

Reactivation of HIV latency by a newly modified Ingenol derivative via protein kinase C δ –NF- κ B signaling

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Objective: Although HAART effectively suppresses viral replication, it fails to eradicate latent viral reservoirs. The ‘shock and kill’ strategy involves the activation of HIV from latent reservoirs and targeting them for eradication. Our goal was to develop new approaches for activating HIV from latent reservoirs.

Design: We investigated capacity of Ingenol B (IngB), a newly modified derivative of Ingenol ester that was originally isolated from a Brazilian plant in Amazon, for its capacity and mechanisms of HIV reactivation.

Methods: Reactivation of HIV-1 by IngB was evaluated in J-Lat A1 cell culture model of HIV latency as well as in purified primary CD4⁺ T cells from long-term HAART-treated virologically-suppressed HIV-infected individuals. The underlining molecular mechanisms of viral reactivation were investigated using flow cytometry, RT-qPCR and chromatin immunoprecipitation.

Results: IngB is highly effective in reactivating HIV in J-Lat A1 cells with relatively low cellular toxicity. It is also able to reactivate latent HIV in purified CD4⁺ T cells from HAART-treated HIV-positive individuals *ex vivo*. Our data show that IngB may reactivate HIV expression by both activating protein kinase C (PKC) δ –nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway and directly inducing NF- κ B protein expression. Importantly, IngB has a synergistic effect with JQ1, a BET bromodomain inhibitor, in latent HIV reactivation.

Conclusions: IngB is a new promising compound to activate latent HIV reservoirs. Our data suggest that formulating novel derivatives from Ingenol esters may be an innovative approach to develop new lead compounds to reactivate latent HIV.

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Introduction

With the advent of HAART, significant advances have been made in controlling HIV-1 (HIV) infection and

improving health of HIV-infected patients. However, HAART fails to eradicate latent HIV reservoirs, and interruption of HAART leads to a rapid rebound of viral loads in both peripheral blood and gut-associated

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lymphoid tissue [1–4]. Therefore, new strategies are needed for eliminating latent HIV pool.

Recent studies suggest that quiescent memory CD4⁺ T cells constitute most of the long-lived viral reservoirs [5–9]. Histone modification-mediated chromosomal suppression of HIV long terminal repeat (LTR) was reported to be critical for establishing HIV latency [10–15]. Histone deacetylases (HDACs) and histone methyltransferases are recruited to HIV LTR and are involved in establishing HIV latency [10,11,16–18]. Disruption of HDACs or enhancer of zeste homolog 2 binding to HIV LTR resulted in reactivation of latent HIV-1 *in vivo* as well as *in vitro* [10,16,19–22]. Resting CD4⁺ T cells harbor low levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor of activated T-cells which may support the establishment of HIV latency [23]. Under most circumstances, in resting CD4⁺ T cells, p-TEFb is restricted in a transcriptionally inactive complex with hexamethylene bis-acetamide inducible 1/bromodomain-containing protein 4/7SK small nuclear RNA for establishing viral latency [24–29]. Therefore, compounds that can disrupt binding or inhibit enzyme activity of HIV-1 transcriptional repressors, such as suberanilohydroxamic acid (SAHA), hexamethylene bisacetamide (HMBA) or a BET bromodomain inhibitor JQ1, or activate NF-κB signaling, such as prostratin, have been considered for inducing reactivation of HIV from latency [30]. A recent study reported reactivation of latent HIV-1 with a single dose of SAHA administration in HIV-infected patients on HAART [31]. Although SAHA induced viral reactivation in patients, identification of novel compounds is important to achieve effective reactivation of latent HIV in the future [31,32]. A new group of compounds, Ingenol derivatives, have been shown to regulate HIV expression *in vitro* by either activating or repressing HIV transcription [33–35]. It is interesting to note that Ingenol esters are structurally analogous to phorbol esters, which are known to reactivate latent HIV reservoirs [36,37].

In this study, we found that a newly modified Ingenol ester compound originally isolated from *Euphorbia tinucalli*, Ingenol B (IngB), effectively promoted reactivation of HIV LTR-induced gene expression through activation of protein kinase C (PKC)δ–Serine 664–NF-κB pathway in the HIV latency culture model of J-Lat A1 cell line and in purified CD4⁺ T cells from HIV-infected individuals under long-term HAART. We also found that IngB had minimal cellular toxicity and higher potency for HIV reactivation than SAHA, JQ1, or prostratin *in vitro*. In addition, IngB had synergistic effects on HIV reactivation in combination with JQ1 or HMBA *in vitro*. In summary, formulation of derivatives from Ingenol family may provide a novel opportunity for HIV reactivation from latent viral reservoirs and potentially supporting viral eradication efforts.

Materials and methods

Cell culture

The J-Lat A1 cells (harboring HIV LTR and green fluorescent protein (GFP) gene under the HIV LTR control) or U1 cells (harboring latent HIV genome) were cultured with RPMI1640 medium with 10% fetal bovine serum at 37°C. For reactivation of HIV LTR, cells were treated with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), SAHA (Santa Cruz Biotechnology, Inc.), JQ1 (Biovision Incorporated, USA), IngB (patent submitted from KyoLab), Prostratin (Sigma-Aldrich), HMBA (Sigma-Aldrich) or TNF-α (BD Biosciences, USA) for 24 h. HIV reactivation was measured by GFP flow cytometry and the data were analyzed using FlowJo Software for J-Lat cells or p24 ELISA for U1 cells. Cell viability was evaluated using Live/Dead dye.

Primary CD4⁺ T-cell isolation

Peripheral blood samples were collected from HIV-infected patients ($n = 7$, all men, age ranged from 40 to 66 years) receiving antiretroviral therapy (ART) for more than 5 years. These individuals had CD4⁺ T-cell numbers ranging from 347 to 1403 cells/μl and plasma viral loads were below 50 copies/ml as measured by qPCR. Patient samples were obtained under informed written consent and a protocol approved by the UC Davis institutional review board. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypopaque as previously described [1]. The CD4⁺ T cells were further purified with EasySep kit from STEMCELL Technologies Inc. (Vancouver, British Columbia, Canada). The purified CD4⁺ T cells were plated at a density of 1×10^6 cells and treated with 200 ng/ml PMA and 2 μmol/l ionomycin, 3 nmol/l IngB, 500 nmol/l SAHA, or 2 μmol/l JQ1 for 6 or 48 h.

To measure changes in the cell activation status in CD4⁺ and CD8⁺ T-cell subsets, PBMCs were isolated from healthy donors and 2×10^6 cells were incubated with DMSO, 200 ng/ml PMA and 2 μmol/l ionomycin, 3 nmol/l IngB, 500 nmol/l SAHA, or 2 μmol/l JQ1 for 24 or 72 h, and immune-stained with anti-CD3, anti-CD4, anti-CD8, anti-CD38, anti-CD69, or anti-human leukocyte antigens (HLA)-DR antibodies (Biolegend, San Diego, California, USA) for 20 min at 4°C. Cells were fixed and analyzed by flow cytometry (FlowJo). In addition, PBMCs were treated with similar regimens for 24–72 h and cytokine was analyzed with ELISA (supernatants) or reverse transcription-quantitative PCR (cells) (Biolegend).

Cell viability and proliferation measurements

Cells were placed in 96-well plates and treated with compounds for HIV reactivation. After 24 or 72 h of incubation at 37°C, cell viability was measured using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Roche Laboratories). Cell

proliferation was used as a measure of cell activation and was detected by determining BrdU incorporation in the S-phase of cell replication using ELISA (EMD-Millipore, QIA58).

Immunoblot analysis

One million J-Lat A1 cells or PBMCs from healthy donors were incubated with IngB for 6 h. Whole cell protein extracts were prepared with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich). Expression of the isoforms of PKC protein or NF- κ B/p65 was evaluated using the PKC Isoform Sampler Antibody Kit (Cell Signaling Technology, 9960S) and anti-NF- κ B/p65 (Abcam). The level of phosphorylation of PKC was determined using anti-Phospho-Ser664-PKC (EMD-Millipore).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed as previously described [38,39]. Briefly, 1×10^6 J-Lat A1 cells were fixed in 1% formaldehyde. The chromatin was sonicated into fragments of 200–1500 nucleotides long and subjected to immunoprecipitation. After incubating with 50 μ l of protein A agarose beads, the immunocomplexes were washed, the chromatin was eluted and reverse cross-linked at 65°C overnight. DNA was extracted with Qiagen PCR purification kit. The upstream primer sequence is 5'-AGCTTGCTACAAGGGACTTTCC-3', and the downstream primer sequence is 5'-ACCCAGTACAGG-CAAAAAGCAG-3'. Quantitative real-time PCR was performed using Agilent Brilliant Ultra-Fast SYBR Green QPCR reagent.

HIV RNA quantification

Total RNA was extracted with the Qiagen RNeasy Kit. Ten microliter and 450 ng RNA were assayed for HIV RNA using TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems at Life Technologies), well conserved primers (HXB2 positions 533–543, 626–643) and fluorescent probe (559–584) from LTR region [40]. Reverse transcription and amplification was performed in a ViiA7 Thermocycler. External genomic HIV RNA standards were prepared from NL4–3 virions obtained from viral stocks expanded in tissue culture *in vitro* and validated via the Abbot Real Time assay. HIV RNA copy numbers were normalized to RNA input. Non-reverse transcription controls were performed using TaqMan Fast Advanced Mastermix (Applied Biosystems at Life Technologies), and HIV DNA co-purified on RNeasy columns was quantified with the same external standards. Final values for HIV RNA expression levels were adjusted by subtraction of HIV DNA copy numbers from total reverse transcription (RT)-PCR readouts.

Statistical analysis

Means and standard errors (SEs) were calculated for all data points from at least three independent experiments in

triplicates. Statistical significance was determined using the Student's *t* test, in which *P* values less than 0.05 were considered significant.

Results

Ingenol B induces reactivation of HIV LTR-regulated GFP expression in J-Lat A1 cell model of HIV latency

An Ingenol ester compound was initially extracted from *E. tirucalli* by KYOLAB Laboratories and was found to share the similar core structure with other Ingenol esters (Fig. 1a). The natural Ingenol was modified to produce a new derivative called Ingenol-3-hexanoate (IngB). Ingenol esters are structurally analogous to phorbol esters, which are known to reactivate HIV from latency. However, there is limited information regarding the effects of Ingenol against viral latency. Therefore, we investigated the ability of IngB to reactivate latent HIV. We opted to evaluate the potency of IngB in the J-Lat A1 cells, a well studied HIV latency cell culture model [41,42]. The J-Lat A1 cells harbor GFP gene under the control of HIV-1 LTR and provide an opportunity to measure activity of compounds that can induce GFP expression by re-activating HIV LTR. Treatment with IngB effectively induced GFP expression in about 50% of the J-Lat A1 cells (Fig. 1b). The ability of IngB to reactivate latent HIV in J-Lat A1 cells was evaluated at concentrations ranging from 23 pmol/l to 6 nmol/l. It effectively activated GFP expression through HIV LTR in more than 50% of the cells at concentrations at 375 pmol/l and above, and it was able to activate GFP expression in 20% of the cells even at the 23 pmol/l concentration. Flow cytometric analysis using live/dead dye showed that IngB caused minimal cellular toxicity on J-Lat A1 cells and had minor effects on cell proliferation at 3–24 nmol/l concentrations (Fig. 1c). Our data showed that IngB is an effective activator of HIV from latency without exerting detectable cellular cytotoxicity.

Ingenol B has higher potency of HIV reactivation compared to other known compounds *in vitro*

Eliminating latent reservoir is challenging since it is very stable and is established very early during HIV infection [43–46]. Because of the high potency of IngB for HIV reactivation, we sought to examine the ability of IngB to reactivate latent HIV in comparison to other compounds previously known to reactivate latent HIV from T-cell reservoirs. These included SAHA, tumor necrosis factor (TNF)- α , prostratin, HMBA and a BET bromodomain inhibitor JQ1. SAHA has been used in clinical trials for reactivation of latent HIV in patients on ART [25,31,47–49]. The J-Lat A1 cells were treated with 500 nmol/l SAHA or 375 pmol/l IngB for 24 h. We found that SAHA reactivated about 5.5% of latent cells, whereas IngB reactivated more than 50% of latent J-Lat cells (Fig. 1d).

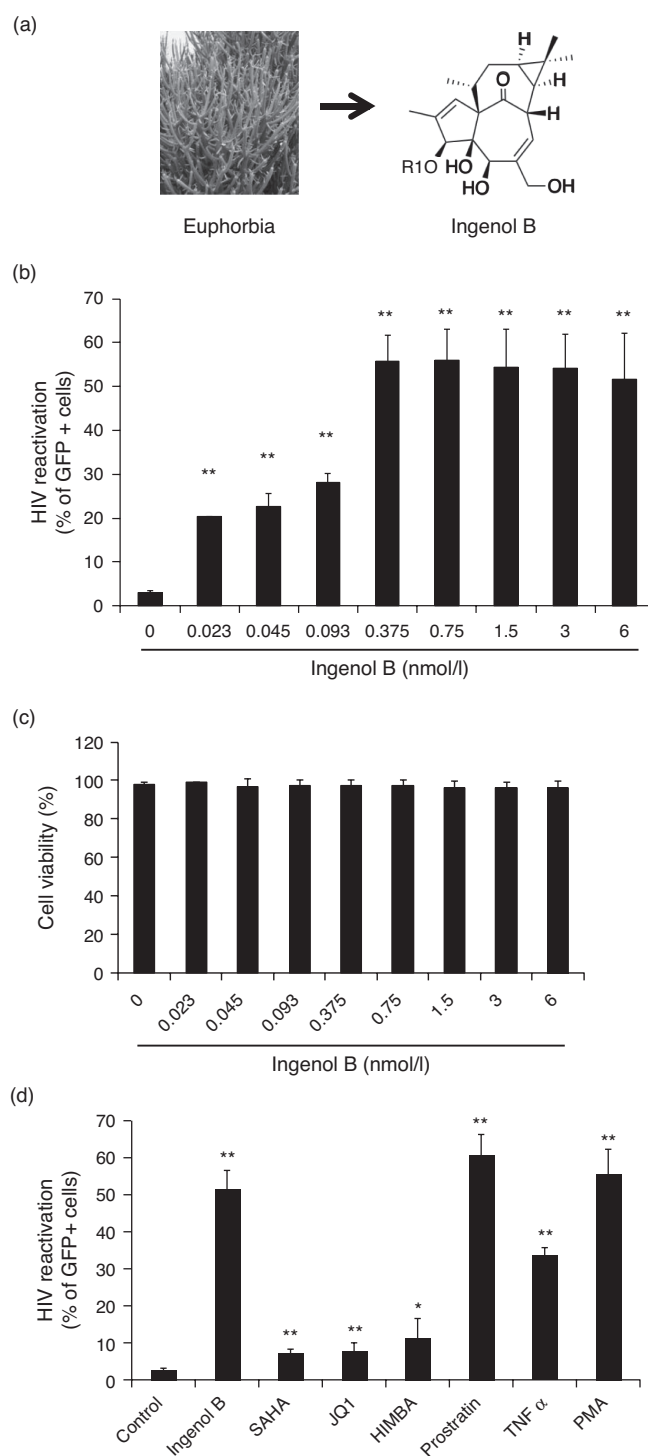


Fig. 1. Ingenuol B is a potent activator of HIV LTR in J-Lat A1 cell line model for HIV latency. (a) Ingenuol B is a modified compound isolated from a Brazilian plant, *Euphorbia tirucalli*. (b) Ingenuol B reactivated latent HIV-1 in J-Lat A1 cells. Increased doses of Ingenuol B, from 23 pmol/l to 6 nmol/l, were added in J-Lat A1 cells for 24 h. Latent HIV-1 reactivation was measured by detecting GFP-expressing cells by flow cytometry. (**) $P < 0.01$. (c) Cell viability during Ingenuol B treatment. Increased doses of Ingenuol B, from 23 pmol/l to 6 nmol/l, were added to J-Lat A1 cells for 24 h and cell viability was measured using live/dead dye by flow cytometry. (d) Ingenuol B has higher potential of HIV LTR reactivation in J-Lat cells compared to SAHA or JQ1. The HIV LTR driven GFP expression in J-Lat A1 cells was determined by flow cytometry at 24 h of treatment with Ingenuol B and other compounds. The dosage for the treatment was: Ingenuol B 375 pmol/l, PMA 5 ng/ml, TNF- α 25 TNF ng/ml, HMBA 5 mmol/l, JQ1 2 μ mol/l, prostratin 10 μ mol/l, SAHA 500 nmol/l. HIV LTR reactivation was analyzed in comparison to untreated J-Lat cell controls. TNF, tumor necrosis factor. (*) $P < 0.05$, (**) $P < 0.01$.

Recently, a bromodomain inhibitor for treatment of male infertility, JQ1, was shown to be a potential candidate for HIV eradication strategy [49–52]. Therefore, we examined the effect of IngB in combination with these previously tested compounds for latent HIV reactivation in J-Lat A1 cells. Treatment with 5 ng/ml PMA served as a positive control in the assay. As shown in Fig. 1d, JQ1 treatment reactivated 7% of latent J-Lat A1 cells and it had potency very similar to that of SAHA [51]. IngB had higher potential of HIV reactivation than SAHA, JQ1, HMBA and TNF- α , except for 10 μ mol/l prostratin. SAHA or JQ1 activated 5–10% of J-Lat A1 cells, whereas IngB activated more than 50% of J-Lat A1 cells. IngB was effective at lower concentrations compared to SAHA or JQ1. Similar results were also seen in U1 cells (data not shown). In summary, our data showed that IngB is a highly potent compound for reactivation of latent HIV in the J-Lat cell line model of HIV latency.

Ingelol B is synergistic with other known compounds for reactivation of latent HIV reservoirs

Because of the high potency of IngB for HIV reactivation, we sought to examine the ability of IngB to reactivate latent HIV synergistically in combination with other compounds previously known to reactivate HIV from latency. Establishment and maintenance of HIV latency are regulated by multiple cellular and molecular pathways. Therefore, it is reasonable to assume that a single drug may not effectively reactivate all the latent HIV reservoirs in patients and may warrant a multimodal approach for HIV eradication as proposed recently [53,54].

Since high concentration of IngB may mask potential synergistic effects of other compounds, we opted to use lower concentration (46 pmol/l) of IngB at which it can reactivate about 20% of latent J-Lat cells. As shown in Fig. 2a, there was no synergistic effect on latent HIV reactivation in presence of prostratin or SAHA. However, IngB was able to synergistically reactivate HIV from latency with the addition of HMBA, TNF- α , or JQ1. Lack of synergistic effect between IngB and prostratin suggested that IngB regulated HIV reactivation may occur through PKC–NF- κ B pathway. As a member of the Ingelol ester family, IngB can modulate PKC signaling and probably may utilize the PKC–NF- κ B signaling in reactivation of HIV from latency [37]. To test this hypothesis, we measured changes in PKC expression by western blot analysis using antibodies specific for four PKC superfamilies (Fig. 2b, left panel). We found that, among the four species of PKCs, expression of PKC μ is barely detected, whereas expression of PKC α and PKC ζ was not altered in J-Lat A1 cells in the presence of IngB. Only PKC δ level was up-regulated, with a peak expression level at 2–4 h following IngB treatment. Further, phosphorylation of PKC δ following IngB treatment was clearly

demonstrated by antiphospho-Ser664-PKC antibody with western blot. PKC δ was phosphorylated at Ser664 as early as 1 h following IngB treatment, peaked at 2 h and returned to baseline at 6 h (Fig. 2b, left panel). Similarly, IngB treatment led to PKC δ phosphorylation in PBMCs from healthy HIV-negative donors (Fig. 2b, right panel). Ingelol was previously reported to up-regulate NF- κ B protein expression [55]. To examine whether IngB modulated NF- κ B protein expression, we performed western blot analysis using anti-NF- κ B/p65 antibody. A rapid increase in the expression of NF- κ B/p65 was seen following the treatment with IngB (30 min–2 h) (Fig. 2c, left panel). Similarly, increased expression of NF- κ B/p65 was detected in the PBMCs from HIV-negative healthy individuals following the IngB treatment (Fig. 2b, right panel). Further, addition of PKC δ inhibitor to J-Lat A1 cells inhibited the magnitude of IngB-induced HIV reactivation from latency (Fig. 2c).

Protein kinase C-induced gene expression is mediated through activation of NF- κ B and its binding to promoter region of the cellular genes [56–58]. To examine whether IngB reactivates HIV latency by promoting PKC δ -induced NF- κ B binding to HIV LTR, ChIP–qPCR assays were performed on J-Lat A1 cells in the presence of 6 nmol/l IngB with or without PKC δ inhibitor. As shown in Fig. 2d, IngB treatment led to an eight-fold increase in NF- κ B/p65 binding to HIV LTR region. This was completely abolished upon the addition of PKC δ -specific inhibitor. Therefore, our findings indicate that IngB regulation of HIV expression is probably through up-regulating PKC δ –NF- κ B/p65 pathway.

We evaluated the effect of IngB on the activation of NF- κ B in primary CD4⁺ T cells purified from HIV-negative healthy donors. We measured changes in the cell surface expression of CD69, a marker of cell activation and proliferation, in PBMCs and isolated CD4⁺ T cells following the treatment with IngB. Since CD69 gene contains three known NF- κ B-binding sites in its promoter region, it can serve as a surrogate marker for in-vivo PKC δ -induced NF- κ B activation [59]. Up-regulation of CD69 expression can occur in primary CD4⁺ T cells stimulated with PKC–NF- κ B activators including PMA and prostratin [60]. As shown in Fig. 2e, in IngB-treated PBMCs, more than 80% of the CD4⁺ or CD8⁺ T cells had cell surface expression of CD69 as compared to untreated controls, with only about 20–30% of CD4⁺ or CD8⁺ T cells expressing CD69. The PMA treatment resulted in induction of CD69 expression in 80% of the CD4⁺ T cells and served as a positive control in the study. These findings suggested that IngB activates PKC δ –NF- κ B signaling not only in J-Lat A1 cell lines but also in primary CD4⁺ T cells. Taken together, our data showed that IngB might reactivate latent HIV through PKC δ –S664–NF- κ B signaling pathway.

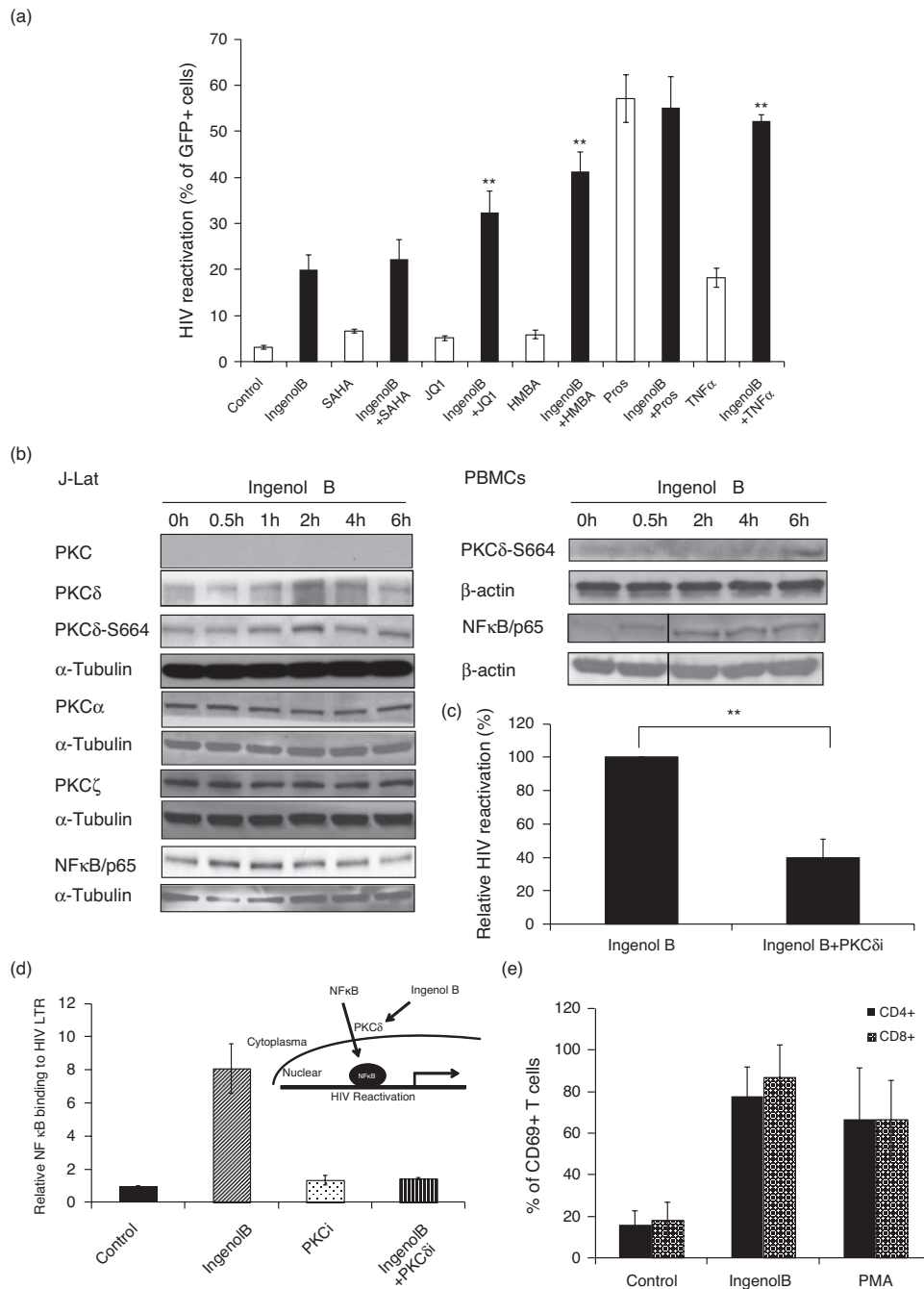


Fig. 2. Ingenuol B reactivates latent HIV LTR-regulated expression through protein kinase C δ -S664-NF- κ B signaling. (a) A single compound or a combination of compounds was added to J-Lat A1 cells for 24 h. HIV-1 LTR-driven GFP expression in the cells was measured by flow cytometry as shown in Fig. 1. Combined treatments included Ingenuol B at 46 pmol/l. (**) $P < 0.01$, compared to single compound. (b) Activation of PKC δ and expression of PKC isoforms in J-Lat cells or human PBMCs from healthy donors. J-Lat A1 cells or human PBMCs were treated with 6 nmol/l Ingenuol B for 6 h. The cells were collected and lysed in RIPA buffer. Western blot analysis was performed to detect expression of isoforms of PKC, expression of NF- κ B/p65, or Ser664 phosphorylation at PKC δ with indicated antibodies. (c) PKC δ inhibitor suppresses Ingenuol B-induced HIV reactivation. J-Lat A1 cells were treated with 3 nmol/l Ingenuol B in the presence of 5 μ mol/l PKC δ inhibitor and examined for GFP expression by flow cytometry. (**) $P < 0.01$. (d) Ingenuol B promotes NF- κ B/p65 binding to HIV LTR through activation of PKC δ . J-Lat A1 cells were treated with 6 nmol/l Ingenuol B with or without 5 μ mol/l PKC δ inhibitor (Millipore). The cells were collected and ChIP-qPCR assay was performed to detect NF- κ B/p65 binding to HIV LTR. Relative NF- κ B/p65 binding to HIV LTR was expressed after normalized to untreated controls. (e) Activation of CD69 expression in PBMC following Ingenuol B treatment. The CD69 expression was analyzed in CD4 $^{+}$ and CD8 $^{+}$ T-cell subsets in PBMC from HIV-negative controls that were treated with PMA or Ingenuol B for 24 h. PMA-treated cells served as the positive control. PKC, protein kinase C.

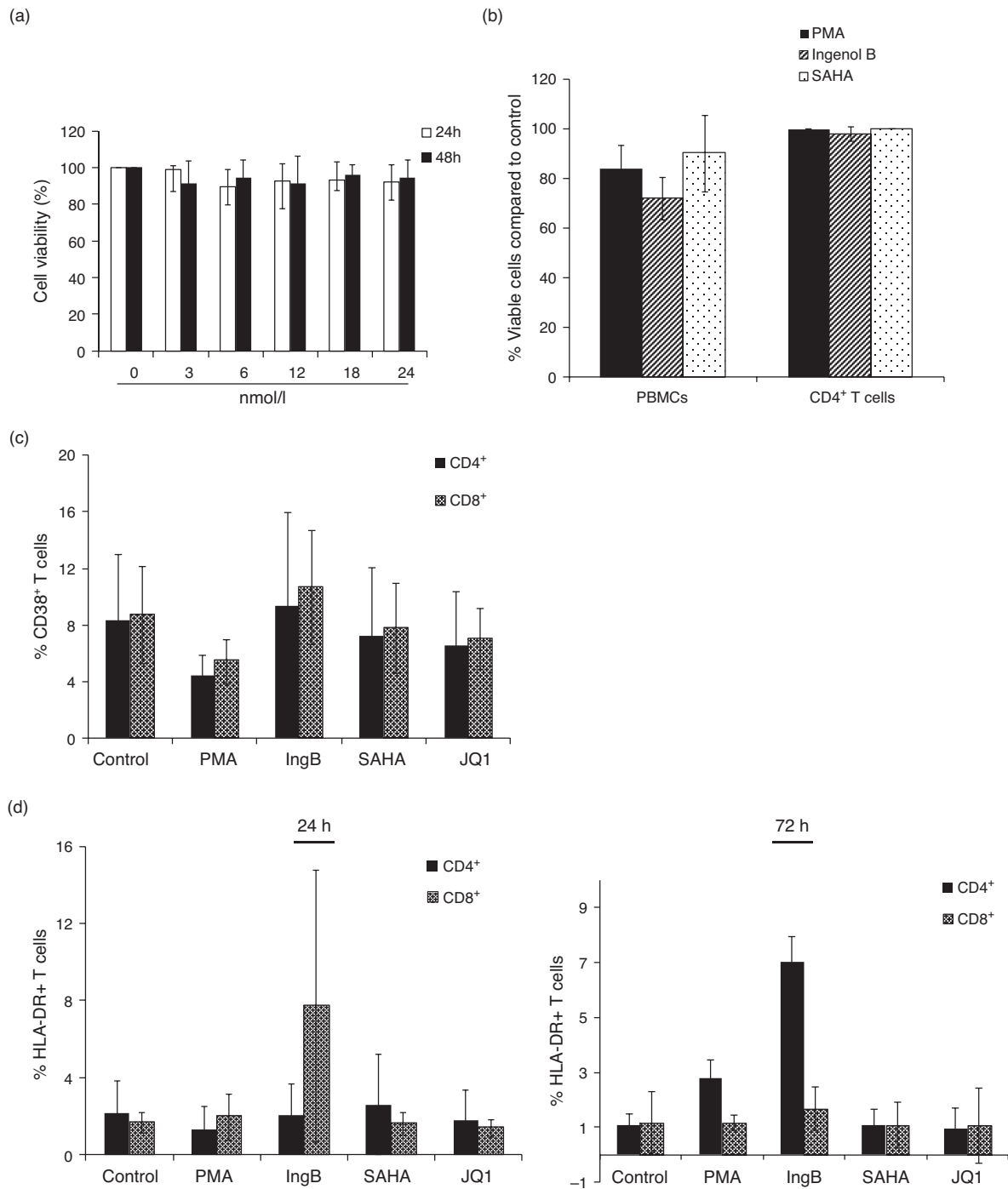


Fig. 3. Ingenol B causes minimal T-cell activation and cell toxicity. (a) Lack of cellular toxicity in J-Lat cells during Ingenol B treatment. J-Lat A1 cells were treated with Ingenol B from 3 to 24 nmol/l for 24 or 48 h, and cell proliferation and viability were measured by MTT. (b) Ingenol B has minimal cell toxicity in PBMCs or isolated CD4⁺ T cells. Human PBMCs or CD4⁺ T cells were isolated from peripheral blood of healthy HIV-negative donors and treated with 5 ng/ml PMA, 500 nmol/l SAHA, or 3 nmol/l Ingenol B for 20 h, and evaluated for cell proliferation/toxicity by MTT. (c) PBMCs were treated with specified agents for 24 h and examined for CD38 expression on CD4⁺ and CD8⁺ T cells by flow cytometry. (d) PBMCs were treated with indicated agents for 24 or 72 h and examined for HLA-DR expression on CD4⁺ or CD8⁺ T cells by flow cytometry. (e) J-Lat A1 cells and CD4⁺ T cells were treated with BrdU with or without Ingenol B or prostratin for 24 h, and BrdU ELISAs were performed with anti-BrdU antibody for quantification of BrdU incorporation. (f–i) Expression of IFN γ , IL-6, IL-2, or TNF- α in PBMCs from healthy donors ($n = 3$) following treatment with 3 nmol/l Ingenol B for 24 h. The expression of IFN γ and IL-6 proteins was detected by ELISA, and RNA expression of IL-2 and TNF- α was detected by RT-qPCR after normalizing the values with glyceraldehyde 3-phosphate dehydrogenase housekeeping gene and untreated controls. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

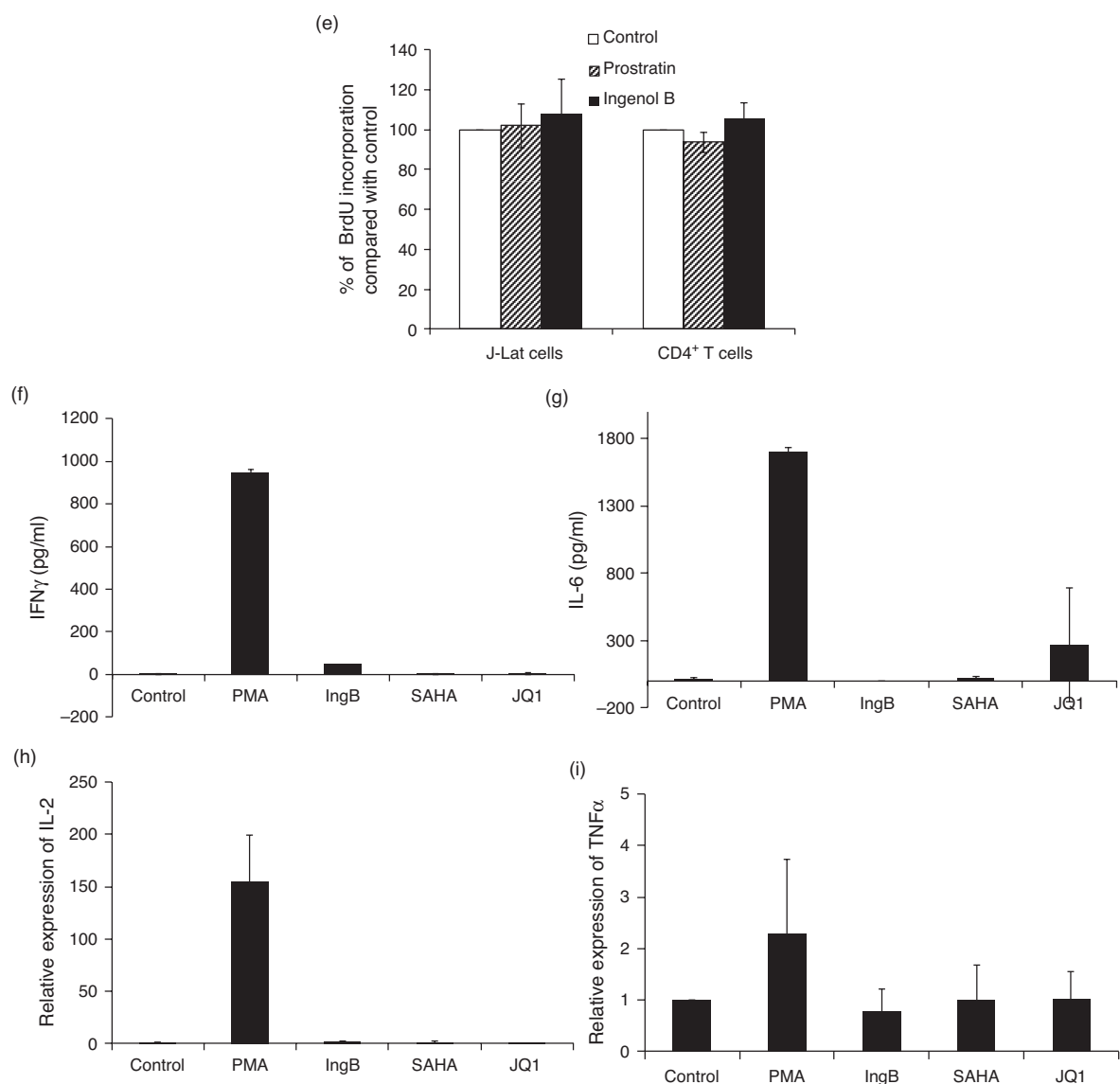


Fig. 3. (Continued)

Ingenol B causes minimal cell activation and cytotoxicity

To determine whether IngB caused any cytotoxic effects on cells, we measured cell viability of J-Lat A1, PBMCs, or purified CD4⁺ T cells following IngB treatment. Minimal cell toxicity was noted in these IngB-treated cells by MTT assay (Fig. 3a, b). This finding is similar to those reported for SAHA treatment.

To investigate whether IngB caused nonspecific T-cell activation, we measured expression of cell activation markers including HLA-DR and CD38 in CD4⁺ and CD8⁺ T-cell subsets from IngB-treated PBMC samples of HIV-negative controls. Compounds causing massive global T-cell activation tend to exert cytotoxicity and may not be suitable for clinical use [61]. Our data showed that IngB did not cause demonstrable up-regulation of expression of CD38 in T-cell subsets (Fig. 3c). However,

IngB treatment caused a minor increase of HLA-DR expression in CD4⁺ T cells and a major increase of CD69 expression in CD4⁺ and CD8⁺ T cells (Figs. 2e and 3d). Our findings raised a concern about potential side effects of this compound. Therefore, we investigated cells in the S-phase of cell cycle using BrdU incorporation assay. However, IngB treatment of J-Lat A1 cells and isolated CD4⁺ T cells from PBMCs did not result in significant changes, which suggested that IngB treatment did not lead to any substantial changes in cell cycle progression or cell proliferation (Fig. 3e). Since cytokine release may induce T-cell activation or be toxic to T cells, we examined levels of several cytokines after IngB treatment [62]. Although IngB induced a low level of interferon (IFN) γ protein production at 24 h, it was about 20-fold lower than the level produced following PMA stimulation (Fig. 3f, h), and it was undetectable after 48 h (data not shown). Both SAHA and JQ1 barely stimulated IFN γ

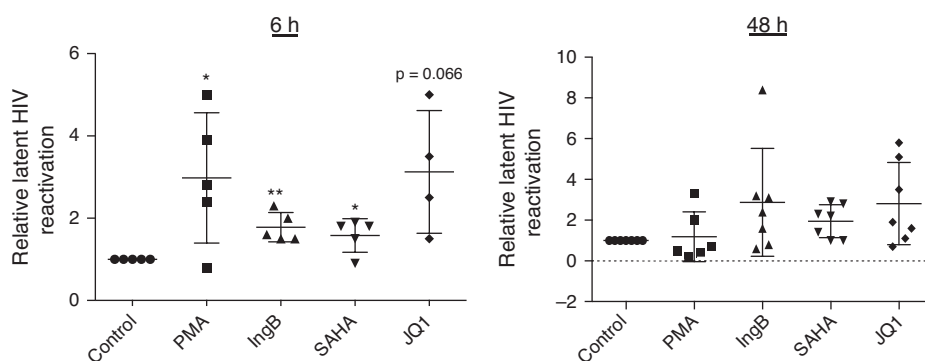


Fig. 4. Ingenol B reactivates latent HIV in primary CD4⁺ T cells from long-term HAART-treated HIV-positive individuals. Primary CD4⁺ T cells were purified from PBMCs isolated from cohorts of HAART-treated virologically suppressed HIV-infected individuals, and incubated with 200 ng/ml PMA and 2 μ mol/l inomycin, 3 nmol/l Ingenol B, 500 nmol/l SAHA or 2 μ mol/l JQ1 for 6 or 48 h. Cells were collected and RNAs were extracted by Qiagen RNeasy kit. RT-qPCR was performed for HIV reactivation analysis after normalized with expression of internal control (18S rRNA). The value was expressed relative to control treatment. (*) $P < 0.05$, (**) $P < 0.01$.

expression, but induced production of interleukin (IL)-6 in two of the three samples, whereas IngB did not have a detectable effect on the expression of IL-6. There was lack of induction of the expression of IL-2 and TNF- α following the treatment with SAHA, JQ1, or IngB. In summary, IngB did not cause massive CD4⁺ T-cell activation or cytotoxicity, and may serve as a suitable candidate for activating HIV from latent reservoirs.

Ingenol B is able to activate HIV expression in purified primary CD4⁺ T cells from HAART-treated HIV-positive individuals

We sought to determine the efficacy of IngB to reactivate HIV in purified CD4⁺ T cells from long-term HAART-treated HIV-positive individuals. Purified CD4⁺ T cells from these patients were treated for 6 or 48 h with 3 nmol/l IngB, 500 nmol/l SAHA, or 2 μ mol/l JQ1, and cellular HIV reactivation was examined by HIV RNA

expression with RT-qPCR. Our data indicated that, at 6 h after treatment, both IngB and JQ1 were able to reactivate latent HIV from all the CD4⁺ T cells isolated from patients, SAHA reactivated latent HIV except one sample; at 48 h after treatment, both IngB and SAHA were able to reactivate latent HIV-4 in seven samples, whereas JQ1 reactivated latent HIV in five of the seven samples (Fig. 4). Thus, IngB is able to reactivate latent HIV from CD4⁺ T cells from HIV-infected patients and is comparable to SAHA or JQ1 in its potency for HIV reactivation. These findings were consistent with our results from J-Lat A1 cell cultures *in vitro*.

Structural similarity of Ingenol ester to phorbol ester can be used for new formulations for HIV reactivation

Ingenol ester is structurally analogous to phorbol esters [36,37] (Fig. 5). Phorbol esters, such as PMA, are highly

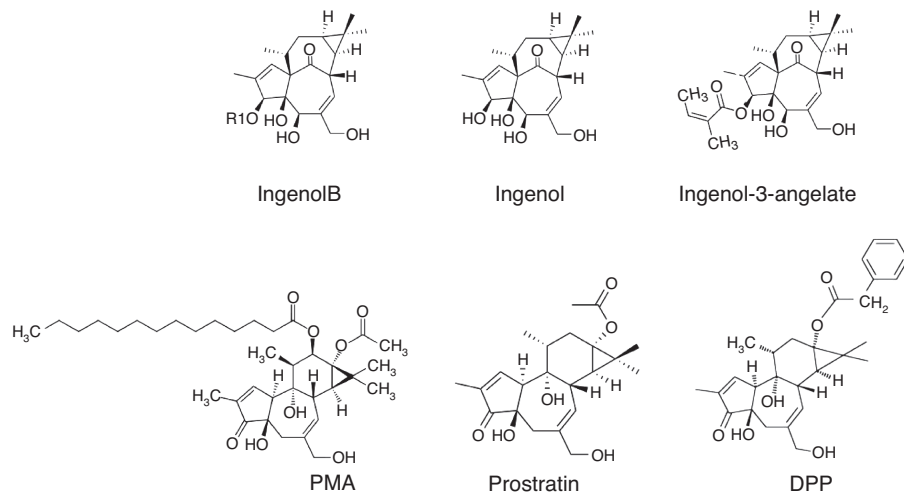


Fig. 5. Core chemical structure of Ingenol B is analogous to phorbol esters. Ingenols share core structure analogous to phorbol esters.

potent to reactivate latent HIV, but toxic to T cells and may promote tumor formation. Prostratin or 12-Deoxyphorbol 13-phenylacetate does not have similar tumorigenic effects [63–65]. Interestingly, only PKC agonists, including prostratin and possibly IngB, showed broad activity in reactivation of latent HIV [66]. Therefore, our data indicate that formulating compounds from Ingenol esters could be a new direction to develop or formulate potent and PKC–NF- κ B pathway-specific small molecules for HIV eradication [67].

Discussion

We found that a newly modified Ingenol ester derivative, IngB, is highly potent in reactivation of latent HIV-1. It is more effective than some of the known compounds, including SAHA, HMBA, JQ1, and prostratin, in HIV reactivation *in vitro*. Importantly, it has limited cellular toxicity in both J-Lat cells and primary human CD4⁺ T cells. We found that IngB reactivates latent HIV through PKC δ –NF- κ B signaling by up-regulating expression of both PKC δ and NF- κ B. However, PKC θ may also be involved in IngB-induced reactivation of HIV latency since 5 μ mol/l of PKC inhibitor could inhibit PKC θ (Fig. 2c and d). It is also possible that activation of PKC δ may induce degradation of I κ B and promote release of NF- κ B from NF- κ B–I κ B complex, which facilitates nuclear translocation of NF- κ B to recruit into HIV LTR. This has been noted in case of activation of other PKCs. Our data show that JQ1 or SAHA alone is not most effective in reactivating latent HIV *in vitro*. The effect of IngB in combination with JQ1 on reversing HIV latency is of interest. Therefore, future evaluation of combined use of JQ1 with IngB in purified CD4⁺ T cells from HIV-infected patients on long-term HAART is warranted. IngB does not modulate proliferation of Jurkat T cells or CD4⁺ T cells, cytokine expression except for the minimal expression of IFN γ . IngB was also shown not to enhance cell proliferation/cell cycle S-phase progression, although IngB can cause increased levels of CD69 expression. We can speculate that higher levels of IngB most probably may not exert serious cytotoxic effects on CD4⁺ T cells. The value of these characteristics of IngB to clinical application remains to be tested.

Recently, an Ingenol ester compound, PICATO (ingenol mebutate), was approved by the US Food and Drug Administration (FDA) for treatment of skin precancer [68] (Fig. 5). It seems that only PKC agonists, such as prostratin, had broad impact on reactivation of latent HIV in currently available in-vitro or ex-vivo HIV latency models [66]. As a PKC agonist, IngB may share similar property with prostratin. It is possible that new Ingenol compounds can be developed through step-economical synthesis [67,69,70]. This will provide an innovative strategy to seek novel compounds that are highly potent

and yet safe PKC–NF- κ B activators for HIV cure in the future.

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Conflicts of interest

There are no conflicts of interest to declare for all authors.

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