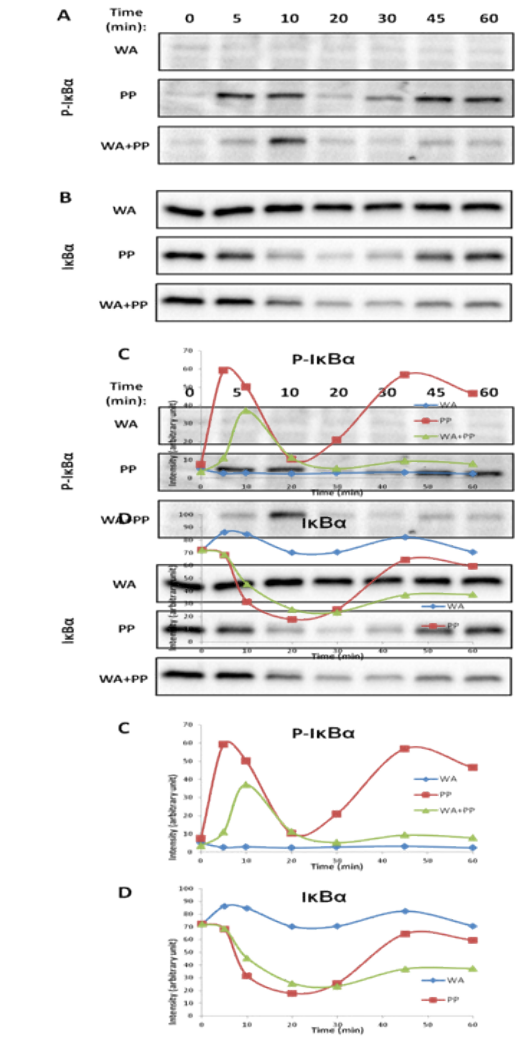

Figure 4: Effect of WA on NF-κB signaling. Jurkat E6.1 cells were incubated with 1 μM WA and/or PMA/PHA (20 ng/ml, 1 μg/ml, respectively) for the indicated periods of time. Cell lysates were analyzed by western blots with the indicated antibodies: (A) anti-phospho-IκB-α, (B) anti-IκB-α. The band intensity was then quantified with ImageLab (BioRad),(C) phosphor-IκB-α, (D) IκB-α. Results are representative of three independent experiments.

reduced the recovery rate of IκB-α protein. Indeed, *de novo* synthesis of this inhibitory protein is modulated by NF-κB through the NF-κB binding sites on the IκB-α promoter. Newly synthesized IκB-α re-enters the nucleus and dissociates the NF-κB-DNA complex, thereby abolishing its activation. This auto-regulatory mechanism provides a feedback control for NF-κB-dependent gene transcription, enabling a rapid yet transient response at the transcriptional level [28].

The inhibition of *de novo* IκB-α synthesis described above could potentially be caused by the action of WA on an upstream process, such as NF-κB translocation, or the formation of the NF-κB-DNA complex. We therefore examined the translocation of NF-κB with EMSA. To test experimentally whether NF-κB can translocate into the nucleus and bind to the HIV-1 LTR, nuclear extracts from Jurkat E6.1 cells stimulated with PMA/PHA and/or WA were incubated with a P^{32}-labeled double-stranded probe containing a consensus sequence of the NF-κB binding site (Figure 5A), as well as one consisting of the -104 to -78 sequence of the HIV-1 LTR (Figure 5B). The DNA-binding complex was identified with specific anti-p65 and anti-p50 antibodies, and the binding specificity was confirmed by cold competition with unlabeled probes. Results indicated the presence of a specific band after 30 min stimulation with PMA/PHA, which was supershifted with antibodies against the p65 subunit or the p50 subunit of NF-κB. This suggests the presence of p65/p50 in the nucleus that is able to bind to the HIV-1 LTR.

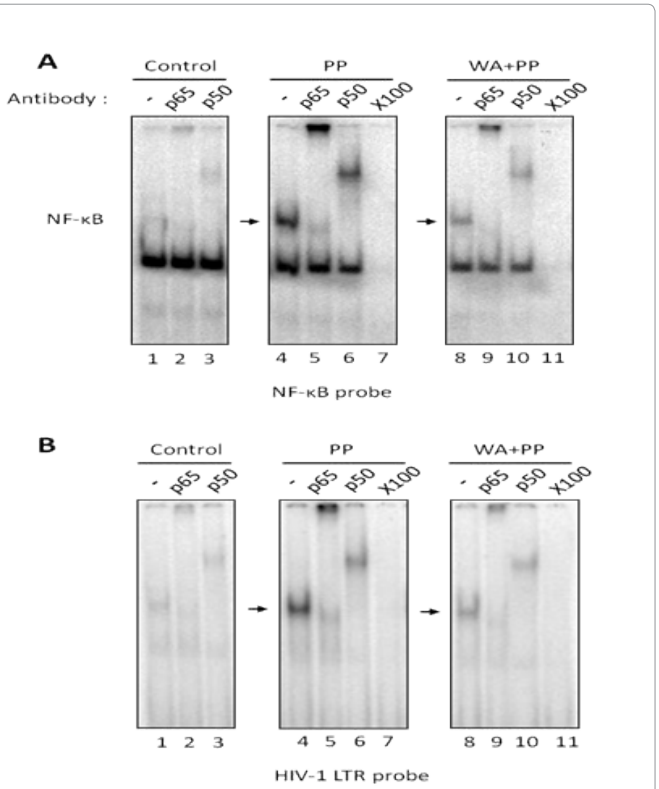

Figure 5: WA inhibits the nuclear translocation of p65/p50 induced by PMA/PHA. Jurkat E6.1 cells were stimulated under different conditions for 30 min, and nuclear proteins were then extracted. (A) A 32P-radiolabeled DNA probe containing the consensus sequence of κB binding sites was incubated with Jurkat E6.1 nuclear extracts, and the resulting complexes were separated on a native polyacrylamide gel. Bound complexes were analyzed by phosphorimaging. For the supershift analysis, 1−2 μg of antibodies against the p65 (lane 2, 5, 9) or p50 (lane 3, 6, 10) subunits of NF-κB were incubated with nuclear extract on ice for 45 min prior to probe incubation. For the negative control, a 100× excess of cold probe (lane 7, 11) was added instead of antibody. (B) As in A except that the DNA probe corresponds to bases -104 to -78 of the HIV-1 LTR. NF-κB complexes are indicated with arrows in both panels.

Moreover, addition of an excess of identical, unlabeled probe effectively blocked formation of the NF-κB-DNA complex in both cases, showing their specificity. Concurrent with the reduced synthesis of IκB-α, WA inhibited the translocation of p65/p50, the predominant form of NF-κB responsible for the activation of various genes, starting from 20 min (Figures 6A and 6B, lane 9-12 versus lane 15-18).

Discussion

Since the discovery of HIV-1 in the early 1980s, extensive efforts have been made to develop effective and innovative strategies for the eradication of this disease, which remains incurable. However, high costs associated with the long-term use of antiretroviral drugs make it difficult for infected individuals to access appropriate antiretroviral treatment in developing countries. Consequently, more effective and financially approachable alternative drugs are urgently needed to control the expansion of HIV-1 infection.

Medicinal plants and natural products have been used to cure various diseases throughout human history, and their use remains important to this day, particularly in eastern countries. Most of these natural compounds have been safely used for centuries, and their often multi-target nature renders them potential candidates for the development of novel anti-cancer, anti-inflammatory, and antiviral agents [29]. WA, a steroidal lactone, is a bioactive molecule that exhibits potent anti-cancer activities, compared with other structurally similar withanolides [14,30]. Here we report that WA exhibits strong inhibition of HIV-1 gene expression in an infected T cell model. Both the lymphocyte T cell line 1G5, which stably harbors a copy of the HIV-1 LTR promoter, and the parental cell line Jurkat E6.1, transiently transfected with pLTR-luc plasmid, showed reduced PMA/PHA-induced activation when the cells were co-treated with WA. Single-round infection assays with wild-type pseudovirus showed similar results. We initially postulated that this reduction in HIV-1 infection could be explained by the inhibition of NF-κB pathway activation.

The transcription factor NF-κB is the major regulatory element involved in HIV-1 replication in CD4 lymphocytes. The two adjacent

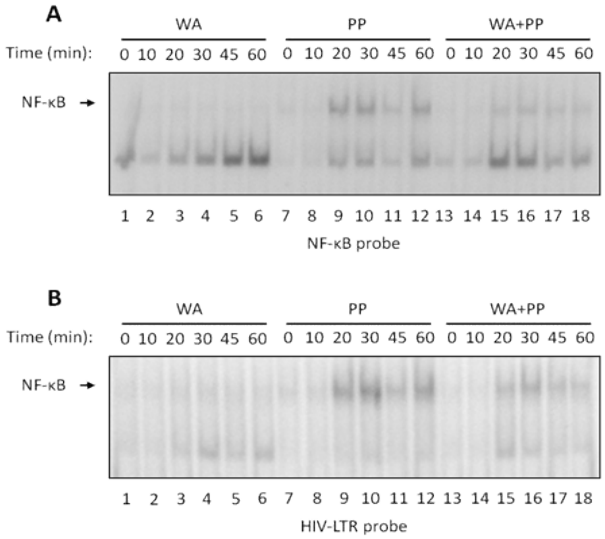

Figure 6: WA reduced the nuclear translocation of NF-κB induced by PMA/PHA. Jurkat E6.1 cells were incubated with WA (1 μM) and/or PMA/PHA (20 ng/ml, 1 μg/ml, respectively) for the indicated periods of time, and nuclear proteins were then extracted. EMSA assays were performed as previously described with 32P-radiolabeled oligonucleotides containing (A) home-made κB binding sites or (B) a sequence corresponding to bases -104 to -78 of the HIV-1 LTR.

NF-κB binding sites in the LTR region are highly conserved among HIV subspecies; deletion or mutation of these sites strongly impairs HIV-1 replication. Under basal conditions, NF-κB is sequestered in the cytoplasm by its interaction with the inhibitory protein IκB-α [31]. Upon activation by inflammatory stimuli, IκB-α is phosphorylated and then degraded by proteasomes, leading to nuclear translocation of NF-κB, which results in the transcription of viral genes. Several research groups have reported a direct interaction between WA and the IKK complex, a kinase upstream of IκB-α that contains several cysteine residues in its catalytic site. Kaileh et al. previously demonstrated that pretreatment of cells with WA hampers TNF-induced IKKβ kinase activity through ser-181 hyperphosphorylation [15], whereas Heyninck et al. showed that cys-179, located in the activation loop of IKKβ, is targeted by WA, and its presence is necessary for optimal phosphorylation of neighboring serine residues [32]. It is therefore possible that WA acts at different steps in the NF-κB pathway in different cell types. Our transfection assays showed that the presence of NF-κB is positively correlated with the inhibitory effect of WA on the LTR; transfection of the pNF-κB-luc plasmid, which contains five consensus sequences of κB binding sites, resulted in consistently higher WA-induced LTR inhibition in transfected cells than in cells lacking the plasmid. Moreover, WA maintained its inhibition in the case of the pκB-TATA-luc plasmid, in which κB binding sites were the only responsive element present in the LTR besides the TATA box.

The results from our single-round infection assays, with pseudotyped viruses bearing mutated NF-κB binding sites in the U3 LTR, suggest that the mutation abolished HIV-1 LTR activity induced by PMA/PHA or TNF-α, which further confirms the implication of NF-κB activation in WA inhibition. It should be noted that the HIV-1 NF-κB mutant created in our lab appears to exhibit a higher expression level than that of the wild-type virus, which suggests a possible negative regulatory role of NF-κB in LTR activation. In concordance with a negative regulatory role for NF-κB, NF-κB1(p50) knock-out mice lack detectable NF-κB DNA-binding activity, yet a higher level of expression of NF-κB-regulated genes was observed than in wild-type animals [33].

The DNA mobility shift assays in the present work suggest that the heterodimer p50/p65 is the active form of NF-κB responsible for HIV-1 LTR activation; rapid translocation of this complex could be observed after 20 min upon PMA/PHA stimulation. This form of NF-κB is in fact the most abundant and common activator of both gene activation and viral transcription, out of all the homo- and heterodimers of the Rel family [34-36]. In most cases, the rapid degradation of the inhibitor protein IκB-α precedes NF-κB translocation. Interestingly, in our study, co-treatment with WA did not reduce PMA/PHA-mediated IκB-α degradation, as shown by western blot assays, whereas the reappearance of IκB-α was hindered to a greater extent by WA. This could be explained by the fact that *de novo* synthesis of IκB-α is regulated by NF-κB itself as part of a feedback inhibition mechanism, in which newly synthesized IκB-α enters the nucleus and binds to NF-κB, thus enabling a rapid yet transient response at the transcriptional level [34,37,38]. We could therefore postulate that the inhibitory role of WA on NF-κB, especially its translocation, leads to IκB-α depletion in the nucleus due to a lack of protein production. Our time-course results with mobility shift assays are also in agreement with the inhibition of IκB-α synthesis observed with western blot assays.

Limitations and Clinical Implications

Our conclusions may have some limitations. Indeed, experiments were performed in T cell lines which may show physiological characteristics different from primary T cells. Also, the role of other transcription factors in the inhibition of HIV-1 transcription by WA

has not been explored. Thus, more experiments are needed to better understand the mechanisms by which, WA inhibits HIV-1.

WA is the first bioactive molecule extracted from *Withania somnifera*, a traditional medicinal plant that has been safely used in India for centuries. Studies have shown that therapeutic doses of WA exert anti-cancer, anti-inflammatory, and anti-angiogenesis effects [9,16,39-41]. Our results suggest that WA, a bioactive compound derived from Indian Ashwagandha, disrupts viral gene expression through the inhibition of NF-κB activation. Targeting NF-κB is thus of particular interest given its central role in numerous diseases and in gene regulation, the latter of which is reflected by its highly conserved nucleotide sequence [42]. Because of the inherent multi-target nature of many plant-derived small molecules, they can interfere with viral proteins and LTR promoters in regulatory regions, which render them unlikely to generate drug-resistant HIV-1 strains. A more thorough understanding of the functions of WA at the molecular level may lead to the development of new strategies for controlling the AIDS epidemic, especially in developing countries.

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Conflict of Interests

The authors declare that there are no conflicts of interest.

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