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As_2O_3 synergistically reactivate latent HIV-1 by induction of NF-κB

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ABSTRACT

None of the current agents can safely and effectively eliminate latent HIV-1 reservoirs, meaning that there is a major barrier to the final cure of AIDS. Arsenic trioxide (As_2O_3), a drug used to treat acute promyelocytic leukemia (APL), was reported to affect the transcription factors and pathways involved in modulating HIV-1 expression. However, little is known about the effect and molecular basis of As_2O_3 in inducing HIV-1 expression in latently infected cells. Using the Jurkat T cell model of HIV-1 latency, we found that As_2O_3 activated latent HIV-1 replication with a similar potency to valproic acid (VPA) and did so in a dose- and time-dependent manner. We also found that As_2O_3 synergistically reactivated latent HIV-1 transcription with prostratin, tumor necrosis factor alpha (TNF-α) or VPA. Moreover, we provide evidence indicating that As_2O_3-induced HIV-1 activation involves the nuclear factor kappa B (NF-κB) signaling pathway. In conclusion, we have found that As_2O_3 can synergistically reactivate latent HIV-1 with other activators, which may provide a new tool to unravel the mechanisms of virus latency and reactivation.

1. Introduction

Despite the remarkable success of chronic suppression of HIV-1 replication by highly active anti-retroviral therapy (HAART), latent reservoirs still remain the primary obstacles to eradication of HIV-1 infection. In the long-term maintained latent reservoirs, the integrated HIV-1 provirus remains transcriptionally silent to evade host immune surveillance and anti-retroviral drugs, but it can resume active infection once HAART is interrupted (Richman et al., 2009; Karn, 2011; Pace et al., 2011). There are multiple complementary mechanisms contribute to maintaining HIV-1 latency, mostly through suppression of transcription. One key factor is cytoplasmic sequestration of the crucial host transcription factors in latent cells, such as nuclear factor kappa B (NF-κB) and nuclear factor of activated T cells (NFAT), which leads to the restricted transcriptional initiation (Nabel and Baltimore, 1987; Kinoshita et al., 1997). Another transcriptional block observed in latently infected cells is the restricted positive transcriptional elongation factor b (P-TEFb) levels, which impede efficient transcription elongation from the HIV-1 promoter (Zhou and Yik, 2006; Contreras et al., 2007). HIV-1 transcription can also be silenced by epigenetic modifications such as histone deacetylation, histone methylation and DNA methylation (Mbonye and Karn, 2011). In addition, posttranscriptional restrictions and miRNAs may also play important roles in maintaining HIV-1 latency (Lassen et al., 2006; Huang et al., 2007; Nathans et al., 2009).

The most widely discussed strategy for eliminating the latent HIV-1 reservoirs is the so-called "shock and kill." To explore potential reactivation strategies, classes of agents targeting the different mechanisms involved in maintaining latency have been explored and can be divided into the following groups: (1) cytokines and chemokines, including agents such as tumor necrosis factor alpha (TNF-α) (Folks et al., 1989); (2) histone deacetylase inhibitors, including suberoylanilide hydroxamic acid (SAHA/vorinostat) (Contreras et al., 2009) and valproic acid (VPA) (Ylisastigui et al., 2004); (3) histone methyltransferase inhibitors, such as BIX01294 (Imai et al., 2010); (4) DNA methyltransferase inhibitors, which are mainly Decitabine (5-azacytidine, 5-Aza) and its analogs (Fenaux, 2005); (5) protein kinase C activators, such as prostratin (Williams et al., 2004) and bryostatin-1 (Mehla et al., 2010); (6) positive transcription elongation factor b activators, including hexamethylene bisacetamide (HMBA) (Vlach and Pitha, 1993) and JQ1(S) (Li et al., 2013); and (7) some unclassified agents, such as disulfiram (Xing et al., 2011). Ideal agents used to eradicate latent HIV-1 should have high efficacy at reactivating latent proviruses without inducing global T cell activation and should exhibit acceptable pharmacologic and toxicologic properties (Xing and Siliciano, 2012). Unfortunately, because none of these agents can safely and effectively attack and eliminate the long-lived HIV-1 reservoirs, new agents or combinations of agents need to be explored.

Arsenic trioxide (As_2O_3) is an FDA-approved drug that is widely used to treat acute promyelocytic leukemia (APL) (Chen et al., 1996; Shen et al., 1997; Soignet et al., 1998). Several lines of evidence have indicated that various signal transduction factors, like

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the mitogen-activated protein kinases (MAPKs), and some transcription factors, such as P53, NF-κB, SP1 and AP-1, can be affected by exposure to As_2O_3 (Sumi et al., 2010). NF-κB is an inducible cellular transcription factor that plays a critical role in the expression of a variety of cellular and viral genes (Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002), including that of HIV. NF-κB belongs to the Rel/NF-κB protein family and forms homodimers or heterodimers by the combination of five different monomers (p65/RelA, RelB, c-Rel, p50/p105, and p52/p100). In non-stimulated cells, NF-κB is cytoplasmically retained in its inactive state through interaction with its specific inhibitors, IκBs (Delhase et al., 1999). Once activated by a wide array of signals, the free NF-κB dimers translocate into the nucleus, bind to the κB-binding site in the promoter region and induce transcription of target genes (Karin and Delhase, 2000). The effect of As_2O_3 on NF-κB activation is inconclusive in the literature, and both inhibitory (Shumilla et al., 1998; Roussel and Barchowsky, 2000) and activating effects (Barchowsky et al., 1999; Hu et al., 2002; Felix et al., 2005) have been reported. The contradictory results in those experiments may be due to the differences in experimental cell types, concentrations or exposure times of the drug.

Although the therapeutic potential of arsenic and its various derivatives has been exploited for hundreds of years, research on their potential use in the treatment of human diseases remains highly promising. In previous studies, As_2O_3 was reported to increase HIV-1 retroviral infectivity in some cases of restricted infection (Sebastian et al., 2006; Pion et al., 2007), to enhance HIV-1 retroviral reverse transcription (Berthoux et al., 2003) and to eliminate potent post-entry restriction of HIV-1 infection by counteracting APOBEC3G-mediated restriction in myeloid dendritic cells (Stalder et al., 2010). Because As_2O_3 affects the transcription factors and pathways involved in modulating HIV-1 expression, we hypothesized that it may be effective at countering HIV-1 latency. However, little is known about the effect and molecular basis of As_2O_3 antagonism of HIV-1 latency. The aim of this study was to investigate the ability and molecular mechanisms of As_2O_3 to induce expression of HIV-1 in latently infected cells and the combined treatment effect of As_2O_3 with other activators on reactivation HIV-1 production. We confirmed that As_2O_3 can synergistically reactivate latent HIV-1 transcription together with prostratin, TNF-α or VPA. We also found that NF-κB transcription factor plays an important role in As_2O_3-mediated regulation of HIV-1 gene expression.

2. Materials and methods

2.1. Reagents and cell culture

As_2O_3, Aspirin and Bay11-7082 were purchased from Sigma–Aldrich (Shanghai, China). VPA was purchased from InvivoGen (San Diego, CA, USA). Prostratin was purchased from LC laboratories (Woburn, MA, USA). Recombinant human TNF-α was purchased from Chemicon International (Temecula, CA, USA). As_2O_3 (100 mM) were prepared in PBS followed by filter sterilization, TNF-α (1 mg/ml), VPA (100 mM), TSA (10 mM), prostratin (10 mM) and aspirin (1 M) were dissolved in anhydrous dimethyl sulfoxide (DMSO) and stored at −20 °C until used. Human FITC conjugated anti-CD25 and PE conjugated anti-CD69 antibodies were purchased from BD-Biosciences (San Jose, CA, USA).

C11 cells, a type of latently infected Jurkat cell, were constructed in our lab and used somewhere else (Ying et al., 2012; Ding et al., 2013). J-Lat clone A7 cells were kindly provided by the NIH AIDS Research and Reference Reagent Program (Dr. Eric Verdin) (Jordan et al., 2003). C11 cells and J-Lat clone A7 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml of streptomycin (Invitrogen, Shanghai, China) at 37 °C under 5% CO_2. Human embryonic kidney 293 cells (HEK 293) were purchased from the American Type Culture Collection and were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% FBS, 100 U/ml penicillin and 100 μg/ml of streptomycin at 37 °C under 5% CO_2.

2.2. Isolation of human peripheral blood mononuclear cells (PBMCs)

Whole peripheral blood from healthy donors was purchased from the Blood Center of Shanghai (Shanghai, China). The PBMCs isolation was made by the difference of gradient density Ficoll-Hypaque (density = 1.077 g/ml, Haoyang Biological manufacture, Tianjin, China). After centrifugation (400g; 30 min at room temperature), the PBMCs were found at the plasma/Ficoll-Hypaque interphase and collected carefully with a Pasteur pipette. After that, the cells were washed in PBS twice (240 × g for 10 min), and resuspended in RPMI 1640 medium containing 4.5 g/l glucose supplemented with 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin and 100 μg/ml of streptomycin.

2.3. GFP expression visualization and flow cytometry analysis

C11 or J-Lat clone A7 cells were incubated with the relevant reagents at the indicated concentrations for the specified times. The expression of GFP as a marker for the reactivation of HIV-1 promoter was observed by fluorescence microscopy. After mock-treated with the same volume of 1 × phosphate buffered saline (PBS) or treated with the indicated concentrations of As_2O_3, cells were viewed using a Nikon fluorescent microscope. All microscope samples were photographed using a Nikon E2 digital camera. The percentage of GFP-positive cells was measured by flow cytometry to determine the level of HIV-1 expression. Cells were washed and resuspended in PBS. The GFP expression was measured by FACScan (Becton Dickinson FACScan Flow Cytometer), and FACS data were analyzed using Cell Quest software (Macintosh, Sunnyvale, CA, USA). Live cells were gated and two parameter analyses were used to differentiate GFP-associated fluorescence from background fluorescence. A total of 10,000 gated events were collected and data represent the percentage of GFP-expressing cells in total gated events. As_2O_3 mediated T-cell activation in PBMCs was monitored after staining for activation markers with FITC conjugated anti-CD25 and PE conjugated anti-CD69 antibodies. Briefly, 1×10^6 cells were washed with PBS and stained with 20 μl of fluorescently labeled antibodies in 100 μl PBS containing 1% FBS for 45 min on ice. Subsequently, cells were washed three times with PBS and finally resuspended in 1 ml PBS containing 1% FBS and 10,000 cells were acquired using FACScan with Cell Quest software. All experiments were performed independently at least three times in triplicate per experimental point.

2.4. In vitro cytotoxicity assay

A Cell Counting Kit-8 (CCK-8) (Dojindo Molecular technologies, Gaithersburg, MD, USA) was used in cytotoxicity assays. CCK-8 utilizes Dojindo's highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], which can be reduced by dehydrogenases in cells to give a yellow-colored product. A WST-8 assay was applied to calculate the number of live cells because the detection sensitivity and reproducibility of WST-8 is higher than other tetrazolium salts such as MTT, XTT, MTS or WST-1 (Isobe et al., 1999; Tominaga et al., 1999). To measure cell viability in the presence of drugs, C11 cells, J-Lat clone A7 cells, Jurkat T cells or human primary PBMCs were subjected to WST-8 assay.

According to the kit protocol, approximately 4×10^4 cells per well (96-well plates were used), were treated with As_2O_3 for 72 h. Then, 10 μl of CCK-8 solution was added to each well of the plate. After 37 °C incubation for 4 h, measures of the absorbance at 450 nm were taken using a microplate reader. The 50% cytotoxic concentration (CC_{50}) was calculated by nonlinear regression analysis using GraphPad Prism 5 software (GraphPad, SanDiego, CA). All experiments were performed independently at least three times in triplicate per experimental point.

2.5. Transient transfection and luciferase assays

HEK-293 cells were plated at 1×10^5 cells/well in 24-well plates 24 h before transfection and transfected by Lipofectamine 2000 following the manufacturer's instruction (Invitrogen). Per well, 1.0 μg HIV-1 LTR-luc (from Dr. Warner C. Greene) (Wang et al., 2005), HIV-1 LTR(ΔκB)-luc, HIV-1 LTR(ΔAP-1)-luc, or HIV-1 LTR(ΔSP1)-luc (from Dr. Andrew D. Badley) (Bren et al., 2008), and 0.1 μg pRL-SV40 (Promega, Madison, WI, USA), formulated into liposomes, were applied. At 24 h post-transfection, the cells were mock-treated or treated with As_2O_3 (8 μM) or TNF-α (10 ng/ml). At 48 h post-treatment, cells were lysed and luciferase activity was measured using Dual-Luciferase Reporter Assay Kit (Promega) and normalized by Renilla luciferase activity, according to the manufacturer's instruction.

2.6. Cell nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from C11 cells with different treatments were obtained as previously described (Gila et al., 2003). Briefly, cells were washed twice with PBS and resuspended in 100 μl ice-cold Buffer A (10 mM HEPES–NaOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM PMSF) and 0.6% of NP-40 for 15 min followed by centrifugation at 15,000*g* for 2 min at 4 °C. The supernatant contained cytoplasmic protein, which was discarded. The precipitated nuclear pellet was once with Buffer A and resuspended in 60 μl ice-cold buffer B (20 mM HEPES–NaOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 25% glycerol). The mixture was incubated on ice for 30 min with intermittent mixing followed by centrifugation at 15,000*g* for 15 min at 4 °C. The supernatant, containing nuclear proteins, was collected and stored at −80 °C for EMSA.

The EMSA for NF-κB was performed by employing LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) as per the manufacturer's protocol. Briefly, biotin-labeled double-stranded NF-κB oligonucleotide: 5′-AGTTGAGGGGACTTTCCCAGG-3′; 3′-TCAACTCCCCTGAAAGGGTCC-5′ was incubated with nuclear extracts at room temperature for 20 min. The samples were then subjected to 5% non-denaturing polyacrylamide gel in Tris/borate/EDTA buffer and then transferred to a nylon membrane. After attaching to the membrane by UV-crosslinking, the DNA–protein complexes were detected by chemiluminescence and analyzed by autoradiography. Cold competition was performed in the presence of 100-fold excess non-labeled consensus oligonucleotides, respectively for 10 min prior to the addition of labeled oligonucleotides.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed according to the manufacturer's protocol (Millipore, Billerica, MA, USA) and a previously described procedure (Ying et al., 2012). Briefly, C11 cells were mock-treated or treated with As_2O_3 (8 μM) or TNF-α (10 ng/ml) for 4 h, then cross-linked with 1% formaldehyde, and the lysates were sonicated to produce 500–1000 bp chromatin fragments. Extracts were then diluted tenfold with immunoprecipitation (IP) dilution buffer. 200 μl of the diluted sample (10%) were used as input controls and 2 ml of diluted sonicated extract for IP. After pre-clearing with Protein G agarose for 1 h at 4 °C with agitation, the supernatant was then incubated at 4 °C overnight with appropriate antibodies [anti-p65 (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-HDAC6 (Active Motif, Carlsbad, CA, USA) or normal mouse IgG (Millipore)]. Protein G agarose beads were added to collect the immune complexes, which was washed with low salt, high salt, LiCl, and Tris–EDTA buffer and eluted in elution buffer. Then the samples were reverse cross-linked by an overnight incubation at 65 °C, and the input controls were treated in the same manner at this point. DNA was isolated and analyzed by PCR for 30 cycles (using the forward 5′-AGACTGCTGACATCGAGCTTTCT-3′ and reverse 5′-GTGGGTTCCCTAGTTAGCCAGAG-3′ primers), which produced a 192 bp PCR fragment containing the NF-κB binding sites. To calculate the relative levels of p65 or HDAC6 binding to LTR, a quantitative real-time PCR assay (ABI Prism 7900 Real Time PCR System, USA) was performed, the amounts of PCR product obtained for immunoprecipitated chromatin samples were normalized against the amounts of PCR product obtained for input DNA. Fold change in occupancy of p65 and HDAC6 at the LTR relative to mock control was calculated. All values represent the average of at least three independent experiments.

3. Results

3.1. As_2O_3 activates latent HIV-1 replication

In order to measure the ability of As_2O_3 to induce expression of HIV-1 in latently infected cells, we used the C11 cells established in our lab, which is a clonal latently infected Jurkat T cell line with a single provirus integrated into intron of RNPS1 and a green florescence protein (GFP) gene under the control of HIV-1 LTR (Ding et al., 2013). The structures of As_2O_3 and VPA are shown in Fig. 1A. After C11 cells were treated with As_2O_3 or VPA at different concentrations for 72 h, fluorescence microscopy detection showed that a fraction of C11 positive cells, representing the (Supplementary Fig. 1). Then, the percentage of GFP-positive cells, representing the transcriptional activity of the HIV-1 promoter, was measured by flow cytometry (Fig. 1B). Three days after treatment with 16 μM As_2O_3, the percentage of GFP-positive cells was found to be as high as 11.8%, similar to the induction activity of 2 mM VPA (13.5%). As showed in Fig. 1C, the percentage of GFP-expressing cells was positively related to the concentration of As_2O_3: when the concentration of As_2O_3 increased from 2 μM to 16 μM, the percentage of GFP-positive cells rose from 2.1% to 11.8%. Although VPA has a slightly higher activation efficiency, it was found to be very toxic to C11 cells at the concentration of 4 mM (Supplementary Fig. 2). These results, therefore, indicate dose-dependent effects of As_2O_3 on HIV-1 reactivation.

To analyze the kinetics of HIV-1 LTR expression induced by As_2O_3, we performed a kinetics experiment in which C11 cells were grown for 1–4 days with As_2O_3 (8 μM) or VPA (2 mM). At each time point, GFP-expressing cells were assayed by flow cytometry. As shown in Fig. 2, after C11 cells were treated with As_2O_3, the percentage of GFP-expressing cells increased over time. Four days after treatment with 8 μM As_2O_3, we observed that the percentage of GFP-expressing cells was about 10.5%. The kinetics of HIV-1 LTR expression induced by VPA (2 mM) showed a rapid rise for the first 3 days and then a plateau by day 4. Unlike cells treated with As_2O_3 or VPA, the mock-treated cells remained low level background GFP expression, indicating a blockage of viral transcription. These results suggest the effects of As_2O_3 on HIV-1 production are time-dependent.

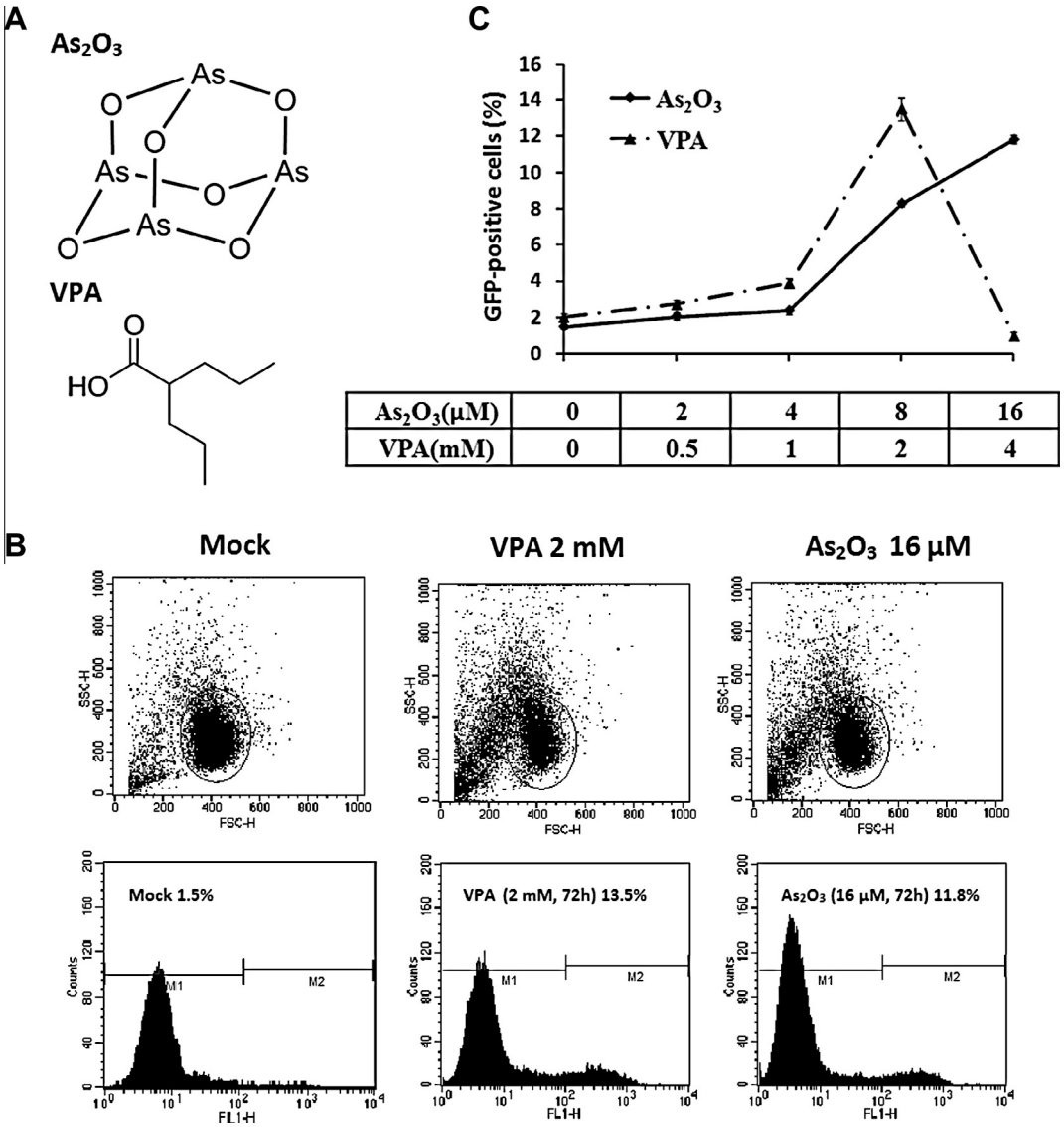


Fig. 1. Reactivation of latent HIV-1 in latently infected C11 cells by As_2O_3 and VPA. (A) The structure of As_2O_3 and VPA. (B) C11 cells were treated with VPA or As_2O_3 at the indicated concentrations. At 72 h post-treatment, the percentage of GFP-positive cells was measured by flow cytometry for GFP expression, which represented the level of HIV-1 transcription. Results are presented as fluorescence histograms. (C) Dose-dependent curve of VPA and As_2O_3 on HIV-1 production in C11 cells. Data show the means ± standard deviations of three independent experiments.

To examine whether similar results could be obtained in other latently infected T cells, we used J-Lat clone A7 cells, which is also Jurkat T cell line latently infected by HIV-1 (Jordan et al., 2003). The results from these cells also indicate that As_2O_3 can potently activate latent HIV-1 replication (Fig. 3 and Supplementary Fig. 3).

3.2. Synergistic activation of HIV-1 production by As_2O_3 and other activators

To investigate whether As_2O_3 synergistically reactivates the HIV-1 promoter when combined with prostratin, TNF-α or VPA, C11 cells were mock-treated or treated with As_2O_3 (8 μM), prostratin (100 nM), TNF-α (10 ng/ml), VPA (2 mM), As_2O_3 (8 μM)/prostratin (100 nM), As_2O_3 (8 μM)/TNF-α (10 ng/ml) or As_2O_3 (8 μM)/VPA (2 mM) for 48 h, respectively. Two activators synergize when their combination produces a greater activation level than the sum of the effects produced by individual activators (Reuse et al., 2009). As shown in Fig. 4, in the absence of stimulation, C11 cells expressed almost no GFP. The stimulation of C11 cells for 48 h with As_2O_3, prostratin, TNF-α or VPA alone induced GFP expression in a small proportion of cells (less than 10%). When cells were co-treated with As_2O_3 and the other activators, we observed synergistic activations of virus production. In these cases, about 20% or more C11 cells became GFP-expressing (Fig. 4). These results indicate that the combinatorial treatment of As_2O_3 with prostratin, TNF-α or VPA cause the synergistic recruitment of unresponsive C11 cells into the expressing cell population.

3.3. Analysis of in vitro cytotoxicity of As_2O_3

To test the cytotoxicity of As_2O_3 *in vitro*, C11 cells, J-Lat clone A7 cells, Jurkat T cells and human primary PBMCs were treated with different concentrations of As_2O_3, VPA or prosratin for 72 h and cell viability was measured. We found a significant correlation between the concentration of As_2O_3 and the viability of these cells. As_2O_3 exhibited higher toxicity than VPA and prosratin at the same concentrations, and high level reductions of cell viability were detected at As_2O_3 concentrations >100 μM (Fig. 5). To further

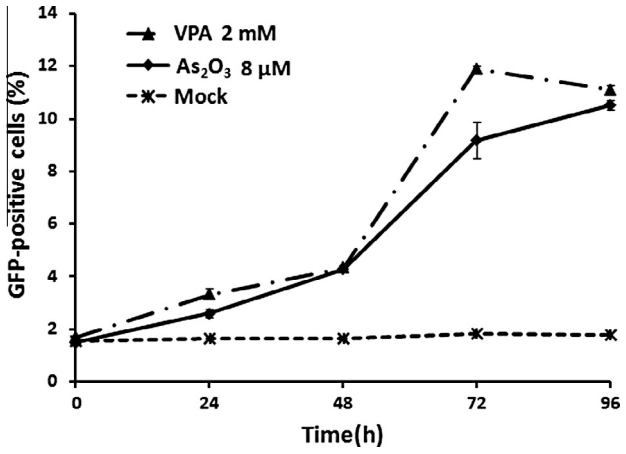


Fig. 2. Time-dependent effects of As_2O_3 and VPA on C11 cells. C11 cells were mock-treated or treated with VPA (2 mM) or As_2O_3 (8 μM) for the indicated times. GFP expression was monitored in gated live cells at 24, 48, 72 and 96 h by standard flow cytometric techniques. Data show the means ± standard deviations of three independent experiments.

evaluate the cytotoxicity, we demonstrated the 50% or 90% cytotoxic concentration (CC_{50} or CC_{90}) of As_2O_3 on these cells in Table 1. We found that the CC_{50} values of As_2O_3 were ranging from 34 μM to 172 μM, while the CC_{90} values were all >1000 μM.

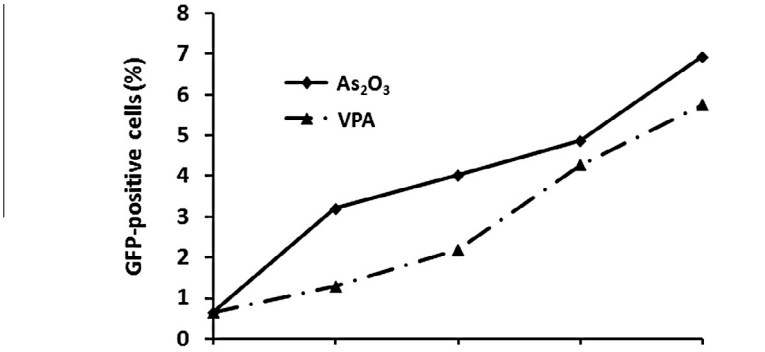
The major disadvantage of some current therapeutic agents is their propensity to non-specifically activate bystander T-cells. We therefore investigated the induction of global T cell activation markers by As_2O_3 treatment. Human PBMCs were treated with As_2O_3 or prostratin for 24 h and the expressions of CD25 and CD69 were detected by flow cytometry using antibodies against CD25 and CD69. In consistency with previously published results (Biancotto et al., 2004), prostratin stimulated expression of activation markers on the surface of PBMCs. In contrast to prostratin, no significant induction of CD25 or CD69 expression was found in the As_2O_3-treated cells at its active concentrations (8 μM and 16 μM), compared to mock-treated cells (Fig. 6). In addition, the lack of CD25 or CD69 expression also was observed with longer As_2O_3 incubation times (48 h and 72 h, Supplementary Fig. 4).

3.4. As_2O_3-mediated activation of HIV-1 involves induction of NF-κB

We next explored the signaling pathway activated by As_2O_3 that mediates activation of the HIV-1 LTR. Binding sites for several inducible transcription factors, including NF-κB, AP-1, and Sp1, can be found in the HIV-1 LTR region. To assess the role of transcription factors in As_2O_3 activation of the HIV-1 LTR, HEK-293 cells were transfected with luciferase reporter plasmids containing the wild type HIV-1 LTR, the LTR lacking the two κB enhancers, the LTR lacking the AP-1 enhancers, or the LTR lacking the Sp1 enhancers and were stimulated with 8 μM As_2O_3 or 10 ng/ml TNF-α. As_2O_3 induced an approximately 2-fold stimulation of the HIV-LTR-Luc reporter relative to mock controls but failed to activate the HIV-1 LTR(ΔκB)-, HIV-1 LTR(ΔAP-1)- or HIV-1 LTR(ΔSP1)-luciferase reporters (Fig. 7A). While TNF-α only failed to activate the HIV-1 LTR(ΔκB)-luciferase reporter. These findings indicate that all these three transcription factors may be involved in As_2O_3-mediated activation of the latent HIV-1 LTR.

The host transcription factor NF-κB is critical for HIV-1 replication. To further confirm the role of NF-κB factor in As_2O_3-mediated activation of the HIV-1 LTR, C11 cells were pretreated with different concentrations of aspirin, which can inhibit TNF-α-induced activation of NF-κB (Kutuk and Basaga, 2004; Zhu et al., 2007), and were subsequently treated with As_2O_3 or TNF-α. Aspirin pretreatment not only inhibited TNF-α-induced GFP expression in a dose-dependent manner, but also strongly inhibited GFP expression induced by As_2O_3 at the concentrations tested (Fig. 7B). On the other hand, pretreatment of another NF-κB inhibitor Bay11-7082 (Pierce et al., 1997) could partially inhibit the As_2O_3-induced GFP expression (Fig. 7C). These results further support the involvement of NF-κB factor in the signaling response of As_2O_3 stimulation.

To assess more directly the role of NF-κB factor in As_2O_3 activation of the HIV-1 LTR, we performed EMSA to investigate whether As_2O_3 stimulation provided sufficient stimulus for NF-κB nuclear translocation and DNA binding. Nuclear extracts from C11 cells stimulated with As_2O_3 or TNF-α were incubated with biotin labeled NF-κB enhancer oligonucleotides. A dose-dependent induction of NF-κB DNA binding was found in the As_2O_3 treated groups compared to the mock-treated cells, and the induction could be entirely suppressed by the NF-κB inhibitor aspirin, which could also inhibit the TNF-α-induced NF-κB DNA binding (Fig. 7D). To investigate whether NF-κB p65 was directly recruited to the HIV LTR *in vivo* following As_2O_3 stimulation, we further performed ChIP assays. Chromatin fragments from C11 cells mock-treated or treated with As_2O_3 or TNF-α were immunoprecipitated with anti-p65 antibodies or normal mouse IgG. DNA was isolated from the immunoprecipitates and was analyzed by PCR using primers specific for HIV-1 LTR. We observed that the amounts of p65 bound



As_2O_3(μM)	0	2	4	8	16
VPA(mM)	0	0.5	1	2	4

Fig. 3. Dose-dependent effects of As_2O_3 and VPA on J-Lat clone A7 cells. J-Lat clone A7 cells were treated with VPA or As_2O_3 at the indicated concentrations. At 72 h post-treatment, the percentage of GFP-positive cells was measured by flow cytometry for GFP expression, and dose-dependent curve of As_2O_3 and VPA on HIV-1 production was represented. Data show the means ± standard deviations of three independent experiments.

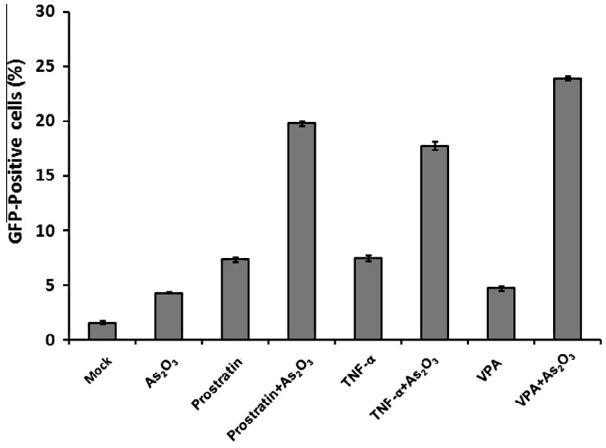


Fig. 4. Synergistic activation of HIV-1 by As_2O_3 and prostratin, TNF-α or VPA in latently infected cells. C11 cells were mock-treated or treated with As_2O_3 (8 μM), prostratin (100 nM), TNF-α (10 ng/ml), VPA (2 mM), As_2O_3 (8 μM)/prostratin (100 nM), As_2O_3 (8 μM)/TNF-α (10 ng/ml) or As_2O_3 (8 μM)/VPA (2 mM). The effects of the synergistic activation of HIV-1 promoter were determined by quantifying the GFP-positive cells using flow cytometry 48 h after treatment. Summary of synergistic activation assays are presented as histograms. Data represent the means ± standard deviations of three independent experiments.

to the LTR region were increased by the treatment of As_2O_3 or TNF-α. No immunoprecipitated DNA fragments were detected by PCR in the normal mouse IgG controls (Fig. 7E). The percentage of input for each immunoprecipitation was calculated and the fold occupancy of p65 relative to mock-treatment was shown. We found that As_2O_3 treatment induced about 2-fold p65 recruited to LTR, while TNF-α treatment increased the p65 recruitment to 3.2-fold (Fig. 7F). In agreement with previous report (Qu et al., 2012), As_2O_3 treatment inhibited the binding of HDAC6 to LTR (Fig. 7E and F), demonstrating the reliability of the ChIP method. Taken together, these results further confirm that NF-κB plays an important role in As_2O_3-mediated regulation of HIV-1 gene expression.

Table 1
The CC_{50} and CC_{90} values of As_2O_3 in different cells.[a]

Cells	CC_{50}[b] (μM)	CC_{90}[b] (μM)
C11	47.23 ± 10.42	>1000
A7	34.22 ± 4.66	>1000
Jurkat	72.92 ± 11.55	>1000
PBMC	171.57 ± 35.23	>1000

[a] The compound was tested in triplicate and the data are presented as means ± SD.
[b] WST-8 assay was used to determine the 50% or 90% cytotoxic concentration (CC_{50} or CC_{90}).

4. Discussion

Currently, extensive efforts are being directed towards the development of effective strategies to eliminate latent HIV-1 reservoirs. The "shock and kill" strategy, which first induces HIV-1 expression in latently infected cells and then purges these cells by antiviral immune responses, viral cytopathic effects or even cell-targeted killing strategies, has recently gained much attention. In devising this strategy, the first step is to find ways to reactivate latent HIV-1 without causing global T cell activation. To this end, so

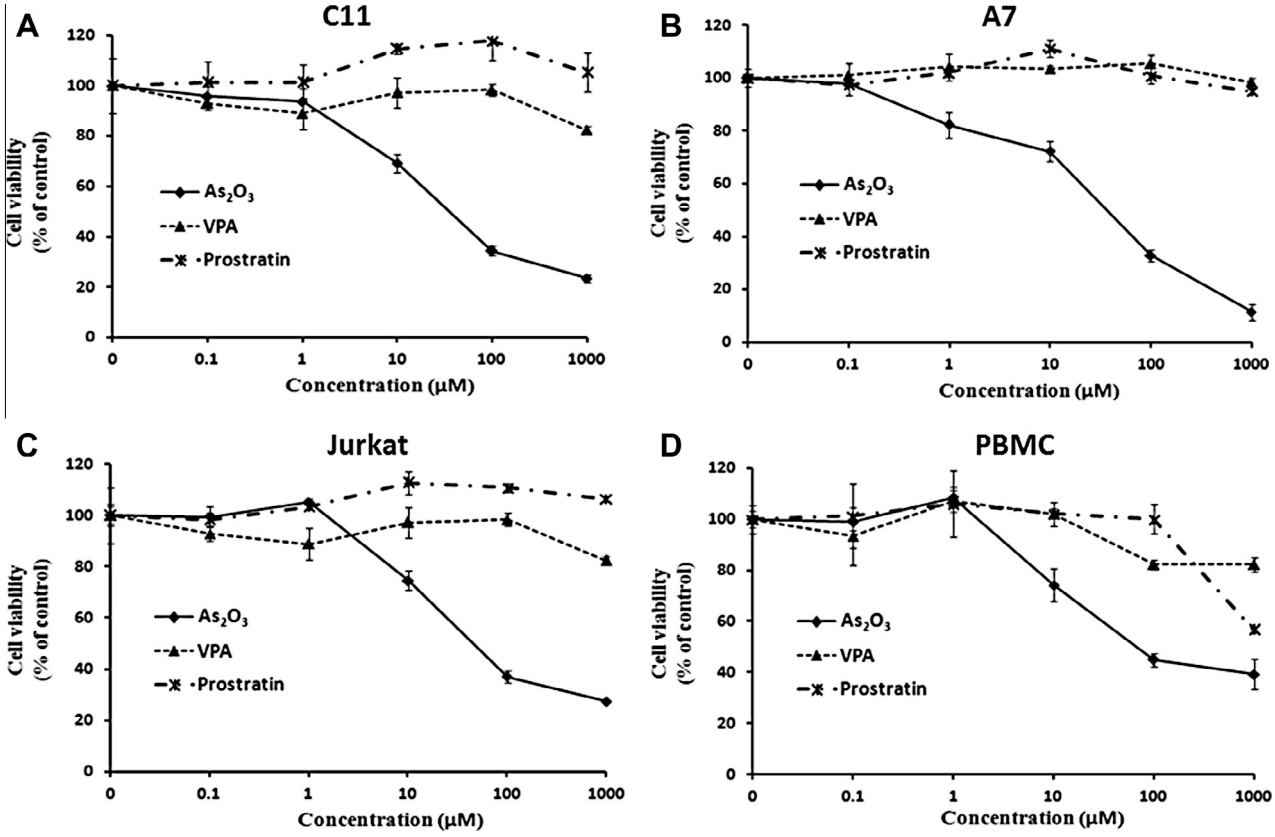


Fig. 5. Effects of As_2O_3 on cell viability of human Jurkat cell lines and PBMCs. C11 cells (A), J-Lat clone A7 cells (B), Jurkat T cells (C) and human PBMCs (D) were treated with As_2O_3, VPA or prostratin at the indicated concentration for 72 h and then cell viability were measured by CCK-8 kit (Dojindo). The division of OD450 between treated and control groups indicate the percentage of cell viability. Data show the means ± standard deviations in three independent experiments.

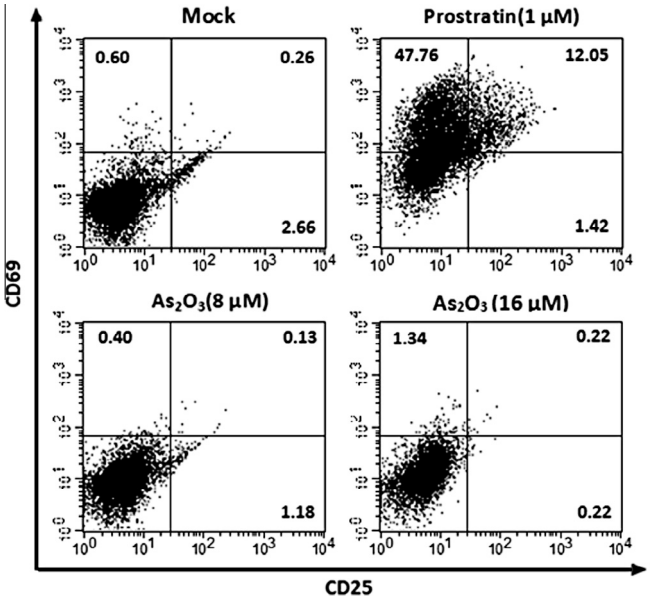


Fig. 6. Effects of As_2O_3 on cell activation marker expression. The effect of As_2O_3 and prostratin on the expression of CD25 and CD69. Human PBMCs were treated with either As_2O_3 (8 μM or 16 μM) or prostratin (1 μM) for 24 h and the expression of CD25 and CD69 was detected by flow cytometry using antibodies against CD25 and CD69. The results are representative of three independent experiments.

many agents targeting different mechanisms of HIV-1 latency have been explored. However, their toxicity and ineffectiveness in trials involving enlarged sample sizes and prolonged treatment limit the clinical applications of such agents (Marsden and Zack, 2009; Remoli et al., 2012; Tyagi and Bukrinsky, 2012; Xing and Siliciano, 2012). For example, the reference substance used in this research, VPA, is a relatively weak and non-toxic HDAC inhibitor that has been approved by the FDA for the treatment of neuropsychiatric conditions as a mood stabilizer and antiepilectic drug (Davis et al., 1994). Although VPA has shown promising effects in depleting latent HIV-1 infection in a small pilot study (Lehrman et al., 2005), further retrospective studies demonstrated VPA had no effects on reducing the number of latently infected resting $CD4^+$ T cells (Siliciano et al., 2007; Archin et al., 2008; Sagot-Lerolle et al., 2008). Thus, better and more specific latency activators are urgently needed.

In order to search new activators of HIV-1 latency, two cell models were used here in this study. Both the C11 and J-Lat clone A7 cells are latently infected Jurkat T cells encoding GFP under the control of HIV-1 LTR as a marker of HIV-1 expression, which can be easily detected by fluorescence microscopy and flow cytometry. In these cell lines, HIV-1 is expressed at a very low level, as measured by GFP marker under basal conditions. However, viral expression can be activated upon appropriate stimulation. This makes the latently infected cell models valuable tools for studying HIV-1 transcriptional silencing mechanism and for screening small molecules that can reactivate latent HIV-1. Considering that the C11 and J-Lat clone A7 cells are infected by different viruses and their provirus integration sites are not the same (Ding et al., 2013; Jordan et al., 2003), we included both cell lines in the studies and found that As_2O_3, a drug used to treat APL, activated latent HIV-1 replication in a dose- and time-dependent manner (Figs. 1–3).

Although the potency of As_2O_3 to reactivate latent HIV-1 is similar to that of VPA, it seems that the efficiency is a little bit low. Combination therapy, in which two or more drugs are used together, is an attractive way to enhance therapeutic index (Fitzgerald et al., 2006). Therefore, we further investigated whether other activators might synergize with As_2O_3. We examined the effects of various cotreatments on C11 cells by combining As_2O_3 with prostratin, a non-tumor-promoting PKC activator (Williams et al., 2004), TNF-α, a monocyte-derived cytokine (Folks et al., 1989), or VPA, an HDAC inhibitor. Our results showed that cotreatments consisting of As_2O_3 and prostratin, TNF-α or VPA can reactivate a higher percentage of latent cells than the sum of the two individual activators (Fig. 4), indicating that As_2O_3 can indeed synergize with different types of activators to reactivate HIV-1 production in latently infected cell lines. The aims of combination therapy are primarily to reduce the chance of evolving drug resistance, to lower the doses of drugs to achieve efficacy with fewer side effects, and to achieve enhanced potency by exploiting additive or greater-than-additive effects of two drugs (Fitzgerald et al., 2006; Tan et al., 2012). Combining As_2O_3 with other activators may achieve some of these goals, but further experiments are needed to explore the molecular mechanisms and correlations between different types of activators. We also found that As_2O_3 showed no synergistic activation when used in combination with 5-Aza, a DNA methyltransferase inhibitor (Fenaux, 2005) (data not shown), possibly because DNA methylation has little relevance to sustaining latent HIV-1 infection in the latently infected C11 cell lines.

These results suggest that As_2O_3 may be useful in combination therapy to antagonize HIV-1 latency only if it has acceptable toxicity. Therefore, we measured the cell viability after treatment with As_2O_3 in C11 cells, J-Lat clone A7 cells, Jurkat T cells and human primary PBMCs, and we found that As_2O_3 exhibited higher cytotoxicity than VPA and prosratin. Because latent viruses respond to T cell activation signals, some researchers have focused on strategies that induce some level of T cell activation as a means of reactivating latent HIV-1 (Prins et al., 1999). Unfortunately, initial attempts to eradicate the latent reservoir using anti-CD3 antibodies and IL-2 proved toxic because of nonspecific T cell activation and release of proinflammatory cytokines. Therefore, an ideal reactivation strategy for virus eradication should reactivate latent HIV-1 but avoid global T cell activation (Yang et al., 2009; Xing et al., 2012; Xing and Siliciano, 2012). Unlike cells stimulated with prostratin, no upregulated surface expression of CD25 or CD69 markers was found in the As_2O_3-treated cells. These results indicate that As_2O_3 can synergistically activate latent HIV-1 without causing global T cell activation.

Next, we explored which signaling pathway is involved in the As_2O_3-mediated activation of the HIV-1 LTR. The expression of integrated HIV-1 in latently infected cells depends initially on LTR activation, which is controlled by cellular transcription factors and the viral transactivator Tat, as well as the chromatin environment (Gaynor, 1992; Wolschendorf et al., 2010; Victoriano and Okamoto, 2012). The HIV-1 LTR contains *cis*-acting elements that are required for transcription initiation and numerous binding sites for many cellular transcription factors, including NF-κB, NFAT, Sp1 and AP-1 (Victoriano and Okamoto, 2012). The activation of HIV-1 gene expression by many extracellular stimuli is critically dependent upon activation of NF-κB, a ubiquitously expressed inducible transcription factor, which binds to two conserved κB sites in the core enhancer region of the HIV-1 LTR (Duh et al., 1989; Griffin et al., 1989). To determine the signaling pathways involved in As_2O_3-mediated activation of the HIV-1 LTR, we first examined the ability of As_2O_3 to activate various signaling pathways using luciferase reporter plasmids. We observed that As_2O_3 effectively activated the wild type HIV-1 LTR-luciferase reporters but displayed no stimulatory effects on the κB or AP-1 or Sp1 enhancers lacking LTR reporter constructs, indicating that HIV-1 reactivation induced by As_2O_3 may involve all these three signaling pathways. Considering its pivotal role in the host innate and adaptive immune responses, we chose NF-κB for further validation. To test whether HIV-1 reactivation in latently infected cells induced by As_2O_3 can be blocked by NF-κB inhibitors, we used

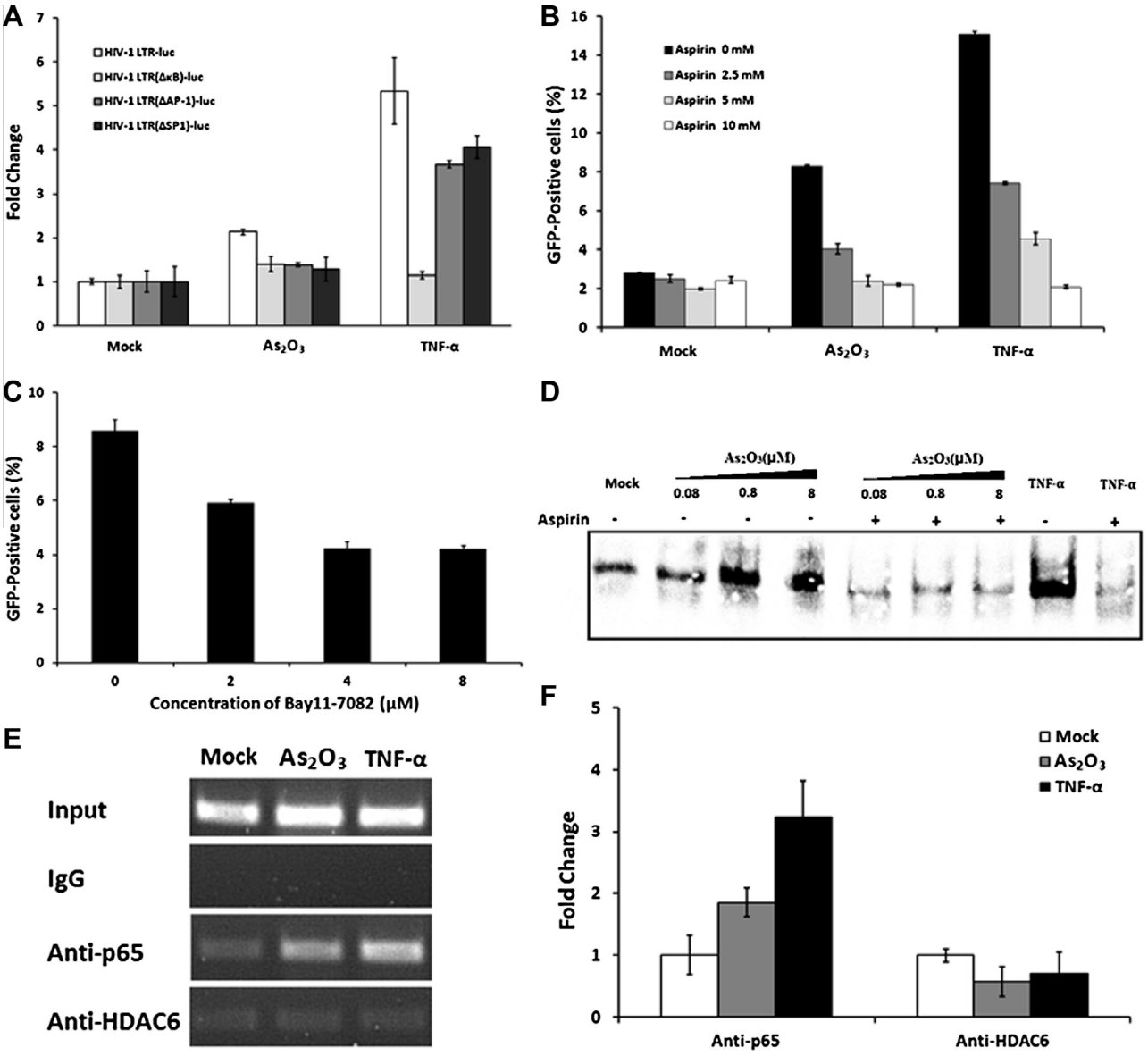


Fig. 7. As_2O_3 activates the HIV-1 LTR through induction of NF-κB. (A) HEK-293 cells were transfected with HIV-1 LTR-, HIV-1 LTR(ΔκB)-, HIV-1 LTR(ΔAP-1)-, and HIV-1 LTR(ΔSP1)-luciferase reporter plasmid DNA. At 24 h post-transfection, the cells were mock-treated or treated with As_2O_3 (8 μM) or TNF-α (10 ng/ml). Luciferase activity was measured after 48 h of stimulation. The error bars indicate standard deviation. (B) C11 cells were pretreated with various concentrations of (0, 2.5, 5 and 10 mM) aspirin for 4 h and subsequently treated with As_2O_3 (8 μM) or TNF-α (10 ng/ml) for 72 h. The percentage of GFP positive cells in As_2O_3 or TNF-α stimulated cells with or without the pretreatment of the chemical inhibitors was measured by flow cytometry. Data represent the means ± standard deviations of three independent experiments. (C) C11 cells were pretreated with the indicated concentrations of Bay11-7082 for 4 h and subsequently treated with 8 μM As_2O_3 for 72 h. The percentage of GFP positive cells was measured by flow cytometry. (D) C11 cells were pretreated with 10 mM aspirin or mock-treated for 4 h and subsequently stimulated with As_2O_3 at the indicated concentrations for 4 h or with TNF-α (10 ng/ml) for 30 min. Nuclear extracts were isolated and subjected to EMSA with biotin labeled NF-κB enhancer DNA probes. (E) C11 cells were mock-treated or stimulated with As_2O_3 (8 μM) for 4 h or TNF-α (10 ng/ml) for 30 min, respectively. ChIP assays were performed using anti-p65 or anti-HDAC6 antibodies or normal mouse IgG. PCR primers for the LTR promoter were used to amplify the DNA isolated from the immunoprecipitated chromatin as described in Materials and methods. (F) Each ChIP experiment was repeated three times to confirm reproducibility of results and real-time quantitation of the fold change relative to untreated control is shown.

aspirin (Brummelkamp et al., 2003; Zhu et al., 2007) and Bay11-7082 (Pierce et al., 1997), agents reported as IκBα phosphorylation inhibitors. We found that both aspirin and Bay11-7082 pretreatment can significantly prevent As_2O_3-induced HIV-1 reactivation, which further supports the involvement of NF-κB signaling pathway in the activation of the HIV-1 LTR by As_2O_3. Then, we performed EMSA to detect the proteins in the nuclear extracts that can bind to the NF-κB oligonucleotides. Our results prove directly that As_2O_3 can stimulate NF-κB's nuclear translocation and DNA binding. At last, we performed ChIP assays and found that As_2O_3 stimulation promoted NF-κB p65 recruitment directly to the HIV LTR *in vivo*. All these results confirm the involvement of NF-κB signaling pathway in the As_2O_3-induced HIV-1 LTR activation.

During the revision of this article, we found that Lusic et al. recently reported treatment of latent cells with As_2O_3 (which induces PML nuclear bodies' degradation) is associated with transcriptional activation of latent HIV (Lusic et al., 2013), further corroborating our results. It was also reported that As_2O_3 could inhibit HDAC6 activity (Qu et al., 2012), which has also been verified in our experiments. As_2O_3 facilitates profound cellular alterations via numerous pathways (Miller et al., 2002), and the NF-κB signaling pathway may be only one of those involved in the As_2O_3-mediated

HIV-1 reactivation. The mechanisms of As_2O_3 to reactive latent HIV-1 still need to be further investigated.

In summary, the latent viral reservoirs remain the major barriers to eradication of HIV-1 infection. Because of the multifactorial nature of latency and the suboptimal efficacy of many latency-reversing agents, the reactivation of the entire latent reservoirs is difficult to achieve. It has become evident that a cocktail of interventions is needed if we want to fully eradicate the virus. In this study, the increased reactivation of latent HIV-1 has been obtained by using combinations of As_2O_3 and other activators, and when these agents are used in combination, lower concentrations could be effective. Another advantage of As_2O_3 is the low risk related to T cell activation. We also provide evidence that the As_2O_3-induced HIV-1 activation involves the NF-κB signaling pathway. While accepting its effectiveness, we should also be aware of the difficulties of implementing an antiviral strategy with As_2O_3 because of its toxicity. However, our study adds As_2O_3 as a tool to unravel the mechanisms of HIV-1 latency and reactivation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.10.010.

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