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Novel histone deacetylase inhibitor NCH-51 activates latent HIV-1 gene expression

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ABSTRACT

Pharmacological manipulations to purge human immunodeficiency virus (HIV) from latent reservoirs have been considered as an adjuvant therapeutic approach to highly-active antiretroviral therapy for the eradication of HIV. Our novel histone deacetylase inhibitor NCH-51 induced expression of latent HIV-1 with minimal cytotoxicity. Using chromatin immunoprecipitation assays, we observed a reduction of HDAC1 occupancy, histone hyperacetylation and the recruitment of positive transcription factors at the HIV-1 promoter in latently infected-cells under the treatment with NCH-51. Mutation studies of the long terminal repeat (LTR) revealed NCH-51 mediated gene expression through the Sp1 sites. When Sp1 expression was knocked-down by small interfering RNA, the NCH-51-mediated activation of a stably integrated HIV-1 LTR was attenuated. Moreover, the Sp1 inhibitor mithramycin A abolished the effects of NCH-51.

1. Introduction

The persistence of cellular reservoirs containing proviruses of human immunodeficiency virus type I (HIV-1) despite prolonged treatment with highly active antiretroviral therapy (HAART) represents the major obstacle in the eradication of HIV. These latently infected cells are permanent sources of viral reactivation and lead to a rebound of the virus load after interruption of HAART [1–4]. Resting memory CD4+ T cells or macrophages lacking proviral gene expression carry an integrated and transcriptionally silent provirus [1,2,5,6].

Restrictive chromatin structures at the HIV-1 long terminal repeat (LTR) contribute to transcriptional silencing leading to latency. The nucleosomal structure, nuc-1, near the viral mRNA start site plays regulatory roles in inducing LTR-driven transcription [7,11]. The compaction of the chromatin during HIV-1 proviral latency and its reversion to a permissive state is directly governed by posttranslational modifications such as acetylation, phosphorylation and methylation [1,2,7–11].

Histone deacetylases (HDACs) have been known to be critical regulators of HIV-1 latency. Accumulated evidence has demonstrated that presence of HDAC1 leads to the repression of HIV-1 LTR, whereas inhibition of HDAC1 results in HIV gene expression [7,11–13]. To date, HDAC1 has been reported to be recruited to the HIV-1 LTR by the NF-κB p50 homodimer, CBF-1 protein, or through binding with complexes containing transcription factors such as YY1, LSF, c-myc and Sp1 [12–16]. We also reported that activator protein 4 (AP-4) acts as a transcriptional repressor by recruiting HDAC molecules and is involved in maintaining viral latency [7].

HDACs have been considered potential targets in the new therapeutic approach against HIV-1 infection because HDAC inhibition results in promoter activation and purges HIV-1 from its latent stage. It has been perceived that inducing viral outgrowth of HIV-1 from persistently infected reservoirs would allow complete eradication of chronic viral infection by the immune system and HAART [1,2]. Previous observations have shown that HDAC inhibitors (HDACis) such as trichostatin A (TSA) and valproic acid (VPA) [2,11,16] are capable of inducing HIV-1 transcription in latently infected cells. Similarly, the FDA-approved drug suberoylanilide hydroxamic acid (SAHA), prescribed for the treatment of cutaneous lymphoma, has also been reported to have successfully reactivated HIV-1 replication from the resting CD4+ T cells and peripheral

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blood mononuclear cells from antiretroviral-treated, aviremic HIV-infected patients [17–19]. However, recent studies have reported that administration of VPA with intensified ART showed limited effects in reducing the latently-infected resting CD4+ T cells or ablating intermittent and low-level viremia [20–23].

In spite of the reported lack of clinical success with VPA, research on this class of agents is still necessary with the goal of developing better therapeutically relevant drugs that can be integrated in a regimen to deplete latent infection. In this study, we examined the effect of novel HDACi NCH-51 on HIV-1 gene expression in latently infected OM10.1 cells. This compound was designed based on the structure of SAHA by replacement of the hydroxamic acid group with acylated thiol group in order to obtain better pharmacokinetics and less toxicity [24]. NCH-51 could inhibit HDACs as strongly as SAHA and inhibited the cell growth of various lymphoid malignant cells and solid tumor cell lines in vitro [24,25]. Here we demonstrate that NCH-51 augmented the HIV-1 production in latently infected OM10.1 cells and such reactivation is associated with a loss of HDAC1 occupancy and subsequent hyperacetylation of histones in nuc-1 at the HIV-1 promoter.

2. Materials and methods

2.1. Cell culture

OM10.1, a derivative of the HL-60 myelomonocytic leukemic cell line containing a single integrated copy of HIV-1_{LAV} provirus [26], and ACH-2, a chronically HIV-1-infected T cell line derived from the parent cell line A3.01 [27,28], were maintained at 37 °C in RPMI 1640 (Sigma) with 10% heat-inactivated fetal bovine serum (Sigma), penicillin (100 units/ml), and streptomycin (100 mg/ml). To maintain the latency of the HIV-1 in OM10.1 and ACH-2 cells, 20 μM AZT was added in the culture medium and was excluded prior to experiments [29]. 293 cells and TZM-bl cells, a HeLa-derived cell line expressing surface CD4, CXCR4, CCR5 and containing a chromatin-integrated HIV-1 LTR [10,19,30,31], were grown at 37 °C in Dulbecco's modified Eagle's medium (Sigma) with supplements.

2.2. Reagents

The synthesis and HDACi activities of NCH-47 and NCH-51 were reported previously [24]. Sodium butyrate (NaB) was purchased from Sigma. Human recombinant TNF-α was purchased from Roche and used at 1.0 ng/ml for NF-κB stimulation. Antibodies for HDAC1, Sp1 acetyl-lysine and acetylated form of human histone H3 were obtained from Upstate Biotech, anti-RNA Pol II and normal rabbit anti-IgG were from Santa Cruz Biotechnology (Santa Cruz, CA), while anti-AP-4 antibody was obtained as previously described [7]. Mithramycin A was purchased from Sigma.

2.3. Plasmids

Construction of HIV-1 LTR-based luciferase expression plasmids, wild type (WT) HIV-1 LTR-Luc (containing the HIV-1 LTR U3 and R), its 5′ truncated mutant CD52-Luc (−117) and CD23-Luc (−65), and NF-κB mutant HIV-1 LTR-Luc, where NF-κB sites were mutated in WT HIV-1 LTR-Luc, were done as previously described [32,33]. Sp1 mutant HIV-1 LTR-Luc plasmid was generated by PCR with WT HIV-1 LTR-Luc DNA as a template and the three

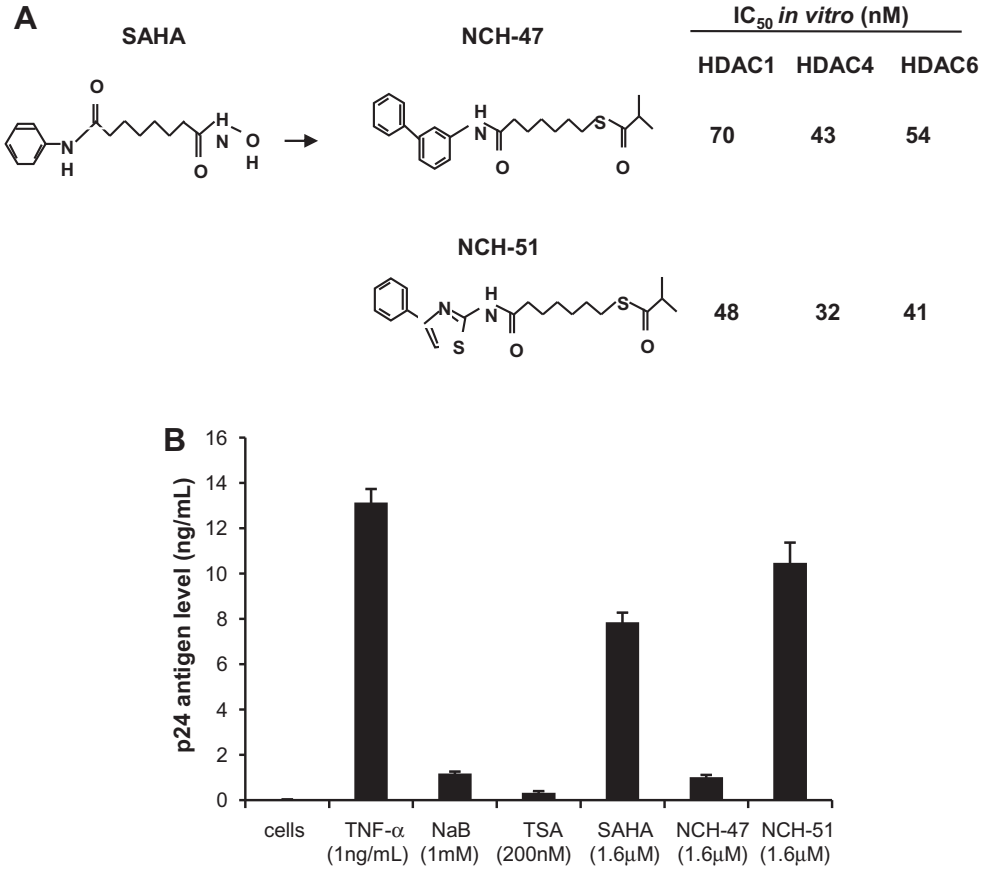

Fig. 1. Screening of the effect of HDAC inhibitors on HIV-1 production. (A) Chemical structures and IC_{50} of HDACis [24]. (B) Induction of viral replication from OM10.1 cells, latently infected with HIV-1. Cells were incubated with various HDACis or TNF-α. The cell culture supernatant was measured for HIV-1 p24 antigen using ELISA. Experiments were performed in triplicates. The means ± S.D. values are indicated. NaB, sodium butyrate; TSA, trichostatin A, SAHA, suberoylanilide hydroxamic acid.

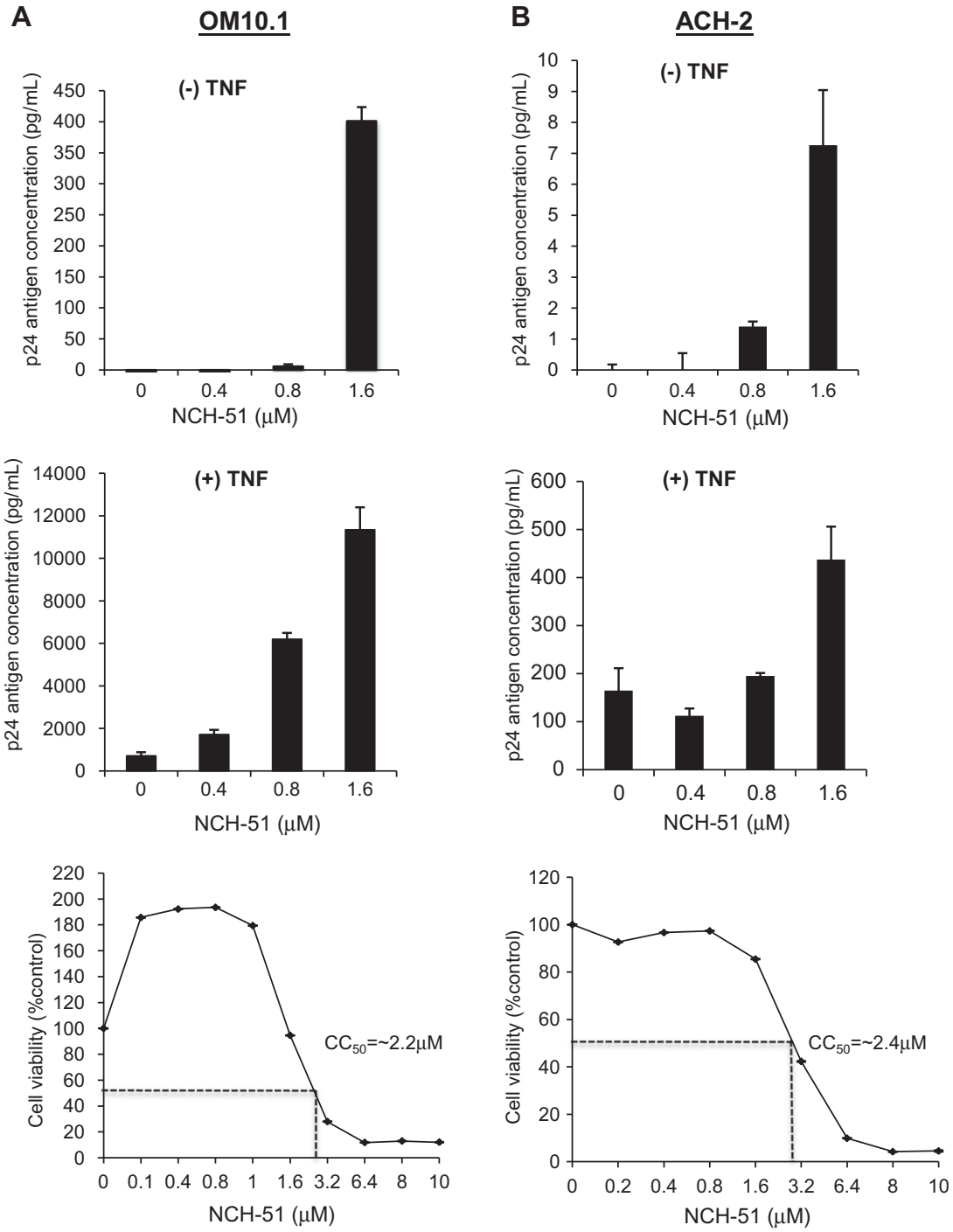

Fig. 2. Effect of NCH-51 on HIV-1 latently/chronically infected cells. (A and B) NCH-51 stimulated HIV-1 production in OM10.1 and ACH-2 cells. OM10.1/ACH-2 cells were treated with NCH-51 for 4 h then exposed to TNF-α (1 ng/ml) for 24 h. Culture supernatants were then collected and assessed for HIV-1 viral p24 antigen determination. (A and B, bottom panels) Cytotoxicity of NCH-51 on OM10.1 and ACH-2 cells. The data are means ± S.D. values of triplicate experiments.

GG sequences (at positions −56 and −57, −67 and −68, −78 and −79) were changed to TT using site-directed mutagenesis as described [7].

2.4. Viral replication assay

The stimulatory effect of HDAC inhibitors in latently infected cells was evaluated based on the extent of activation of HIV-1 viral p24 core antigen production in OM10.1 cells as previously described [7,33]. Briefly, cells were treated with or without NCH-47 or NCH-51 for 4 h then stimulated with or without TNF-α for 24 h at 37 °C. The culture supernatants were then collected and assayed for viral p24 antigen using the commercially available Retrotek HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Cellular Products, Buffalo, NY). The cytotoxicity of the test compounds was measured by WST-1 method (Roche) [33]. Fifty percent cytotoxic concentration (CC_{50}) was defined as the compound concentration that reduced cell viability by 50%. In another experiment, OM10.1 cells were pretreated with varying concentrations of mithramycin A for 2 h and then stimulated with

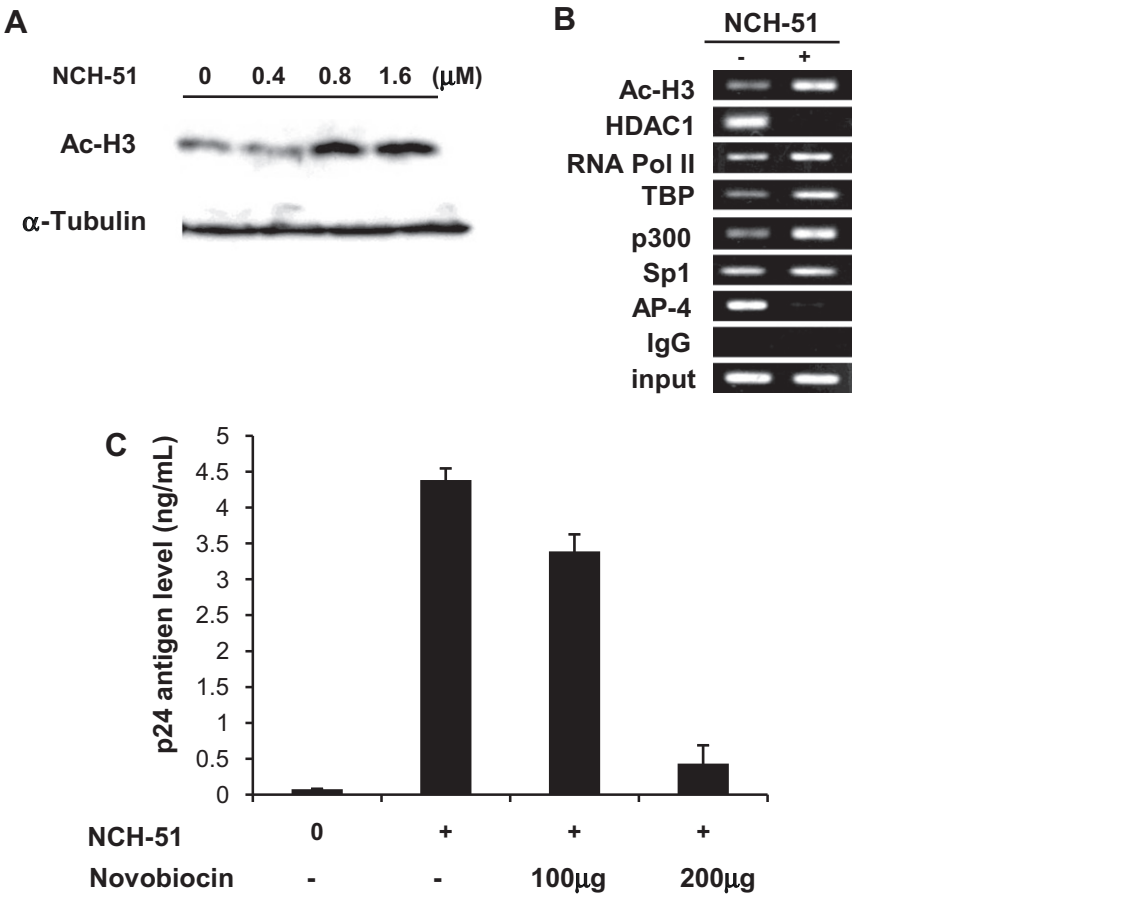

Fig. 3. NCH-51 increases acetylation of nuc-1 and decreases occupancy of HDAC1 at the HIV-1 LTR. (A) OM10.1 cells were treated with the desired concentrations of NCH-51 for 24 h and cell lysates were collected and subjected to Western blotting. Protein concentration was determined to ensure equal loading of proteins. (B) Recruitment of transcription factors and the acetylation of histones at the HIV-1 LTR. OM10.1 cells treated with or without NCH-51 (1.6 μM) were assayed by chromatin immunoprecipitation with designated antibodies. Input DNA (input) represents 10% of total input chromatin DNA while immunoprecipitation with IgG serves as a negative control. (C) Effects of novobiocin on the NCH-51-mediated HIV-1 production. OM10.1 cells were pretreated with novobiocin (100 and 200 μg/ml), a topoisomerase DNA II inhibitor, for 1 h and then stimulated with NCH-51 (1.6 μM) for an additional 24 h. Viral production was assessed as described in Section 2. The data are means ± S.D. of triplicate experiments.

NCH-51 for 24 h. HIV-1 p24 antigen levels were determined as described above.

2.5. Transfection and luciferase assay

Transfections were carried out in 293 cells (0.1 μg plasmid DNAs) and Jurkat T cells (0.3 μg plasmid DNAs) using Fugene 6 Transfection Reagent (Roche Applied Science) and Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions, respectively. HIV-1 LTR-based reporter plasmids described above were transfected for 24 h and then treated with NCH-51 (1.6 μM) or TNF-α (5 ng/ml), as a positive control, for an additional 12 or 24 h. Transfected cells were harvested and the extracts were subjected to luciferase assay using the Luciferase Assay System (Promega). Luciferase activity was normalized with protein concentration. Protein concentration was measured with BCA protein assay kit (PIERCE).

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously described [7,8,33]. Briefly, OM10.1 cells were cross-linked with formaldehyde for 10 min at 37 °C and reaction was stopped with 125 mM glycine. Cells were lysed and the cross-linked chromatin was sheared by sonication 10 times for 30 s each time at the maximum power with 30 s of cooling on ice between pulses (Bioruptor; COSMO Bio, Tokyo). Sheared chromatin fractions were collected and pre-cleared with salmon sperm DNA protein G-agarose beads (Upstate Biotech) for 1 h. Immunoprecipitation was then carried out with the desired antibodies overnight at 4 °C with rotation. The immuno-precipitates were collected using μMACS magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and washed sequentially with specified buffers. Immune complexes were eluted, reverse cross-linked at 65 °C for 5 h and treated with proteinase K at 45 °C for 1 h. DNA was purified by QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. PCR (31–33 cycles) was performed as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The primer sequences used for PCR spanning −109 to +79 nucleotides within HIV-1 LTR were the following: forward (5′-TAC AAG GGA CTT TCC GCT GG-3′) and reverse (5′-TTG AGG CTT AAG CAG TGG G-3′) [7]. For each reaction, 10% of the original sheared chromatin DNA was similarly reverse-crosslinked, purified, and the recovered DNA was used as input control.

2.7. siRNA transfection

The short interference (si)RNAs against Sp1 and control gfp were purchased from Santa Cruz Biotechnology Inc. and Dharmacon, respectively. TZM-bl cells were cultured in 24-well plates

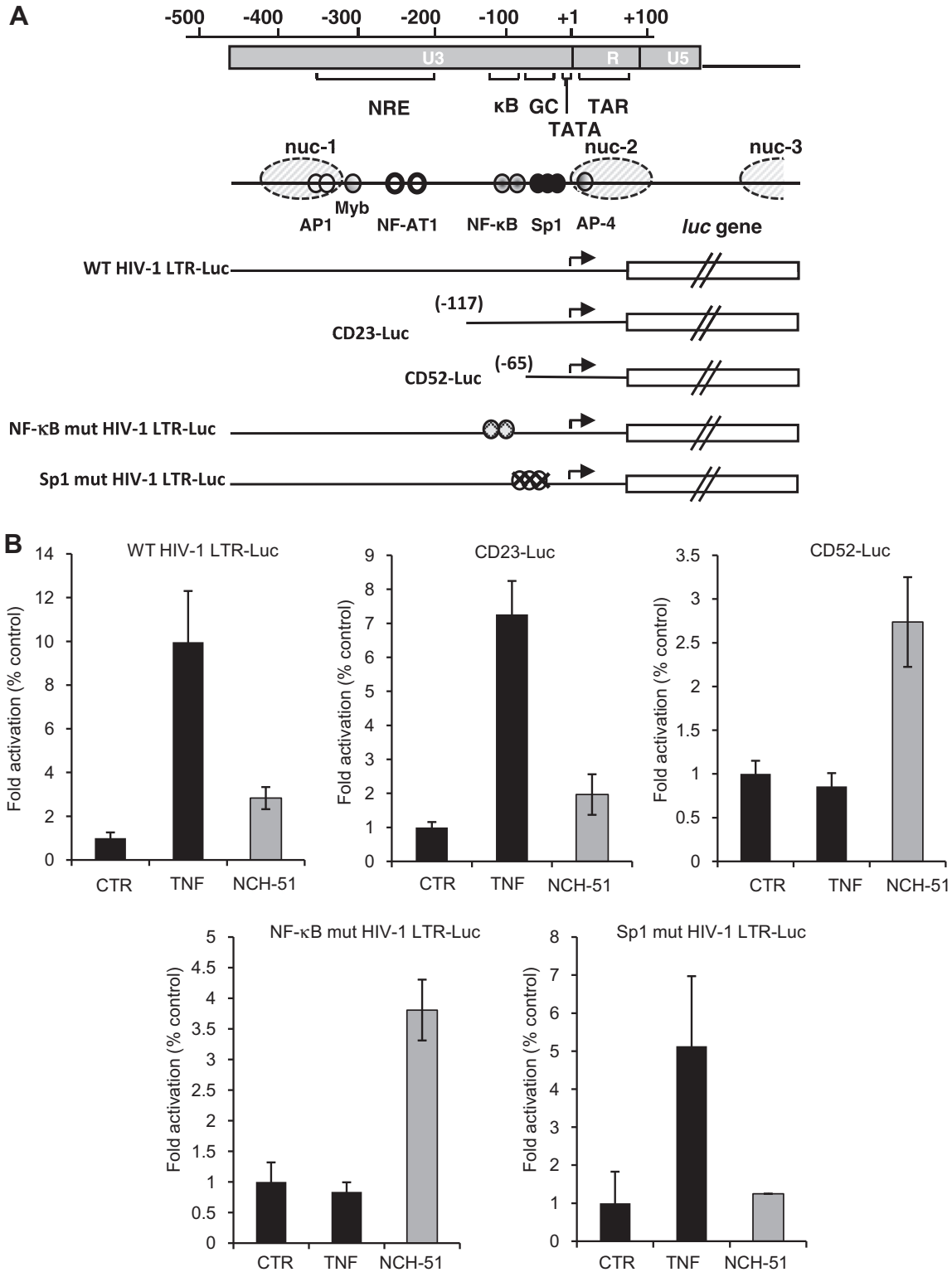

Fig. 4. NCH-51 activates transiently transfected HIV-1 LTR. (A) A schematic diagram of the WT HIV-1 LTR-based reporter plasmids used in determining the contribution of various portions of the HIV-1 promoter. (B and C) The HIV-1 LTR promoter constructs described in A were transiently transfected in 293 cells and Jurkat T cells, treated with NCH-51 as described in Section 2, and harvested for luciferase assay. Luciferase activity was normalized by the protein concentration of the lysate. The data are means ± S.D. of triplicate experiments.

and treated with 50 nM siRNAs using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen) and incubated for 48 h. To ensure the knockdown of Sp1 protein, Western blotting was performed with anti-Sp1 antibody. The transfected cells were harvested and subjected to luciferase assay.

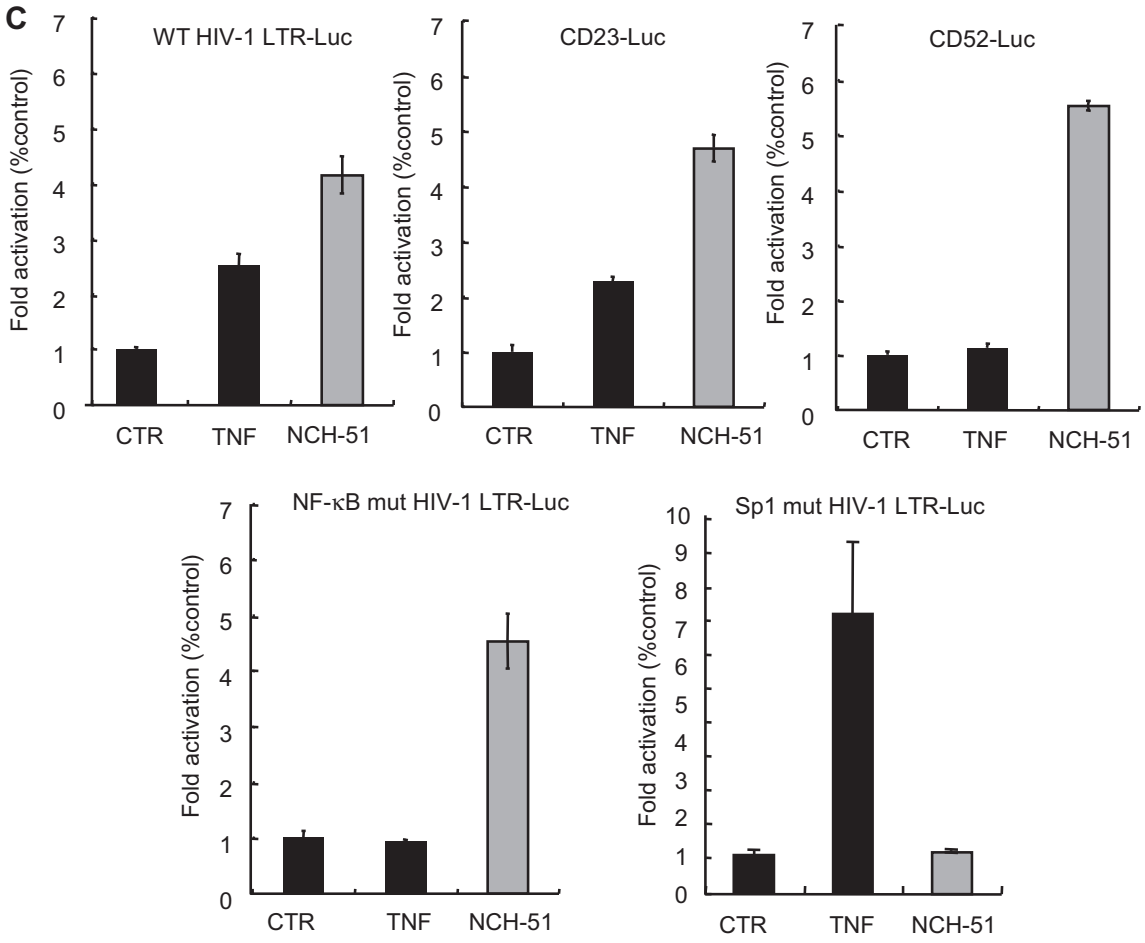

Fig. 4 (*continued*)

3. Results

3.1. NCH-51 activates HIV-1 production in latently infected cells

HDAC inhibitors induce acetylation of histones consequently propelling the activation of gene transcription. To assess whether our novel HDACis, NCH-47 and NCH-51, can activate the latent HIV-1 proviruses in infected cells, we initially tested these compounds in OM10.1 cells as well as other known HDACis such as SAHA, NaB and TSA (Fig. 1B). Strikingly, although there is a little difference in the structures of NCH-47 and NCH-51, we found that NCH-51 markedly augmented HIV-1 production compared with NCH-47. The effect of NCH-51 was even greater than other known HDACis such as SAHA, NaB and TSA at tested concentrations. Thus, we investigated further the activity of NCH-51 in the following experiments.

As shown in Fig. 2A, treatment with NCH-51 alone induced the production of HIV-1 in OM10.1 cells. This effect was further augmented by TNF-α. We performed similar experiments using ACH-2 cells, a chronically HIV-1-infected T cell line, and found that NCH-51 could upregulate virus production in these cells as well (Fig. 2B). At the concentrations tested, 48 h incubation with NCH-51 elicited a slightly higher cell death in ACH2 than OM10.1 cells (Fig. 2A and B, bottom panels). CC_{50} values for both cell lines were estimated to be approximately 2 μM. These results demonstrate that NCH-51 can efficiently reactivate expression of HIV-1 from latently/chronically infected cells under non-cytotoxic concentrations.

3.2. NCH-51 facilitates HIV-1 replication through chromatin remodeling

Transcriptional silencing during HIV-1 latency is controlled by the local environment of the integrated virus as well as the presence of other activating or repressing viral or host factors [34]. Initially, we examined the histone acetylation state of whole cell lysates from OM10.1 cells. As demonstrated in Fig. 3A, NCH-51 treatment increased the acetylation of histone H3 at either K9 or K14 in a dose-dependent manner. To confirm this observation, we employed a chromatin immunoprecipitation assay (Fig. 3B). Consistent with results obtained by Western blotting, hyperacetylation of histone H3 at nuc-1 region of the HIV-1 LTR occurred upon NCH-51 treatment with simultaneous depletion of HDAC1 occupancy. In addition, assessment of the differential recruitment of other transcription factors revealed an enrichment of positive transcription factors such as RNA polymerase II and TBP (TFIID), whereas the negative transcription factor AP-4 was abolished. NCH-51 treatment of TZM-bl cells [10,19] containing chromatin-integrated HIV-1 LTR yielded similar results (data not shown).

We then examined whether the NCH-51-induced HIV-1 virus production in OM10.1 cells would be abrogated by the topoisomerase II inhibitor, novobiocin [35]. Topoisomerase II is a nuclear matrix-associated enzyme that cleaves and religates dsDNA, an event required for the structural reorganization of nuclear chromatin, and thus plays an essential role in the effects of HDACi [35]. As demonstrated in Fig. 3C, we found that the virus production induced by NCH-51 in OM10.1 cells was inhibited by novobiocin

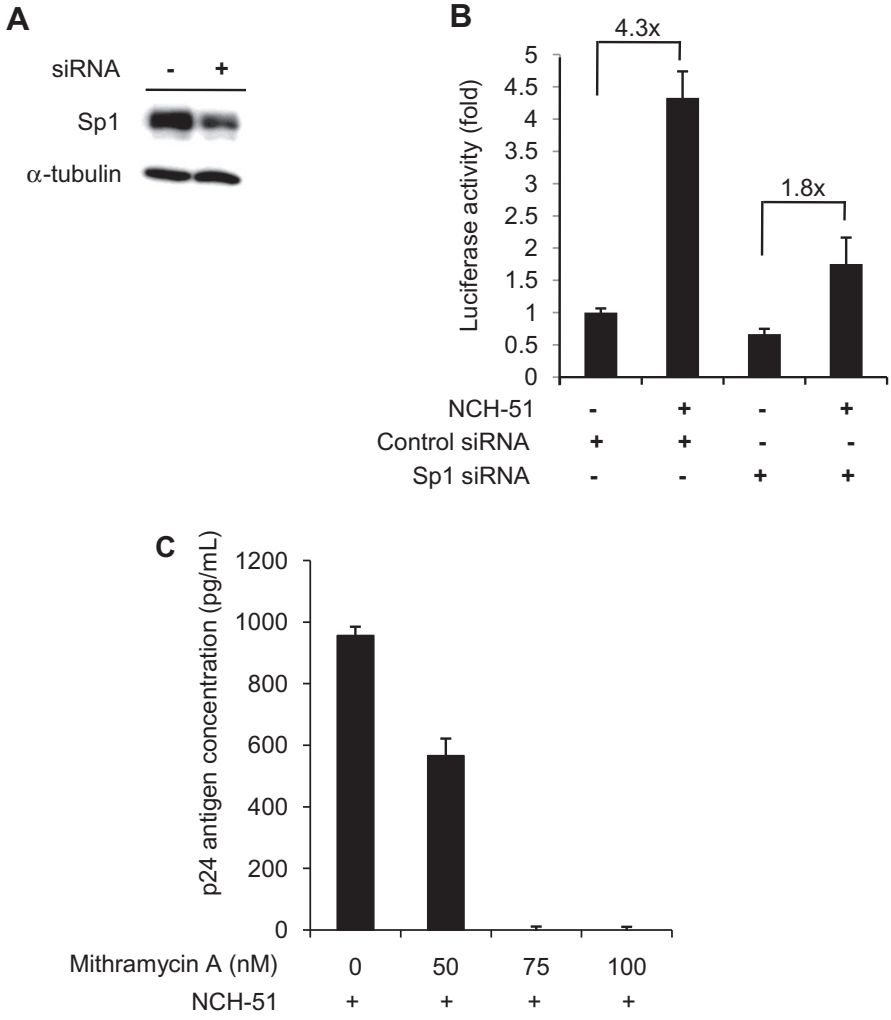

Fig. 5. Sp1 is essential for latent HIV-1 LTR gene expression. (A) Western blot of Sp1 protein levels after Sp1 siRNA treatment of TZM-bl cells. (B) Knockdown of Sp1 by siRNA reduced HIV-1 LTR promoter activity by NCH-51 (1.6 μM). The data are means ± S.D. of triplicate experiments. (C) Effects of mithramycin A on the viral production induced by NCH-51. OM10.1 cells were pretreated with the specific Sp1 inhibitor mithramycin A at the indicated concentrations for 2 h then treated with NCH-51 (1.6 μM) for another 24 h. Cell supernatants were collected and assayed for HIV-1 p24 antigen levels. The data are means ± S.D. of triplicate experiments.

suggesting that NCH-51 have contributed to changes in chromatin structure and promoted HIV reactivation.

Overall, these results show that the induction of viral replication from latently infected OM10.1 cells by NCH-51 correlates with the hyperacetylation of histones and the simultaneous removal of HDAC1 on the HIV-1 promoter.

3.3. NCH-51 activates transcription from HIV-1 LTR

SAHA was previously reported to induce HIV-1 transcription [17–19]. To determine whether NCH-51 activates transcription from HIV-1 LTR, we generated various plasmid constructs, as shown in Fig. 4A, transiently transfected them into 293 cells and determined the levels of HIV-1 gene expression by luciferase assay.

NCH-51 stimulated wild type (WT) HIV-1 LTR expression as well as the CD23-Luc HIV-1 LTR promoter (Fig. 4B). When NF-κB binding sites in the LTR were absent (CD52-Luc) or mutated (NF-κB mut HIV-1 LTR-Luc), a marked transcriptional activation by NCH-51 was still observed whereas it totally abolished the effects of TNF-α. (Fig. 4B, top right and bottom left panel). We thus speculated that Sp1 might be accountable for the reactivation potential of NCH-51 because of the reported role of Sp1 sites for transcriptional activation of HIV-1 LTR by another HDACi valproic acid [36]. Thus, we created a plasmid construct of HIV-1 LTR with all three Sp1 binding sites mutated (Fig. 4C, bottom right). Indeed, despite the presence of intact NF-κB binding sites, the mutation of all Sp1 binding sites severely weakened the effects of NCH-51. A similar effect by NCH-51 was observed using Jurkat T cells (Fig. 4C). We noted that longer exposure (24 h) to NCH-51 promoted higher transcriptional activity of the HIV-1 LTR. Meanwhile, we also observed that TNF-α stimulation caused higher promoter activation in Sp1-mutant HIV-1 LTR than the wild type (Fig. 4C, first and right-bottom panel), indicating that without Sp1 sites transcription is carried out mainly by the NF-κB elements. These findings indicate that Sp1 appears to be crucial in the transcriptional activation of HIV-1 LTR by NCH-51.

3.4. The effect of Sp1 knockdown

To examine the effect of endogenous Sp1, TZM-bl cells, containing a stable chromosomally-integrated HIV-1 LTR, were treated with a siRNA designed to knock-down Sp1 gene expression. Transfection with 50 nM Sp1 siRNA markedly reduced but did not completely deplete Sp1 protein expression, most likely because Sp1 is ubiquitously expressed (Fig. 5A). Sp1 siRNA treatment, however, significantly suppressed the effects of NCH-51 in inducing the tran-

scriptional activity of the HIV-1 LTR promoter, as shown in Fig. 5B (1.8× compared to 4.3× with control siRNA). To evaluate further the involvement of Sp1 in the NCH-51-mediated activation, we treated OM10.1 cells with varying concentrations of mithramycin A, an Sp1 antagonist [38]. We found that mithramycin A inhibited the HIV-1 p24 expression induced by NCH-51 (Fig. 5C), thus indicating the importance of Sp1 for this response.

4. Discussion

Despite advances in HAART, the presence of drug-inaccessible sanctuaries that harbor latent proviruses impedes HIV eradication. An alternative approach of inducing the expression of quiescent HIV-1 proviral genomes, but has limited activating effects, has been sought with the prospect that it will allow expression of latent HIV and avoid the pitfalls of global T-cell activation [1,2,23,36]. This clinical strategy has been initiated by Lehrman et al. [37] using the combination of the HDACi valproic acid with intensified HAART and was found successful in accelerating the clearance of HIV-1-infected cell population from resting CD4+ T cells in HIV-1 infected patients. Since then, many have continuously searched for the appropriate pharmacological activator(s) and/or combinatorial drug regimen that may efficiently deplete latent reservoirs.

We provide additional evidence demonstrating the efficacy of HDACi NCH-51 in reactivating HIV-1 replication in latently/chronically infected cells such as OM10.1 and ACH-2. This compound has been shown to have better pharmacokinetics and less cytotoxicity than the parent compound SAHA [20]. NCH-51 could clearly upregulate gene expression from a latently infected HIV provirus by a mechanism, though not fully elucidated yet in our system, which considerably utilizes the Sp1 sites and the removal of the constitutively associated repressors (e.g., HDAC1 and AP-4) and the subsequent recruitment of positive transcription factors (e.g., p65, TFIID and p300) to the HIV-1 promoter region.

The nucleosome nuc-1, immediately downstream of the transcription start site of proviral HIV-1, plays a crucial role in the generation of post-integration latency. Nuc-1 is in a hypoacetylated state exhibiting a repressive configuration of the local chromatin [2,9,11]. Such suppressive chromatin structure must be remodeled prior to transcriptional activation by NF-κB or by other positive transcription factors such as Tat. HDAC inhibition induces acetylation of core histones that relaxes chromatin structure, making the DNA more accessible to the transcriptional machinery and co-activators [7,9,18,19,39]. Our findings have shown that the alteration in the chromatin structure by NCH-51 resulted in the dynamic recruitment of positive transcription factors and epigenetic changes on the HIV-1 LTR promoter thereby initiating directed transcriptional activation.

We found that Sp1 sites are considerably involved in the NCH-51-mediated activation of the HIV-1 promoter as depicted in our transient transfection studies and Sp1 siRNA knockdown experiment. Previous studies have indicated that Sp1 sites can recruit transcriptional repressor factors/complexes as well as activating factors [11,13,14,40]. Sp1 appears to contribute to the proviral latency by recruiting HDAC1 and/or HDAC2 [10,14,41] to the HIV-1 LTR. Treatment by NCH-51 could most likely disrupt the formation of this repressor complex by displacing HDAC1 from the HIV-1 LTR as evidenced in our ChIP results. Thus, in the absence of Sp1 binding sites (Fig. 4) or Sp1 protein (Fig. 5) the HDAC1 could not be recruited and no effect could be observed. We also speculate that in the latency model system that we used, the de-repression of HDAC activity on Sp1 upon NCH-51 treatment most likely regulates Sp1 function through the consequential recruitment of transcriptional co-activators such as p300 [42,43] and P-TEFb [44]. p300 exhibiting intrinsic HAT activity could regulate the transcriptional activity of Sp1 directly or by acting as a scaffold for other co-activators of Sp1 [34,42,43]. The enrichment of TBP/TFIID level in our ChIP assay also suggests its direct or indirect association with Sp1 or with other cellular proteins that are responsible for initiation of transcription [7,45,46]. Moreover, NCH-51 induction might, though not yet fully elucidated here, induce other posttranslational modifications of Sp1 apart from acetylation wherein no remarkable change was found after an hour of treatment with NCH-51 (data not shown). This possibility needs further clarification because posttranslational modification of Sp1 was previously reported to influence its protein stability and transcriptional activity [47].

The effective reactivation of latent HIV-1 gene expression by the simultaneous treatment of NCH-51 and TNF-α suggests that our HDACi has the potential for combinatorial therapeutics against HIV latency. However, at present, we need to carefully evaluate the limitations of such approach due to a possible sequential generation of viral escape mutants or uncontrolled spread of HIV-1 in anatomic sites where HDACis can penetrate but inaccessible to HAART. Moreover, investigations on the effect of NCH-51 on global gene expression changes and on host genes required for HIV replication must be considered to ensure its applicability and safety.

Acknowledgments

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