

## Supplemental Digital Content 1

### Supplemental Material and Methods

**Cell culture.** The TH4-7-5 cell line (established in author's laboratory [1]) is a clonal astrocytoma cell line persistently infected with HIV-1 IIIB that contains a single integrated proviral DNA copy [2]. HEK293T (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures; DSMZ No. ACC 635) and TH4-7-5 were cultured in DMEM (Gibco) with 10% FCS and 1% Antibiotic Antimycotic solution (Sigma Aldrich). J-Lat 8.4 is a clonal cell line of Jurkat T cells latently infected with the molecular HIV-1 clone HIV-R7/E-/GFP, which is *env*-defective and contains a GFP open reading frame instead of *nef* [3]. J-Lat 8.4 were obtained from the NIH AIDS reagents program (Cat. Nr. 9847) and cultivated in RPMI (Gibco) with 10% FCS and 1% PenStrep (Sigma-Aldrich). The HIV-1 indicator cell line LC5-RIC was used to determine levels of infectious virus particles in culture supernatants [4]. LC5-RIC cells were cultured in DMEM containing GlutaMAX-1 (Gibco) supplemented with 10% FCS and 1% Antibiotic-Antimycotic solution and supplemented with 0.74 mg/ml Geneticin (G418 sulfate; PAA Laboratories) and 0.125 mg/ml hygromycin B (PAA Laboratories) to maintain selection pressure for transgenes [4]. All cells were cultivated at 37 °C and 5% CO<sub>2</sub>.

**Plasmids used for lentiviral production.** The following plasmids with *env*-defective HIV-1 proviruses were used to produce lentivirus particles: pSG3.1Δ*env* (NIH AIDS reagents program, Cat. Nr. 11051, [5]), which contains a 4 nucleotide insertion and a stop codon within *env* and pNL4-3-deltaE-EGFP (NIH AIDS reagents program, Cat. Nr. 11100,[6]), in which the coding sequence for an enhanced variant of the green fluorescence protein (eGFP) replaces a portion of the *env* gene. pMDG2 expresses the surface protein of the vesicular stomatitis virus (addgene, 12259).

**Intracellular FACS staining to measure GFAP expression.** For analysis of intracellular levels of the glial fibrillary acidic protein (GFAP), fixed cells were washed with PBS, permeabilized with 0.5% saponin in PBS for 10 minutes at room temperature, washed again and incubated in FACS buffer (1% FCS in PBS) for 20 minutes. Cells were then incubated with primary anti-GFAP antibody (mouse

mAB anti-GFAP, clone GA5, NEB) for 1 hour and secondary labeled antibody (anti-mouse Alexa 488, Invitrogen) in FACS buffer. After final washing, cells were resuspended in PBS and 50000 cells analysed by flow cytometry.

**Reverse transcription.** For generation of cDNA, RNA was isolated from cells with the RNeasy Kit (QIAGEN, Hilden) according to the manufacturer's protocol. DNA digestion was performed as recommended on column with the DNase Set (QIAGEN, Hilden). 0,5 – 1 µg RNA was reverse transcribed using random hexamers with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Residual RNA from reverse transcription was eliminated by digestion with RNase H (NEB) for 20 min at 37 °C.

**qPCR.** For absolute quantification of viral DNA copies, genomic DNA of cells was isolated with the DNA QIAamp DNA Mini Kit (QIAGEN), according to the manufacturer's protocol. The primer pair BK-1 F 5'-GTAATACCCATGTTTTCAGCATTATC-3' and BK-1 R 5'-TCTGGCCTGGTGCAA TAGG-3' was used to amplify a *gag* segment [7]. As a reference gene,  $\beta$ -globin was amplified ( $\beta$ -globin-for 5'-ACACAAGTGTGTTCACTAGC-3' and  $\beta$ -globin-rev 5'-CAACTTCATCCACGTT CACC-3'). A standard curve was generated by qPCR analysis of serial dilutions of genomic DNA from TH4-7-5 cells, which contain a single integrated DNA copy [2]. 30 ng genomic TH4-7-5 DNA was set to 10000 genomes and 5000 virus equivalents/cells (1 diploid cell contains ~ 6 pg DNA). For relative quantification of total HIV-1 transcripts, qPCR was performed with cDNA generated by reverse transcription, using primers that bind to *nef* and amplify all HIV-1 transcripts (forward primer 5'-GTGTGTGGTAGATCCACAGATCAAGG-3' and reverse primer 5'-CCAGTCACACCTCAGGT ACCTTTAAGACC-3' [8]. Sequences of the house keeping gene *RNA polymerase II* (RPII) were amplified in parallel using the primers RPIIs 5'-GCACCACGTCCAATGACA-3' and RPIIs 5'-GTGCGGCTGCTTCCATAA-3' [9]. A standard LightCycler protocol was used and data was analysed with the second derivative maximum method. Expression levels were calculated with the  $2^{-\Delta\Delta CT}$  method as described by Livak and Schmittgen [10], with  $\Delta C_T$ -values representing  $C_T(target\ gene) - C_T(house\ keeping\ gene)$ .

**Immunofluorescent staining and confocal microscopy.** Cells of interest were washed and fixed with 2% PFA for 30 min at RT or at 4 °C overnight. After washing with PBS, cells were quenched with 50 mM NH<sub>4</sub>Cl/20 mM Glycine, permeabilized with 0.1% Triton X and unspecific binding sites were blocked with 5% BSA in PBS. Each step was done for 10 min at room temperature, followed by several washing steps with PBS. For analysis of the nuclear translocation of p65, stainings with primary (rabbit anti-p65, ab7970, Abcam) and secondary antibody (anti-rabbit Cy3, Dianova) were performed in 5% BSA in PBS for 1 hour and 30 minutes respectively and were completed with extensive washing steps with PBS. After staining of nuclei with DAPI for 10 min at room temperature, cells were washed again and mounted on glass slides with Mowiol (Roth, Germany). Samples were dried for 24 hours at room temperature in the dark and then stored at -20 °C until analysis by fluorescence microscopy (Nikon TiE equipped with Perkin Elmer UltraView Vox System) was performed. GFP was excited with a 488 nm laser, Cy3 with a 561 nm laser and DAPI with a 405 nm laser. For imaging GFP expression, the 20x objective, numerical aperture 0,75 was used with air; imaging of Cy3 expression was performed with the 60x objective, numerical aperture 1,49 and oil. Calculations of Pearson`s correlation for at least 200 cells was done using Volocity 6.2.1-software (Perkin Elmer).

**Chromatin Immunoprecipitation (ChIP).** For CHIP assays chromatin was prepared from  $1 \times 10^7$  cells and divided into 3 samples used for precipitation with either 2 µg of rabbit anti-p65 antibody (ab7970, Abcam) or with normal rabbit IgG (Cat. Nr.: 12-370, Millipore) as control. The third sample was used to quantify input material. Quantification of precipitated DNA was performed by standard qPCR protocol with the LightCycler 480 (Roche), using following primers: gapdh-for 5'-TACTAGCGGTTTTACGGGCG-3', gapdh-rev 5'-TCGAACAGGAGGAGCAGAGAGCGA-3', LTRκB-for 5'-AGGTTTGACAGCCGCCTA-3' and LTRκB-rev 5'-AGAGACCCAGTACAGGC AAAA-3'. DNA levels corresponding to each of the 2 DNA regions of interest (NF-κB binding sites of the HIV-1 LTR and GAPDH locus) were determined in each precipitate and the proportion of the precipitated DNA relative to the DNA in the input material calculated. Values for specifically

precipitated DNA were determined by normalizing the proportions of the DNA precipitated with specific antibodies (i.e. anti-p65) to the proportions of the DNA in the precipitate of the unspecific IgG control antibody. The results are given as the ratio between the values for the specifically precipitated LTR and *gapdh* DNAs, multiplied by 100 (i.e. % *gapdh*).

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## Supplemental Digital Content 2

Table S1 - Compounds evaluated for HIV-1 activation in latently infected HNSC populations

Compound	Conc. (this study)	Ref.	Reference cells	Reference concentrations
SAHA	5µM	[1, 2]	Latent SupT1 with reporter provirus (24STNLESG cells) ACH2, U1, J89, J-Lat, JdeltaK, TZMbl	5 µM 0.1 – 10 µM
TNF-alpha	10 ng/ml	[3]	Diverse J-Lat clones; other latent T-cell models	10 ng/ml
Sodium Butyrate (NaB)	500 µM	[2, 4]	J89GFP U1, ACH-2, J-Lat	1 mM 2.5 µM - 5 mM
Valproic acid (VPA)	1 mM	[2, 4, 5]	CEM-SS; PBMC J89GFP ACH2, U1, J89	0.25 – 1 mM 1 mM 0.1 – 5 mM
Prostatin	1 µM	[3, 6]	Various J-Lat clones J-Lat	1 µM 1.1 – 10 µM
Phorbol myristat acetate (PMA)	50 ng/ml	[7]	Various J-Lat clones	50 ng/ml
Trichostatin A (TSA)	100 nM	[2, 4, 8]	J89GFP J-LAT8.4 A7, J89, U1, J-Lat	50 nM 500 nM 1 nM – 2 µM
Aza deoxycytidine (5-Aza)	1 µM	[9]	Latently infected Jurkats	1 µM

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Table S2 - NF- $\kappa$ B inhibitors used to inhibit HIV-1 reactivation

Compound	Conc. (this study)	Mode of action	Reference cells/concentrations	Beneficial effects in animal models for the CNS	Therapeutic relevance
<b>BMS-345541</b>	7.5 $\mu$ M	Allosteric inhibitor of IKK [1]	THP-1; EC50 1-5 $\mu$ M for inhibition of cytokine production and inhibition of TNF- $\alpha$ stimulated phosphorylation of I $\kappa$ B- $\alpha$	Ameliorates ischemic brain damage in mice [2]	Anti-cancer agent [3]
<b>Bortezomib</b>	1 nM	Proteasomal inhibitor, blocks degradation of I $\kappa$ B- $\alpha$ [4]	Multiple myeloma cell lines (5 nM) [5]; PAM-LY2 (metastatic variant of transformed keratinocytes) (10 nM) [6]	Inhibits autoimmune encephalomyelitis in mice (model for MS) [7]	Approved for treatment of multiple myeloma (Velcade; [8])
<b>Pyrrolidine dithiocarbamate (PDTC)</b>	50 $\mu$ M	Inhibits I $\kappa$ B-ubiquitin ligase, prevents degradation of I $\kappa$ B- $\alpha$ [9]	L929 cells (mouse fibroblasts)/ 200 $\mu$ M [9]; Inhibition of expression of influenza virus RNAs in A549 cells (lung cancer cells) 50 $\mu$ M [10]	Reduced inflammation and oxidative stress in rats in a hypoxemia-ischemia model, improving neurological and cognitive functions [11]	n.e.
<b>Withaferin A (WTA)</b>	2 $\mu$ M	Reviewed in Vanden <i>et al.</i> [12]; proteasomal inhibitor [13]; negatively affects activity of the IKK complex [14, 15]; prevents TNF- $\alpha$ expression [16]	Human PBMC 0.2-1 mg/ml [17]; MDA-MB231 (human breast cancer cells); Inhibition of IL-6 transcription 2.8 $\mu$ g/ml (5.9 $\mu$ Mol) [16]; L929sA cells (murine fibroblasts stably transfected with IL-6 reporter plasmid) IC <sub>50</sub> = 250 nM [15]	Reduces ALS symptoms in ALS-mouse model (TDP-43 transgenic) [18]; anti-brain tumor activity in a glioma xenograft model [19]	n.e.

n.e. not established

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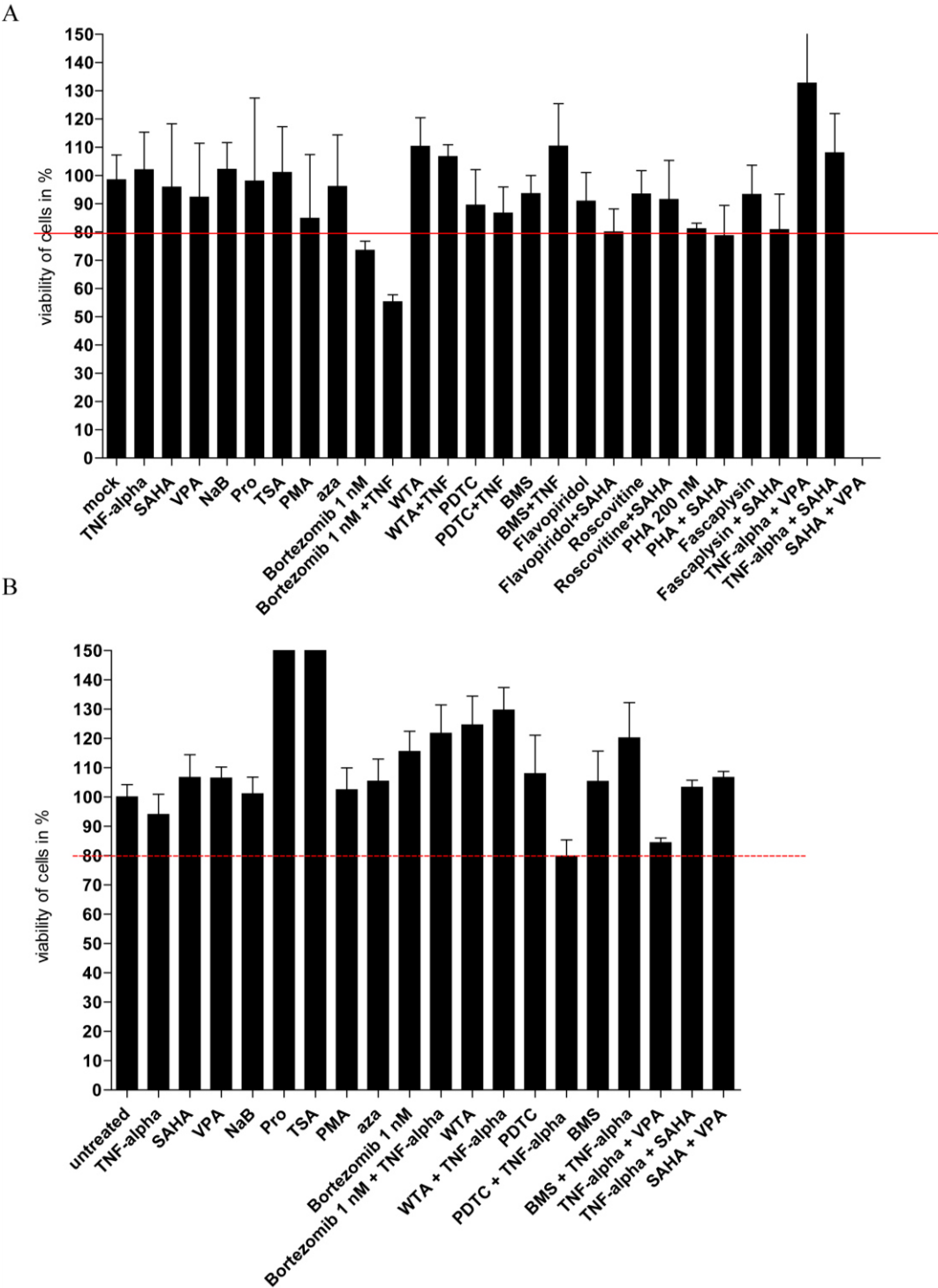
Table S3 - Cdk inhibitors used to inhibit HIV-1 reactivation

Compound	Conc. (this study)	Target Cdks	Reference cells/concentrations	Therapeutic relevance	Comments
<b>R-Roscovitrine (Seliciclib; Cyc202)</b>	10 $\mu$ M	Cdk 1, 2, 5, 7, 8, 9 [1, 2]	HIV-1 infected T-cell (OM10.1) and monocytic (U1) cell lines activated with TNF-alpha; IC50 $\leq$ 2 $\mu$ M; acute infection of PBMC, inhibition at 10 $\mu$ M [3]	Phase II clinical trials with patients with solid tumors [4]	Roscovitrine treatment (intracerebroventricular injection) has beneficial effects in mouse models for traumatic brain injury [5]
<b>Flavopiridol</b>	50 nM	Cdk 1, 2, 4, 6, 7, 9 [2]	IC50 in acutely infected PBMC $\leq$ 40nM; MDM ( $\leq$ 60 nM); HeLa cells 9.5.nM; RT activity in culture supernatant [6]; Sx-22-1 cells (HeLa with HIV-LTR- $\beta$ gal) and Jurkat T-cells (RT activity) < 25 nM [7]	Phase II (various hematological malignancies) [4]	Low therapeutic index because of cytotoxicity
<b>PHA767491</b>	200 nM	Cdk 7,9	IC50 in various human myeloma cell lines; myeloma primary samples: 1 – 3.5 $\mu$ M [8]	Experimental drug multiple myeloma	
<b>Fascaplysin</b>	350 nM	Cdk 4	IC50 0.35 $\mu$ M; inhibition of CDK4 activity [9]; IC50 $\leq$ 1.3 $\mu$ M for small lung cancer cell lines [10]	Experimental drug	

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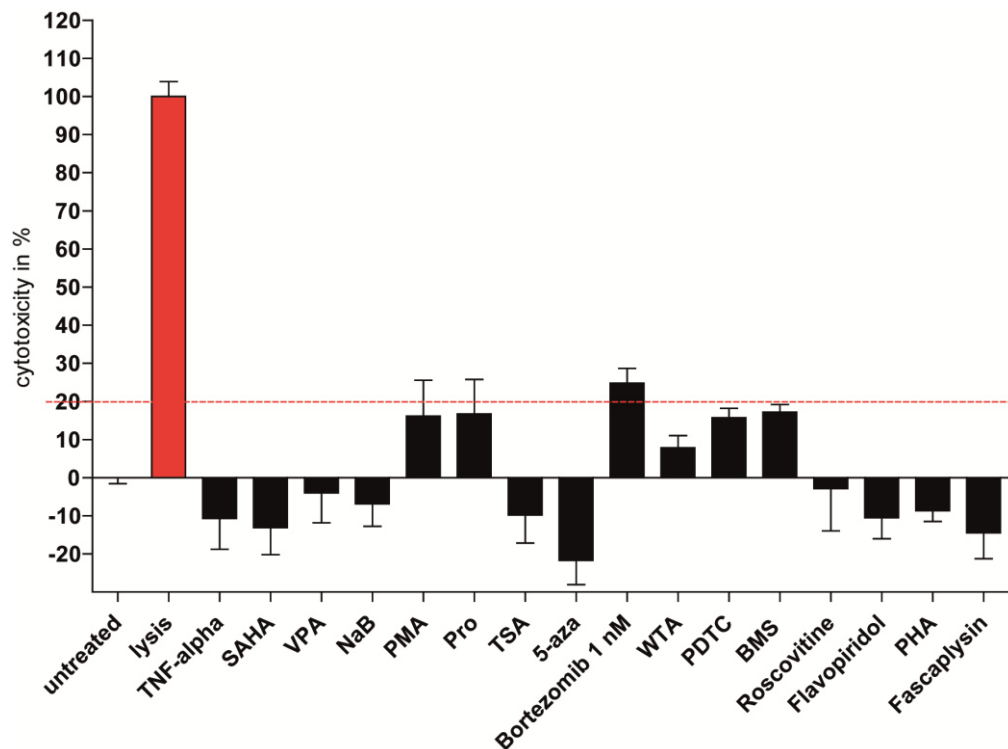


Figure S1



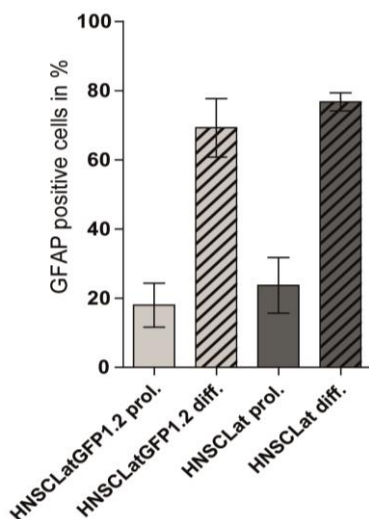
**Figure S1. Cell viabilities of proliferating and differentiated HNSCLatGFP1.2 after treatment with various compounds.** Proliferating (A) and differentiated (B) HNSCLatGFP1.2 were treated with compounds at concentrations indicated in Tables S1, S2 and S3 of SDC2 for 30 hours. Cell viability was assessed by MTT assays as described in Material and Methods.

**Figure S2**



**Figure S2. Treatment of J-Lat 8.4.** J-Lat8.4 were treated with compounds at the same concentrations used for treatment of latently infected HNSC for 30 hours. Cell viability was assessed by CellTox™ Green Cytotoxicity Assay as described in Material and Methods.

**Figure S3**



**Figure S3. Differentiation increases expression of the astrocyte marker GFAP in latently infected HNSCs.** Expression of GFAP in progenitor and differentiated HNSCLatGFP1.2 and HNSCLat was determined by intracellular immunofluorescent staining with antibodies against GFAP and flow cytometry analysis as described in Material and Methods.

