**Procyanidin Trimer C1 Derived from *Theobroma cacao* Reactivates Latent Human
Immunodeficiency Virus Type 1 Provirus**

Takanori Hori¹, Jacob Barnor², Tung Nguyen Huu³, Osamu Morinaga³, Akiko Hamano¹,
Jerry Ndzinu^{1,2}, Angela Frimpong², Keren Minta-Asare², Mildred Amoa-Bosompem²,
James Brandful², John Odoom², Joseph Bonney², Isaac Tuffour², Baffour-Awuah
Owusu², Mark Ofosuhene², Philip Atchoglo², Maxwell Sakyiamah⁴, Richard Adegle⁴,
Regina Appiah-Opong², William Ampofo², Kwadwo Koram¹, Alexander Nyarko², Laud
Okine⁴, Dominic Edoh⁴, Alfred Appiah⁴, Takuhiro Uto³, Yoshiyuki Yoshinaka¹, Shin
Uota¹, Yukihiro Shoyama³, and Shoji Yamaoka¹

1 Tokyo Medical and Dental University, 2 Noguchi Memorial Institute for Medical
Research, 3 Nagasaki International University and 4 Centre for Plant Medicine
Research

T.H. and J.B. contributed equally to this work.

Corresponding author: Shoji Yamaoka

Address: Department of Molecular Virology, Tokyo Medical and Dental University,
Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8519, Japan

Tel.: +81 3-5803-5181, e-mail: shojmmb@tmd.ac.jp

Abstract

Despite remarkable advances in combination antiretroviral therapy (cART), human immunodeficiency virus type 1 (HIV-1) infection remains incurable due to the incomplete elimination of the replication-competent virus, which persists in latent reservoirs. Strategies for targeting HIV reservoirs for eradication that involves reactivation of latent proviruses while protecting uninfected cells by cART are urgently needed for cure of HIV infection. We screened medicinal plant extracts for compounds that could reactivate the latent HIV-1 provirus and identified a procyanidin trimer C1 derived from *Theobroma cacao* as a potent activator of the provirus in human T cells latently infected with HIV-1. This reactivation largely depends on the NF- κ B and MAPK signaling pathways because either overexpression of a super-repressor form of I κ B α or pretreatment with a MEK inhibitor U0126 diminished provirus reactivation by C1. A pan-PKC inhibitor significantly blocked the phorbol ester-induced but not the C1-induced HIV-1 reactivation. Although C1-induced viral gene expression persisted for as long as 48 h post-stimulation, NF- κ B-dependent transcription peaked at 12 h post-stimulation and then quickly declined, suggesting Tat-mediated self-sustainment of HIV-1 expression. These results suggest that procyanidin C1 trimer is a potential compound for reactivation of latent HIV-1 reservoirs.

Key words: HIV, latency, reactivation, procyanidin

Introduction

Combination antiretroviral therapy (cART) can successfully control the viral load of human immunodeficiency virus type 1 (HIV-1) in most HIV-1 infected patients and has led to a significant decrease in the number of deaths related to acquired immunodeficiency syndrome (AIDS) [1]. Although HIV-1 infection is now managed as a chronic disease, persistence of HIV-1 in reservoirs, such as in resting memory CD4⁺ T cells, is a major obstacle for HIV eradication [2]. Poor adherence to cART often permits the emergence of mutant virus from reservoirs; therefore, new approaches to purge latent reservoirs and to discontinue cART (a functional cure) are urgently needed. Such an approach is expected to activate the latent provirus to promote death of latently infected cells through viral cytopathic effects or killing by host cytotoxic T lymphocytes (CTLs).

To reactivate HIV-1, a wide variety of approaches have been proposed that employ histone deacetylase inhibitors (HDACIs), methylation inhibitors, NF- κ B activators, protein kinase C (PKC) modulators, Akt/Hexim-1 modulators, and BET bromodomain inhibitors [3]. Clinical trials with HDACIs are underway, e.g., vorinostat (SAHA), panobinostat (LBH589), and romidepsin (FK288) [4]. PKC agonists that activate latent proviruses through NF- κ B activation and are considered promising for killing of HIV-1

reservoirs include prostratin and bryostatin, which have non-carcinogenic properties and are capable of inducing HIV-1 transcription. A clinical trial is in progress to determine whether bryostatin reduces latent HIV-1 reservoirs. Plants are good source of structurally diverse compounds and can therefore also serve as a potential source of novel therapeutic agents. Here we describe a new class of a plant-derived molecule that activates the latent HIV-1 provirus through the ERK and NF- κ B pathways in human T cells independently of PKC.

Material and Methods

Cells

Jurkat-derived cell lines and ACH-2 cells were maintained in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. HEK293T cells were propagated in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin.

Reagents

Tumor necrosis factor α (TNF- α) was purchased from Repro Tech (Rocky Hill, NJ).

Trichostatin A (TSA) was purchased from Sigma Aldrich (St. Louis, MO), and a mitogen-activated protein kinase (MEK) inhibitor U0126 was purchased from Promega (Madison, WI). Pan-PKC inhibitor Gö6983 was purchased from Calbiochem (San Diego, CA), and phorbol myristate acetate (PMA) was purchased from Enzo Life Sciences (Farmingdale, NY). Cells were incubated with inhibitors for 1 h prior to activation. TNF- α was resuspended in the RPMI medium, and other compounds were resuspended in DMSO and stored as recommended by manufacturers.

Plasmids

The lentiviral vectors capable of expressing an NF- κ B-driven firefly luciferase, CS- κ B-R2.2 [5], and an EF1 α -promoter-driven *Renilla* luciferase, pCS-EF-1 α -Rluc-puro (pCERp) [6] were described previously. pNL4-3luc [7], the pCMV- Δ R8.2 packaging construct, and a vector capable of expressing the vesicular stomatitis virus G protein (VSV-G), pHCMV-VSV-G, were kind gifts from Dr. I. S. Y. Chen (UCLA, Los Angeles, CA, USA). For construction of the pCSII-EF-SR-IB vector, the entire coding sequence of SR-I κ B α was amplified by PCR from pMRX-SR-I κ B α -GFP [8] using the following primer set: Forward, 5'-CACCATGTTCCAGGCGGCCGA-3' and reverse,

5'-TCATAACGTCAGACGCTGGCCT-3'. The PCR product was cloned into the entry vector pENTRTM/D-TOPO[®] (Invitrogen, Carlsbad, CA). The SR-IκBα gene was transferred by recombination with the LR clonase reaction (Invitrogen, Carlsbad, CA) into the lentiviral destination vector pCSII-EF-IB-RfA [9]. The pCSII-EF-MCS-IB plasmid was used as a control vector.

Preparation of virus stocks

The production of VSV-G-pseudotyped NL4-3luc and other lentiviral vectors was described previously ([10],[5] and [6]). The virus supernatant was harvested at 48 and 60 h post-transfection and was filtered through a 0.45 μm-pore syringe filter.

Generation of the JLR2 cell line and its derivatives

To generate reporter T cells latently infected with HIV-1, we infected Jurkat cells with the VSV-G-pseudotyped NL4-3luc virus and cultured cells until surviving cells proliferated sufficiently. Single-cell clones were isolated by limiting dilution, and those capable of inducing expression of the HIV-1 provirus in response to PMA were selected. One such cell clone was transduced with the pCERp lentiviral vector, and JLR2 cells were established. To establish JLR2 cells expressing SR (JLR2 SR), JLR2 cells were

infected with the VSV-G-pseudotyped pCSII-EF-SR-IB lentiviral vector and selected with 4 µg/mL of blasticidin S. Control cells (JLR2 EV) were established in a similar manner by transduction with the CSII-EF-MCS-IB lentiviral vector. To establish JKR cells, Jurkat cells were infected with VSV-G-pseudotyped CS-κB-R2.2 and pCERP lentiviral vectors as described previously [6].

Extraction and isolation of the trimer procyanidin C1

Air-and-shade-dried cocoa beans (700 g) were milled and then extracted with 50% aqueous EtOH (2.0 L, four times) under sonication at 40°C. The combined extracts were concentrated under reduced atmospheric pressure yielding a crude residue (42.1 g), which was defatted by partition between H₂O and CHCl₃ (500 mL, three times). The aqueous fat-free cocoa extract (500 mL) was subjected to a Diaion HP-20 column after washing with approximately 10 L of H₂O. The column was then eluted by MeOH (2.0 L) followed by evaporation and lyophilization, which yielded a deep brown powder of total polyphenols (15.6 g). This powder (10.0 g) was dissolved in MeOH (20 mL) and then applied to a 40 × 500 mm column containing the Toyopearl HW-40F gel and eluted with the same solvent to give 16 fractions (CC-1 through CC-16). Fraction CC-6 was rich in trimers according to liquid chromatography with mass spectrometry (LC-MS)

analysis and was also found to exhibit marked reactivation of the latent HIV-1 provirus. Therefore, fraction CC-6 was subjected to further chromatography on a reversed-phase C₁₈ column (YMC C₁₈ ODS-A gel, 50 μ m, 25 $\times$ 400 mm) with MeOH:H₂O (3:5, v/v) as the mobile phase followed by a Sephadex LH-20 column (20 $\times$ 450 mm) with MeOH as an eluent. The last procedure yielded 85 mg of trimeric procyanidin C1 (>95% purity by HPLC). Nuclear magnetic resonance spectra and high-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESI-TOF MS) experiments were performed to identify procyanidin C1 using JEOL ECX 400 NMR and JEOL Accu TOFTM LC 1100 mass spectrometers (JEOL, Tokyo, Japan).

Epicatechin-(4 β $\rightarrow$ 8)-epicatechin-(4 β $\rightarrow$ 8)-epicatechin (procyanidin C1) had the following characteristics: brown powder; *R_f* 0.42 [C₁₈-TLC, MeOH-H₂O (2:3, v/v)]; ¹³C-NMR (100 MHz, CD₃OD) δ 29.8 [C-4 bottom unit (b)], 37.4 [C-4 middle (m) and top (t) unit], 66.8 (C-3 b), 72.9 (C-3 m), 73.5 (C-3 t), 77.0 (C-2 m and t), 79.7 (C-2 b), 96.2 (C-8 t), 96.6 (C-6 m and t), 97.6 (C-6 b), 100.6 (C-4a b), 102.3 (C-4a m and t), 107.7 (C-8 b and m); B-ring carbons: 115.3 (C-2'), 116.0 (C-5't), 119.1 (C-6'), 132.1 (C-1'), 132.5 (C-1'), 144.3 (C-3'), 144.8 (C-3'), 145.6 (C-4'), and 145.8 (C-4'); A-ring carbons: 154.7, 156.6, 156.8, 157.3, and 157.9; positive HR-ESI-MS *m/z* 867.2154 [M + H]⁺ (calculated for C₄₅H₃₉O₁₈, 867.2136).

Western blotting

Whole-cell lysates were prepared as described previously [10]. Approximately 30 µg of whole-cell lysates were subjected to SDS-PAGE, and then the proteins were transferred to a polyvinylidene difluoride (PVDF) membranes and analyzed by standard immunoblotting procedures using the following antibodies: anti-phosphorylated-ERK1/2 (anti-p-ERK1/2) and anti-ERK1/2 (#9101 and #9102, respectively, Cell Signaling Technology, Inc., Beverly, MA), anti-IκBα (C-21, sc-371, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-HIV1 p24 [39/5.4A] anti-GAPDH (ab9071 and ab8245, respectively, Abcam, Cambridge, England). The membranes were then incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibody (#7074S, Cell Signaling Technology, Inc., Beverly, MA or A206PS, American Qualex Antibodies, Inc., San Clemente, CA, respectively), and proteins were visualized using the Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore Corporation, Billerica, MA) or enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL).

The dual luciferase assay and an enzyme-linked immunosorbent assay (ELISA)

Approximately 2.5×10^5 cells were stimulated, and then firefly and *Renilla* luciferase activities were measured using the GloMAX multidetection system (Promega Corp, Madison, WI) according to the manufacturer's instructions. The firefly luciferase activity was normalized to *Renilla* luciferase activity. The amount of the Gag protein in cell lysates was quantitated by an HIV-1 CA (p24) ELISA kit (ZeptMetrix Corporation, Buffalo, NY).

Results

Screening of herbal extracts

One hundred and thirteen extracts were prepared using 50% ethanol from 86 selected medicinal plants found in Ghana. The extracts were used in the range of 3.125–100.000 µg/mL for screening. To screen the selected Ghanaian medicinal plants for compounds that could activate expression of the latent HIV-1 provirus, we established a subline of the Jurkat T-cell line as a human T-cell latency model. HIV-1 gene expression can be induced in latently infected cells by stimulation with phorbol esters, HDACIs, or cytokines, such as TNF- α . Treatment of JLR2 cells with PMA, TSA, or TNF- α induced the expression of the proviral reporter gene (Supplementary Fig. 1A). JLR2 cells were treated with solutions containing 50 µg/mL of crude plant extracts dissolved in 50%

ethanol. We determined the ratiometric expression of the firefly luciferase relative to the *Renilla* luciferase after 20 h of incubation. Ten of 113 extracts prepared from 86 plants reproducibly enhanced the viral reporter gene activity in JLR2 cells (data not shown). In one of the screened plants, *Theobroma cacao*, activity-guided fractionation by column chromatography procedures identified procyanidin trimer (Tc-1), tetramer (Tc-2), and pentamer (Tc-3) as active compounds (Supplementary Fig. 2A). Stimulation of JLR2 cells with Tc-1 induced expression of the HIV-1-linked luciferase gene in a dose-dependent manner (Supplementary Fig. 2B).

We selected Tc-1 for further experiments because it activated the latent provirus at a lower concentration than did other oligomers. NMR studies revealed that the structure of Tc-1 is identical to a previously reported compound C1[11] (Fig. 1A); thus, Tc-1 was designated as C1 hereafter. Because the firefly luciferase gene was inserted into the position of *Nef* of HIV-1, which is expressed from a fully spliced HIV-1 mRNA, we considered the expression of p24 and Pr55-Gag as another indicator of provirus stimulation from latency. Immunoblotting experiments and ELISA showed that C1 induced the expression of p24 or Pr55-Gag in JLR2 cells (Fig. 1C and Supplementary Fig. 3A). To confirm that the reactivation of the latent HIV-1 provirus with C1 is not a cell type-specific event, similar experiments were performed on another

human T-cell line (ACH-2) that was latently infected with HIV-1 [12]. Immunoblotting experiments and ELISA showed that C1 induced the expression of p24 or Pr55-Gag in ACH-2 cells (Fig. 1D and Supplementary Fig. 3B).

NF- κ B mediates the procyanidin C1-induced reactivation of HIV-1

The long terminal repeat (LTR) of HIV-1 contains binding sites for a variety of transcription factors, including NF- κ B, NF-AT, AP-1, and Sp1 [13]. Because C1 was recently reported to activate NF- κ B through phosphorylation of I κ B α and translocation of RelA [11], we analyzed the role of NF- κ B in the reactivation of the latent HIV-1 provirus by C1. For this purpose, Jurkat cells were transduced with two lentiviral vectors expressing firefly luciferase driven by an NF- κ B-dependent promoter and an EF1 α promoter-driven *Renilla* luciferase, and the resultant reporter cells were designated as JKR cells. JKR and JLR2 cells were stimulated with PMA as a control or with C1 to monitor the kinetics of NF- κ B-driven reporter gene expression or HIV-1 LTR-driven reporter gene expression. In JKR cells, induction of the expression of the NF- κ B-driven luciferase reporter gene was apparent within 3 h after addition of C1 to the culture medium and thereafter increased until it reached a peak 12 h after stimulation (Fig. 2C). This C1-induced NF- κ B-dependent transcriptional activity

quickly decreased to baseline 36 h after stimulation. PMA-stimulated cells showed robust induction of the reporter gene expression, which reached a maximum at 12 h after stimulation and remained high throughout the entire experimental period. In JLR2 cells, the level of NL4-3 luciferase reporter gene expression reached a maximum between 24 h and 36 h after stimulation with C1 and remained high throughout the entire experimental period. The kinetics of PMA-induced reporter gene expression in JLR2 cells were similar to those caused by C1, but the extent of gene induction by PMA was more than 10-fold greater than that caused by C1. These findings provided evidence that procyanidin C1 activated NF- κ B quite transiently but caused persistent activation of HIV-1 gene expression.

Next, to determine how NF- κ B contributed to the production of the latent provirus induced by C1, JLR2 cells were transduced with a lentiviral vector expressing a super-repressor form of I κ B α (SR), which has mutations S32A and S36A rendering the molecule refractory to phosphorylation by I κ B kinases, thereby specifically suppressing NF- κ B activation. In control vector-transduced JLR2 cells (JLR2 EV), PMA and C1 induced the HIV-1 gene expression as expected (Fig. 2A). In contrast, cells expressing SR (JLR2 SR) failed to substantially increase the reporter gene expression in response to either C1 or PMA (Fig. 2A). Immunoblotting analyses

revealed that expression of HIV-1 Pr55-Gag and p24 was barely induced by either C1 or PMA in the presence of SR in JLR2 cells (Fig. 2B). These findings indicated that NF- κ B activation played an important role in the C1-mediated activation of the latent HIV provirus in JLR2 cells.

The MAPK pathway is involved in procyanidin C1-induced provirus activation

C1 was reported to induce phosphorylation of ERK in mouse macrophages [11]. To assess the importance of the MAPK pathway in the C1-induced activation of the latent HIV-1 provirus in JLR2 cells, cells pretreated with the MEK inhibitor U0126 were stimulated with C1 or PMA, and the reporter luciferase activity was measured. The U0126 treatment of JLR2 cells diminished phosphorylation of ERK induced by either C1 or PMA and profoundly suppressed induction of the reporter gene expression (Figs. 3A and B), suggesting that the MEK activation mediated the C1-induced activation of the HIV-1 provirus in JLR2 cells.

A pan-PKC inhibitor Gö6983 does not inhibit C1-mediated provirus activation

We next tested whether PKC activation is involved in the C1-mediated provirus reactivation. Pretreatment of JLR2 cells with a pan-PKC inhibitor Gö6983 potently

inhibited the PMA-induced provirus gene expression in a concentration-dependent manner but did not affect the C1-mediated provirus gene expression at the highest concentration, indicating that C1 reactivated the latent HIV-1 provirus independently of PKC (Fig. 3C). This notion was further supported by the synergistic reactivation of the HIV-1 provirus by lower concentrations of C1 and PMA (Supplementary Fig. 4A). C1 also synergistically worked with TSA in inducing the provirus gene expression (Supplementary Fig. 4B). These results reinforced the notion that reactivation of the HIV-1 provirus by C1 is mechanistically different from reactivation induced by PMA or an HDAC inhibitor.

Discussion

Although various stimuli, including inflammatory cytokines, phorbol esters, HDAC inhibitors, and PI-3 kinase agonists, have been reported to reactivate the latent HIV-1 provirus, we report here for the first time that a procyanidin trimer called C1 triggers production of the latent HIV-1 provirus in human T cells. Our results indicate that C1 activates the NF- κ B and MAPK pathways, both of which perform crucial functions in reactivation of the latent HIV-1 provirus by C1, and that PKC activity is not important for this C1-mediated provirus activation. Procyanidins have long been implicated in the modulation of inflammatory responses, including production of inflammatory mediators such as cytokines and eicosanoids as well as regulation of cellular signal transduction pathways (see the review by Khan et al., 2014) [14]. For instance, Hou et al. showed that proanthocyanidins with the galloyl moiety, but not prodelphinidin without this moiety, inhibit cyclooxygenase 2 expression in lipopolysaccharide (LPS)-activated mouse macrophages by blocking the NF- κ B and MAPK pathways (C1 lacking this moiety was not tested) [15]. Mackenzie et al. reported that B dimeric procyanidin inhibits PMA-induced NF- κ B activation in Jurkat cells [16]. The effects of the C1 trimer reported in this study, which are apparently opposite to those of the B2 dimer in the Jurkat cell line, may result from differences in the oligomerization status of

procyanidins. Terra et al. reported that procyanidins B1 dimer and C1 trimer inhibit activation of I κ B kinase β in LPS-stimulated THP-1 cells pretreated with PMA for induction of differentiation [17]. They also demonstrated inhibition of MAPK activation by these procyanidins. It should be noted that THP-1 cells in that study were pretreated with a high concentration of PMA for 24 h before procyanidin treatment of THP-1 cells. It was reported that PMA induces differentiation of THP-1 cells into macrophage-like cells, where NF- κ B activation is required for survival and differentiation. Thus, it is conceivable that THP-1 cells that were pretreated with PMA already have an elevated level of NF- κ B activity when treated with procyanidin B1 or C1, which may have resulted in a different outcome in their study. Most recently, Sung et al. reported that C1 activates the NF- κ B and MAPK pathways leading to production of inflammatory cytokines in mouse macrophages [11]. Similarly, our results clearly show that C1 reactivates latent HIV-1 proviruses in human T cells via a mechanism dependent on the NF- κ B and MAPK pathways, although the cellular receptor mediating the C1-induced activation of these pathways is yet to be identified.

The transient NF- κ B activation and prolonged induction of viral gene expression by C1 are noteworthy properties of this compound because persistent NF- κ B activation is likely to induce expression of proinflammatory cytokines, an undesirable

but likely effect of provirus activators. As reported by Wolschendorf et al. for the HIV-1-reactivating protein factor secreted by the nonpathogenic bacterium *Massilia timonae* [18], the C1-mediated transient NF- κ B activation is expected to initially produce a small but sufficient amount of viral mRNA encoding Tat, which in turn greatly enhances viral RNA production. This virus-specific regulatory mechanism is believed to sustain a high level of viral gene expression in C1-stimulated cells.

We detected synergistic activation of the HIV-1 provirus after combined treatment of JLR2 cells with procyanidin C1 and a low dose of either TSA or PMA. However, this synergistic reactivation is not surprising because mechanisms of action of these three stimuli are different from one another.

In conclusion, we isolated and characterized procyanidin C1 as a novel activator of the latent HIV-1 provirus. Although it remains to be determined how C1 stimulates the NF- κ B and MAPK signaling pathways, the transient nature of NF- κ B activation and sustained induction of provirus gene expression are desirable properties of a provirus-reactivating agent, dissociation of viral and cellular gene induction.

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References

- [1] P.G. Yeni, S.M. Hammer, M.S. Hirsch, M.S. Saag, M. Schechter, C.C. Carpenter, M.A. Fischl, J.M. Gatell, B.G. Gazzard, D.M. Jacobsen, D.A. Katzenstein, J.S. Montaner, D.D. Richman, R.T. Schooley, M.A. Thompson, S. Vella, P.A. Volberding, Treatment for adult HIV infection: 2004 recommendations of the International AIDS Society-USA Panel, *Jama* 292 (2004) 251-265.
- [2] A. Alexaki, Y. Liu, B. Wigdahl, Cellular reservoirs of HIV-1 and their role in viral persistence, *Curr HIV Res* 6 (2008) 388-400.
- [3] A.D. Badley, A. Sainski, F. Wightman, S.R. Lewin, Altering cell death pathways as an approach to cure HIV infection, *Cell Death Dis* 4 (2013) e718.
- [4] C. Van Lint, S. Bouchat, A. Marcello, HIV-1 transcription and latency: an update, *Retrovirology* 10 (2013) 67.
- [5] Y. Saitoh, V.J. Martinez Bruyn, S. Uota, A. Hasegawa, N. Yamamoto, I. Imoto, J. Inazawa, S. Yamaoka, Overexpression of NF-kappaB inducing kinase underlies constitutive NF-kappaB activation in lung cancer cells, *Lung Cancer* 70 (2010) 263-270.
- [6] S. Uota, M. Zahidunnabi Dewan, Y. Saitoh, S. Muto, A. Itai, A. Utsunomiya, T. Watanabe, N. Yamamoto, S. Yamaoka, An IkappaB kinase 2 inhibitor IMD-0354 suppresses the survival of adult T-cell leukemia cells, *Cancer Sci* 103 (2012) 100-106.
- [7] V. Planelles, F. Bachelierie, J.B. Jowett, A. Haislip, Y. Xie, P. Banooni, T. Masuda, I.S. Chen, Fate of the human immunodeficiency virus type 1 provirus in infected cells: a role for vpr, *J Virol* 69 (1995) 5883-5889.

- [8] N. Hironaka, K. Mochida, N. Mori, M. Maeda, N. Yamamoto, S. Yamaoka, Tax-independent constitutive IkappaB kinase activation in adult T-cell leukemia cells, *Neoplasia* 6 (2004) 266-278.
- [9] M. Higuchi, C. Tsubata, R. Kondo, S. Yoshida, M. Takahashi, M. Oie, Y. Tanaka, R. Mahieux, M. Matsuoka, M. Fujii, Cooperation of NF-kappaB2/p100 activation and the PDZ domain binding motif signal in human T-cell leukemia virus type 1 (HTLV-1) Tax1 but not HTLV-2 Tax2 is crucial for interleukin-2-independent growth transformation of a T-cell line, *J Virol* 81 (2007) 11900-11907.
- [10] T. Hori, H. Takeuchi, H. Saito, R. Sakuma, Y. Inagaki, S. Yamaoka, A carboxy-terminally truncated human CPSF6 lacking residues encoded by exon 6 inhibits HIV-1 cDNA synthesis and promotes capsid disassembly, *J Virol* 87 (2013) 7726-7736.
- [11] N.Y. Sung, M.S. Yang, D.S. Song, E.B. Byun, J.K. Kim, J.H. Park, B.S. Song, J.W. Lee, S.H. Park, H.J. Park, M.W. Byun, E.H. Byun, J.H. Kim, The procyanidin trimer C1 induces macrophage activation via NF-kappaB and MAPK pathways, leading to Th1 polarization in murine splenocytes, *Eur J Pharmacol* 714 (2013) 218-228.
- [12] K.A. Clouse, D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barstad, J. Kovacs, A.S. Fauci, T.M. Folks, Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone, *J Immunol* 142 (1989) 431-438.
- [13] K.A. Jones, B.M. Peterlin, Control of RNA initiation and elongation at the HIV-1 promoter, *Annu Rev Biochem* 63 (1994) 717-743.
- [14] N. Khan, O. Khymenets, M. Urpi-Sarda, S. Tulipani, M. Garcia-Aloy, M. Monagas, X. Mora-Cubillos, R. Llorach, C. Andres-Lacueva, Cocoa polyphenols and inflammatory markers of cardiovascular disease, *Nutrients* 6 (2014) 844-880.
- [15] D.X. Hou, S. Masuzaki, F. Hashimoto, T. Uto, S. Tanigawa, M. Fujii, Y. Sakata, Green tea proanthocyanidins inhibit cyclooxygenase-2 expression in LPS-activated mouse macrophages: molecular mechanisms and structure-activity relationship, *Arch Biochem Biophys* 460 (2007) 67-74.
- [16] G.G. Mackenzie, F. Carrasquedo, J.M. Delfino, C.L. Keen, C.G. Fraga, P.I. Oteiza, Epicatechin, catechin, and dimeric procyanidins inhibit PMA-induced NF-kappaB activation at multiple steps in Jurkat T cells, *Faseb j* 18 (2004) 167-169.
- [17] N. Martinez-Micaelo, N. Gonzalez-Abuin, X. Terra, C. Richart, A. Ardevol, M. Pinent, M. Blay, Omega-3 docosahexaenoic acid and procyanidins inhibit cyclo-oxygenase activity and attenuate NF-kappaB activation through a p105/p50 regulatory mechanism in macrophage inflammation, *Biochem J* 441 (2012) 653-663.

- [18] F. Wolschendorf, A. Duverger, J. Jones, F.H. Wagner, J. Huff, W.H. Benjamin, M.S. Saag, M. Niederweis, O. Kutsch, Hit-and-run stimulation: a novel concept to reactivate latent HIV-1 infection without cytokine gene induction, *J Virol* 84 (2010) 8712-8720.

Figure legends

Fig. 1. Procyanidin C1 purified from *Theobroma cacao* activates the latent HIV-1 provirus. (A) Chemical structure of Tc-1 (C1). (B) JLR2 cells were stimulated with the indicated concentrations of C1 or PMA. Luciferase activity was measured 20 h post-stimulation. The mean luciferase value in DMSO treated control cells was arbitrarily set to 1.0. The fold induction and standard deviations in three independent experiments are shown. (C and D) JLR2 (C) or ACH-2 (D) cells were stimulated with the indicated concentrations of C1 or PMA for 20 h. The Gag protein expression in JLR2 cells was detected by immunoblotting analysis with an anti-p24 or anti-GAPDH antibody.

Fig. 2. Reactivation of the latent HIV-1 provirus by C1 requires NF- κ B activation. (A) JLR2 cells transduced with the vector pCSII-EF-IB (JLR2 EV) or pCS-EF-SR-IB (JLR2 SR) were stimulated with the indicated concentrations of C1 or 4 nM phorbol myristate acetate (PMA). Luciferase activity was measured 20 h post-stimulation. The mean luciferase value in DMSO-treated control cells was arbitrarily set to 1.0. (B) Immunoblotting analysis of JLR2 EV or JLR2 SR cells stimulated with 25 or 50 μ M C1 or 4 nM PMA. Approximately 30 μ g of each whole-cell lysate that was prepared 20 h after the stimulation was subjected to immunoblotting analyses using anti-p24,

anti-I κ B α , or anti-GAPDH antibodies. The data shown are representative of three independent experiments. (C) Kinetics of HIV-1 LTR- or NF- κ B-driven reporter gene expression after stimulation with either C1 or PMA. JLR2 (left) and JKR (right) cells were incubated with DMSO (diamonds), 25 or 50 μ M C1 (crosses and triangles, respectively), or 4 nM PMA (squares). Luciferase activity of each sample was measured at the indicated time points. The mean luciferase activity level at time 0 was arbitrarily set to 1.0. Error bars indicate standard deviations in three independent experiments.

Fig. 3. The MAPK inhibitor U0126 inhibits C1-mediated provirus activation, but the pan-PKC inhibitor Gö6983 does not inhibit. (A) Immunoblotting analysis of JLR2 cells stimulated with the indicated concentrations of C1 or 4 nM PMA in the presence or absence of 10 μ M U0126. The cells were harvested 2 h post-stimulation. Approximately 30 μ g of each whole-cell lysate was subjected to immunoblotting analyses using anti-pERK1/2 (pp44/42 MAPK) (Thr202/Tyr204), or anti-ERK1/2 (p44/42 MAPK) antibodies. (B) Luciferase activity in JLR2 cells stimulated with 25 or 50 μ M C1 or 4 nM PMA in the presence or absence of 10 μ M U0126 was measured 20 h post-stimulation. The mean luciferase value in DMSO-treated control cells was arbitrarily set to 1.0. Error bars indicate standard deviations in three independent

experiments. (C) JLR2 cells were pretreated with the indicated concentrations of the pan-PKC inhibitor Gö6983 1 h before stimulation with 25 or 50 μ M C1 or 4 nM PMA. In each condition, the mean luciferase activity level in DMSO-pretreated control cells was arbitrarily set to 100. The mean luciferase values and standard deviations from three independent experiments are shown.

Fig. 4. Possible model of the C1-mediated reactivation of the latent HIV-1 provirus. C1 activates the ERK/MAPK and NF- κ B pathways in a PKC-independent manner. Both the ERK/MAPK and NF- κ B pathways are necessary for the reactivation of the HIV-1 provirus.

Figure

Figure 1

Figure 2

Figure 3

Figure 4