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HIV Replication Is Increased by RNA Methylation METTL3/METTL14/WTAP Complex Activators

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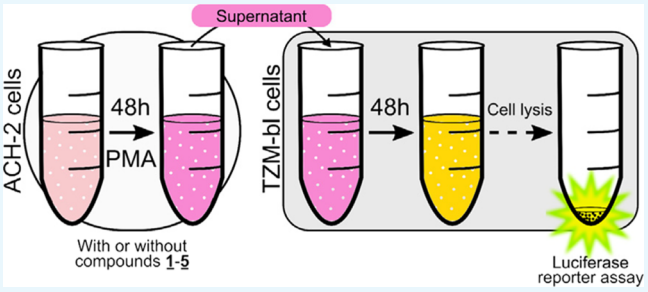
ABSTRACT: The N6-methyladenosine (m^6A) modifications in both viral and host cell RNAs play an important role in HIV-1 virus genome transcription and virus replication. We demonstrate here that activators of the METTL3/METTL14/WTAP RNA methyltransferase complex enhance the production of virus particles in cells harboring HIV-1 provirus. In parallel, the amount of m^6A residues in the host cell mRNA was increased in the presence of these activator compounds. Importantly, the m^6A methylation of the HIV-1 RNA was also enhanced significantly (about 18%). The increase of virus replication by the small-molecule activators of the METTL3/METTL14/WTAP complex excludes them as potential anti-HIV-1 drug candidates. However, the compounds may be of large interest as activators for the latent HIV-1 provirus copies deposited in host cells' genome and the subsequent virus eradication by an antiviral compound.



INTRODUCTION

Modification of viral RNAs by methylation of the amino group at the 6-position of adenosine (m^6A) has been known for some time.[1,2] Recently, it was shown that the presence of m^6A in viral RNA regulates HIV-1 replication and gene expression.[3−5] Therefore, it is of significant interest to study how the regulation of HIV-1 life cycle is affected by compounds influencing the activity of proteins and enzymes in the m^6A RNA-methylation pathway.

Three types of such proteins can be targeted for this purpose as the methylation of adenosine is dynamically regulated in mammalian cells by (1) RNA methyltransferases or "writers," (2) demethylases or "erasers," and (3) m^6A-recognizing proteins or "readers."

The N6-methylation of adenosine is catalyzed by a 200 kDa methyltransferase heterodimer complex consisting of the methyltransferase-like protein 3 (METTL3), methyltransferase-like protein 14 (METTL14) and the associated proteins Wilms tumor 1 associated protein (WTAP), RBM15/RBM15B, and KIAA1429,[6,7] and the m^6A methyltransferase (METTL16).[8,9] METTL3 embraces the active center of the RNA m^6A methyltransferase, while METTL14 together with RBM15/RBM15B plays an important role in substrate RNA recognition and binding.[10,11] The primary function of WTAP is to localize METTL3 and METTL14 to nuclear speckles.[12] Removal of the methyl group from m^6A is catalyzed by two RNA demethylases, the fat mass and obesity-associated protein (FTO) and α-ketoglutarate-dependent dioxygenase homolog 5 (ALKBH5).[13,14] The m^6A modification in mRNA is specifically recognized by members of the YT521-B homology (YTH) family of proteins.[7,15] Three YTHDF (YTH domain family) members, YTHDF1, YTHDF2, and YTHDF3, are localized in the cytoplasm[16,17] and have been associated with different regulatory processes. For instance, binding of YTHDF1 enhances the translational rates of its mRNA targets,[18] whereas binding of YTHDF2 induces mRNA degradation.[19] The reader protein YTHDF3 promotes translation when associated with YTHDF1, and mRNA decay when associated with YTHDF2.[17] Two YTHDC (YTH domain-containing) nuclear proteins have been also identified as m^6A readers. YTHDC1 is located in nucleus,[20] and YTHDC2 is present both in the nucleus and in the cytoplasm.[21] The effects of a third class of m^6A readers (IGF2BP1−3) have been suggested to present an alternative pathway by which m^6A affects mRNA stability.[22]

The presence of m^6A in viral RNA has been observed in numerous viruses.[23−30] It has been established that m^6A residues both in the viral RNA and host cell RNA play important roles in HIV-1 provirus transcription and virus replication.[3−5,31−33] The m^6A modifications have been

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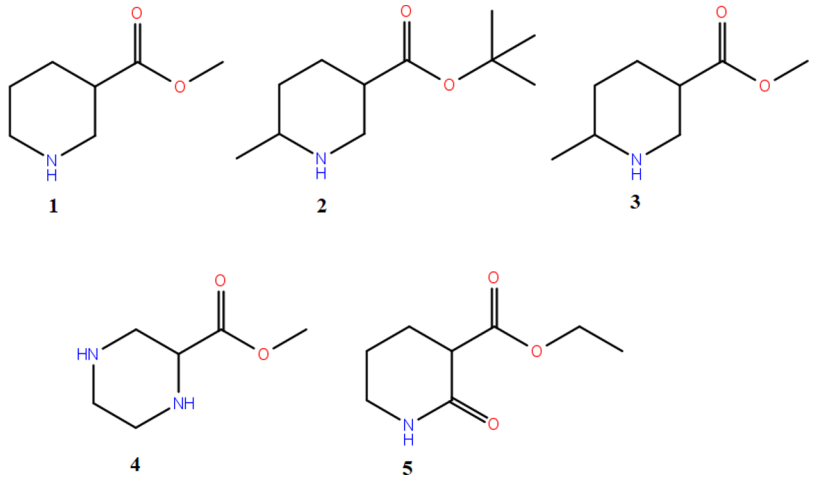

Figure 1. RNA m^{6}A methyltransferase complex METTL3/METTL14/WTAP activator compounds.

identified in both the 5′ and 3′ untranslated regions (UTRs), as well as in the *rev* and *gag* genes of the HIV-1 genome.[5] Interestingly, viral infection itself triggers an increase in m^{6}A in both host and viral mRNAs.[4] The HIV-1 infection of primary CD4+ T-cells or Jurkat cells significantly increases m^{6}A levels of cellular RNA independently of viral replication.[34] Silencing of either METTL3 or METTL14 in the human T-cell lymphotropic virus-I (HTLV-I) carrying human T-cell line, MT4, significantly decreased HIV-1 replication, and an additive effect was observed when both proteins were simultaneously knocked down. Conversely, ALKBH5 silencing caused an increase in viral replication.[4] In addition, it has been reported that overexpression of YTHDF1, YTHDF2, and YTHDF3 (Y1−3) proteins in HEK-293 T-cells leads to the suppression of HIV-1 infection by primarily decreasing HIV-1 reverse transcription, while knockdown of these endogenous proteins in Jurkat or primary CD4+ T-cells increases HIV-1 infection.[5] The overexpression of the Y1−3 proteins in HIV-1 target cells decreases viral genomic RNA (gRNA) levels and inhibits reverse transcription.[35]

Recently, we reported the discovery of small molecules that activate the RNA m^{6}A methyltransferase METTL3/METTL14/WTAP complex and increase the m^{6}A level in cell mRNA.[36] These activators have a unique mechanism of action, working as artificial coenzymes to the enzymatic methylation reaction. The molecular dynamics simulations reveal that simultaneous binding of these compounds and the methylation agent *S*-adenosylmethionine (SAM) in the active center of the enzyme complex significantly increases the binding efficiency of the latter. This leads to increased efficiency of methylation by the METTL3/METTL14/WTAP complex.[36] Biologically, the activators of the m^{6}A writer complex provide the first upstream means for increasing cellular m^{6}A amounts. The compounds are esters and presumably in the hydrolyzed form (carboxylic anions) in the cell culture medium. We have measured the half-life of compound **4** in human serum ($t_{1/2}$ = 6 h). Contrary to FTO or ALKBH5 inhibitors that rely on the baseline activity of m^{6}A writing to be effective, these small-molecule m^{6}A writer activators can help targeted guidance of cells to specific phenotypes. Based on the available knowledge on the m^{6}A dynamics in HIV-1 infection as briefly described above, the increase of m^{6}A in host cells and/or HIV RNAs should enhance virus replication. Thus, in general, instead of having possible HIV-1 inhibitor activity, compounds that activate m^{6}A writers could serve as reactivators of cells with latent HIV-1 proviruses deposited in the host cell genome.[37,38] Putatively, together with appropriate anti-HIV medications, this could pave the way for HIV-1 eradication from the host.

In the current work, we have studied the activity of the small-molecule activators of the RNA m^{6}A methyltransferase METTL3/METTL14/WTAP complex on HIV-1 replication. In parallel, we monitored the dynamics of m^{6}A as influenced by the activation of m^{6}A methylation in both HIV-1 viral and cellular host RNAs.

RESULTS AND DISCUSSION

The effect of the METTL3/METTL14/WTAP activators (compounds **1**−**5**, Figure 1[36,39]) on the gene expression from HIV-1 provirus and formation and release of HIV-1 virions in ACH-2 cells were measured using viral p24 ELISA assay. In the case of compound **1**, no statistically significant effect was observed (Figure 2A). Increased production of virions was observed for the activator compounds **2**, **3**, **4**, and **5** (Figures 2B−E, respectively). For compounds **3**, **4**, and **5**, the increase in virion production was concentration-dependent; for some active compounds (**2** and **3**), a significant increase in the virion production was detected already at 0.01 μM concentration. The excessive production of viral capsid protein most likely represents a consequence of multiple effects of the N6-methylated adenosine in RNAs. For example, the gag-pro, a precursor for capsid proteins and reverse transcriptase, is translated from nonspliced genomic RNAs; therefore, the effect of m^{6}A residues on splicing may promote production and nuclear export of full-length HIV-1 genomes activating also production of proteins translated directly from these RNAs.

In order to reveal whether or not activator compounds increase the amount and/or infectivity of infectious HIV-1 virions, the aliquots of the supernatants collected from the ACH-2 cells treated with the compounds at 10 μM concentration were used to infect the TZM-bl reporter cells; after incubation for 48 h at 37 °C, the luciferase reporter activities that are proportional to the amount of HIV-1 proviruses integrated into the genome of reporter cells were measured (Figure 3). Coherently, with the observed increase of the release of p24, a substantial increase in the virion production and/or their infectivity was observed for ACH-2

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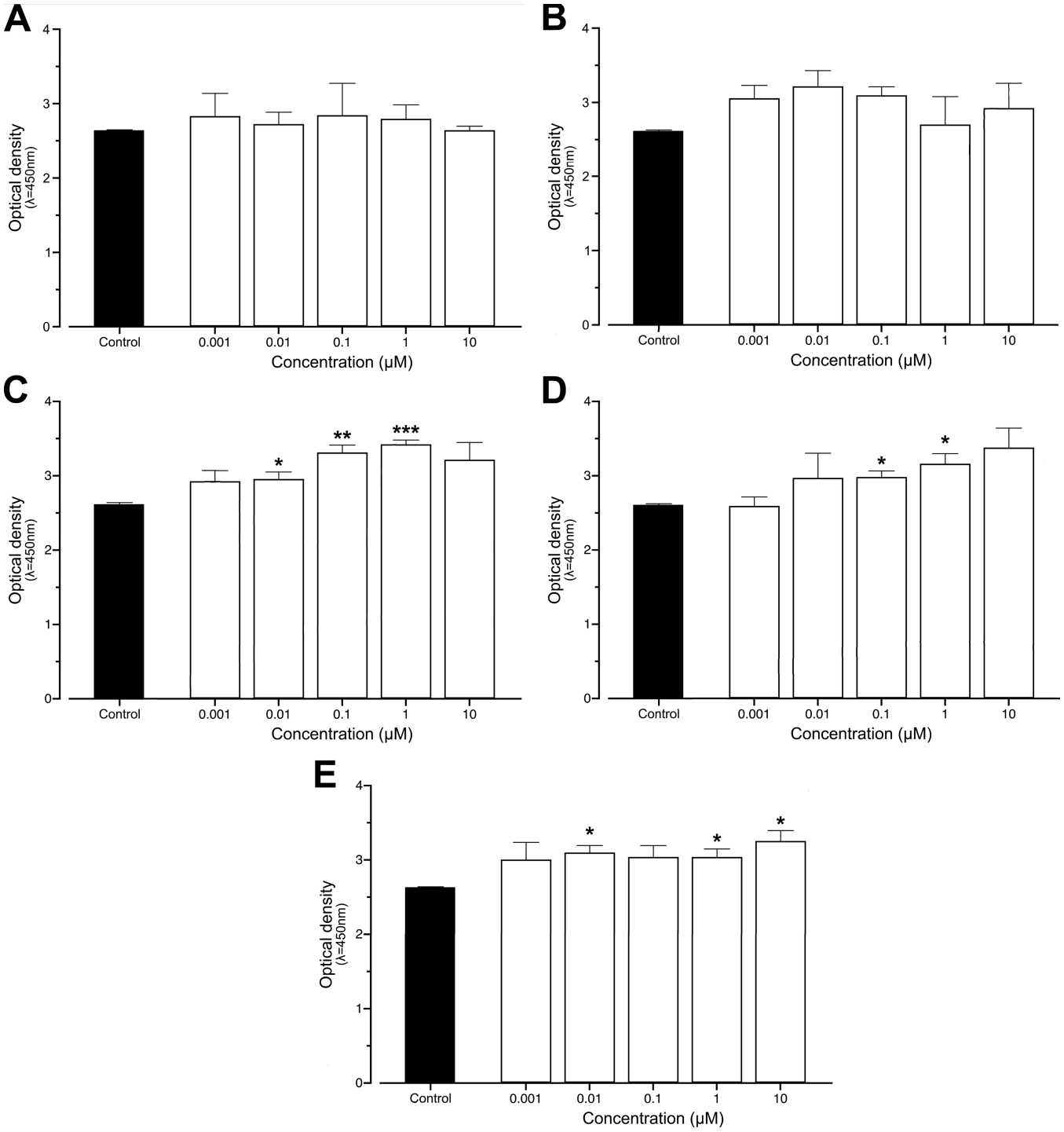

Figure 2. Dependence of the amount HIV-1 p24 released to the growth medium after treatment of ACH-2 cells with the METTL3/METTL14/WTAP activator compounds at different compound concentrations. A compound **1**; B compound **2**; C compound **3**; D compound **4**; and E compound **5**. The optical density at 450 nm (OD450) measured using the Abcam HIV-1 p24 ELISA Kit (ab218268) is proportional to the HIV-1 p24 concentration. The results are shown as means ± standard deviation (S.D) from three independent experiments ($n = 3$); *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

cells treated with active compounds **3**, **4**, and **5**. For compounds **1** and **2**, such effect is lacking (Figure 4), reflecting their inability to activate HIV-1 provirus (Figure 2A,B). Thus, the activator compounds did not have a negative impact on the infectivity of released HIV-1 virions nor did their presence in the used inoculum have a negative impact on the infection of TZM-bl cells. Thus, the encapsidated RNA, presumably containing excessive m^{6}A methylation, did function as a viral genome, i.e., was reverse transcribed in infected TZM-bl cells.

Therefore, we studied the effect of our METTL3/METTL14/WTAP activator compounds on the level of m^{6}A methylation in the viral RNA as well as in cellular mRNA. The results for the following experimental scenarios are presented in Figure 5: (i) HIV-1-infected cells in the absence of the compound **4**, (ii) HIV-1-infected cells in the presence of the

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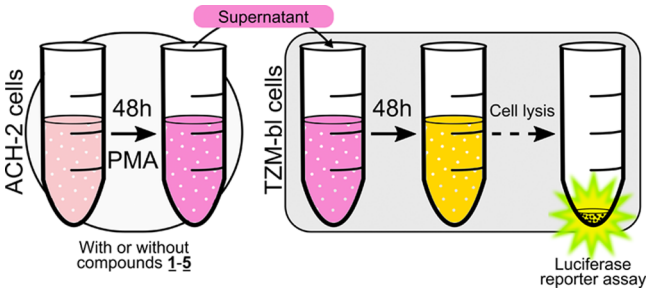

Figure 3. Scheme of the measurement of the replication of HIV-1. ACH-2 cells were treated with the METTL3/METTL14/WTAP activator compounds at 10 μM concentration, and cells used to obtain the control stock no compound activator were added. Subsequently, obtained stocks were used to infect TZM-bl cells, and the amount of infectious virions in stocks was estimated by measuring Luc activity.

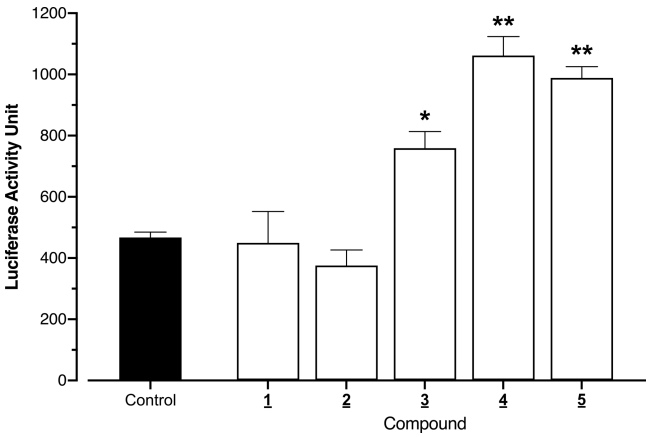

Figure 4. Compounds **3**, **4**, and **5** increase production of infectious HIV-1 virions in ACH-2 cells activated by PMA. Luc activities in lysates of TZM-bl reporter cells that were infected with media collected from ACH-2 cells treated with METTL3/METTL14/WTAP activator compounds. Activity of Luc (in Luciferase Activity Units, vertical axes) is proportional to the amount of infectious HIV-1 virions in the used inoculum. The results are shown as means ± S.D. of three independent experiments; $*p < 0.05$, $**p < 0.01$.

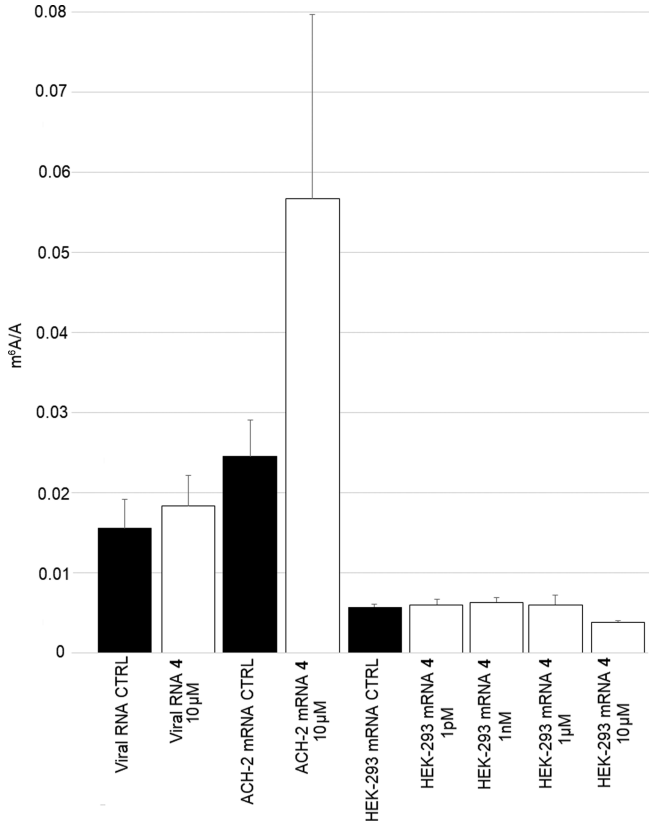

Figure 5. Effect of compound **4** on the LC/MS measured ratio of N6-methyladenosine and adenosine (m^6A/A) in HIV-1 viral RNA, ACH-2 cell mRNA, and HEK-293 cell mRNA.[36]

compound **4**, (iii) noninfected cells in the absence of the compound **4**, and (iv) noninfected cells in the presence of compound **4**. The results for the last two scenarios are taken from our earlier publication[36] and relate to the HEK-293 cells. First, it can be seen that the relative amount of m^6A in the cell mRNA is increased by the HIV-1 infection, in congruence with the earlier observation by Lichinchi et al.[4] The treatment of ACH-2 cells containing HIV-1 provirus with the METTL3/METTL14/WTAP activator compounds increases further the mRNA N6-adenosine methylation level in these cells more than twice (Figure 5). This effect cannot be attributed to increased activation of HIV-1 provirus as it has been previously reported that the infection of Jurkat and primary CD4+ T-cells with HIV-1 did not significantly affect the percentages of total m^6A peaks mapped to the human genome in the 5′ UTR, coding DNA sequence (CDS), 3′ UTR, and noncoding regions (<1%).[5] Moreover, importantly, a significant increase of the m^6A methylation of the virus RNA genome itself (about 18%, Figure 5) was also observed. This is the first demonstration that the m^6A methylation level of a viral RNA can be changed by using a small-molecule external agent (METTL3/METTL14/WTAP activator). Therefore, if such effect will be valid in the case of other RNA viruses such as flaviviruses, coronaviruses, hepatitis C virus, and others, it would open a completely new way for the development of antiviral compounds that affect the virus life cycle through the methylation or demethylation of viral RNAs.

Whereas the increase of HIV-1 virus production by the small-molecule activators of the METTL3/METTL14/WTAP complex discards them as possible antivirals, the compounds may be of large interest as supporters for the removal of the latent HIV provirus copies deposited in host cells' genome.[38,40] Cells containing activated provirus can be subsequently targeted by use of conventional anti-HIV-1 drugs, i.e., using the so-called "shock-and-kill" approach.[37,41,42]

CONCLUSIONS

The importance of viral RNA modifications in the virus life cycle has been known for some time. The presence N6-methyladenosine, m^6A, modification has been demonstrated in the RNA of HIV-1[32] and viruses with RNA genomes such as flaviviruses (dengue virus, Zika virus, and West Nile virus), hepatitis C virus,[43] and human respiratory syncytial virus.[28] In the present work, we have shown that the treatment of cells harboring HIV-1 provirus with the small-molecule RNA m^6A methyltransferase complex METTL3/METTL14/WTAP activator compounds causes a notable increase of N6-adenosine methylation in the viral RNA genome. This effect is accompanied with increased infectious virus formation. The increase of virus replication by the small-molecule activators of the METTL3/METTL14/WTAP complex excludes them as potential anti-HIV-1 drug candidates. However, the com-

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pounds may be of large interest as activators for the latent HIV-1 provirus copies deposited in host cells' genome and the subsequent virus eradication by an antiviral compound.

MATERIALS AND METHODS

Compounds. Methyl piperidine-3-carboxylate hydrochloride (**1**) (ArkPharm, Inc., Catalog Number: AK-86479, Purity >97%).

Tert-Butyl 6-methylpiperidine-3-carboxylate (**2**) (Life Chemicals Inc. Product ID: F2163−0155, Purity >95%).

Methyl 6-methylpiperidine-3-carboxylate (**3**) (ArkPharm, Inc., Catalog Number: AK103663, Purity >95%).

Methyl piperazine-2-carboxylate (**4**) (ChemDiv, Inc., Catalog Number: FF20−0374, Purity >90%).

Ethyl 2-oxopiperidine-3-carboxylate (**5**) (Enamine, Ltd., Catalog Number Z397585734, Purity >90%).

Cell-Culture-Based Assay of HIV-1 Activity. *Cell Lines.* The ACH-2 cell line was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: ACH-2[40,44] Dr. Thomas Folks. ACH-2 cells were grown in Roswell Park Memorial Institute medium 1640 (RPMI 1640) (Sigma-Aldrich) supplemented with 25 mM HEPES, 0.3 g/L L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), and penicillin−streptomycin.

The TZM-bl cell line was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc.[20,45,46] TZM-bl cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS and penicillin−streptomycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Assays Using HIV-1 Virions. ACH-2 cells were seeded at a concentration of 2×10^5 cells in 200 μL into wells of a 96-well plate, and HIV-1 virion production was induced by the addition of 100 nM PMA. The cells were incubated for 48 h with added compounds at given concentrations (Milli-Q water was used as a vehicle control), and subsequently, the media containing HIV-1 virions were collected. The amount of HIV-1 p24 protein released into the media was measured using an HIV-1 p24 enzyme-linked immunosorbent assay (ab218268, Abcam plc, Cambridge, UK).

TZM-bl cells express firefly luciferase (Luc) under control of the HIV-1 long terminal repeat (LTR) promoter. The promoter is activated by the Tat protein produced by the integrated HIV-1 provirus.[47−49] In this assay, TZM-bl cells were seeded on a 96-well plate (2×10^5 cells per well). The next day, media was removed, and 50 μL of the supernatant of the incubation media containing the virus treated with the compounds at 10 μL concentration and polybrene (6 μg/mL) was added to TZM-bl cells. An equal amount of virus was used in the case of each compound. The plate was incubated 2 h at 37 °C and 150 μL DMEM medium was added to each well. The cells were thereafter incubated for 48 h at 37 °C. The supernatant was removed and 50 μL of lysis buffer was added to each well. The wells were then incubated for 15 min and irradiated with UV light for 5 min to eliminate infectious virus. Then, the virus titer in the used inoculum was estimated by measuring luciferase activity in the cell lysate using a Luciferase Assay System and Glomax 20/20 Luminometer (both from Promega Corp., Madison, WI).

m^{6}A Detection in RNA. *Isolation of RNA from HIV-1 Virions and ACH-2 Cells.* A total of 2×10^8 ACH-2 cells were seeded on a 175 cm^2 cell culture flask in 100 mL RPMI 1640 medium, and HIV-1 virion production was induced by the addition of 100 nM PMA. The activated cells were incubated for 48 h in the presence or absence of compound **4** at 10 μM concentration. The media containing HIV-1 virions was collected and centrifuged for 10 min at 3050 ×*g*. Thereafter, 25 mL 50% PEG6000 and 1.87 mL 5 M NaCl were added to the 100 mL supernatant and incubated for 24 h. The virions were precipitated using centrifugation for 10 min at 5500 ×*g* at 4 °C and the viral RNA was separated using the TRIzol kit (Gibco, ThermoFisher Scientific, Waltham, MA).

To obtain mRNAs, the ACH-2 cells treated as described above were collected; total polyadenylated RNA was obtained using the Dynabeads mRNA DIRECT Micro Kit (ThermoFisher).

mRNA and Viral RNA Enzymatic Digestion. Viral RNA (250 ng) or cellular mRNA was digested by nuclease P1 (2 U, Fujifilm Wako Pure Chemical Corp., Osaka, Japan) in 25 μL of buffer containing 25 mM NaCl and 2.5 mM $ZnCl_2$ at 37 °C for 2 h followed by the addition of NH_4HCO_3 (1 M, 3 μL) and alkaline phosphatase (0.5 U). After an additional incubation at 37 °C for 2 h, the sample was dissolved in 50 μL of Milli-Q water and filtered (0.20 μm pore size, 10 mm diameter, Merck Millipore, Burlington, MA).[50]

LC/MS Detection of m^{6}A. Analysis of nucleosides was performed with Agilent 1290 UHPLC and Agilent 6460 Triple Quadrupole MS (both from Agilent Technologies Inc., Santa Clara, CA). The injected sample volume was 5 μL. Chromatographic separation of adenosine and N6-methylated adenosine was done with a reversed phase column (2.1 × 100 mm, 1.7 μm Waters). UHPLC eluents were A, 10 mM ammonium formate at pH 5 and B, methanol. Gradient elution was from 5 to 25% B in 5 min followed by 4 min at 5% MeOH, total flow being 400 μL/min. Retention times of monitored adenosine nucleosides were 3.6 and 5.6 min, respectively. A mass spectrometer was set to the positive electrospray ionization mode with the daughter ion analysis mode (MS/MS) (Ade 268 → 136 *m/z* and 6mAde 282 → 150 *m/z*) using collision energy 7 and 21, respectively. Ion optimization was done using automatic tuning with source capillary temperature at 400, and 250 °C was used as transfer line temperature. A mixture of nitrogen and air was used as electrospray ionization gases, and argon was used as collision gas. Quantification of sample analysis was done with the instrument's quantitation program for adenosine at 1−10,000 nM and for N6-methylated adenosine at 0.5−3000 μM concentration ranges.

Quantification and Statistical Analysis. Statistical significances of differences observed in virus production and m^{6}A measurement experiments were assessed using one-way ANOVA and unpaired Student's *t*-test with Excel software (Microsoft Corp., Redmond, WA). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$. Results were considered statistically significant at p values lower than 0.05.

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Author Contributions

M.K. and A.M. designed and directed the study. S.S. and E.Ž. carried out the experimental work with virus. S.S. and K.H. carried out the LC/MS measurements, and N.S. provided funding acquisition. All authors analyzed the data and discussed the results. S.S., M.K., A.M., and E.K. prepared the manuscript.

Notes

The authors declare no competing financial interest.

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